Identification of Serine 119 as an Effective Inhibitor Binding Site of M. tuberculosis Ubiquitin-like Protein Ligase PafA Using Purified Proteins and M. smegmatis

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A B S T R A C T

Owing to the spread of multidrug resistance (MDR) and extensive drug resistance (XDR), there is a pressing need to identify potential targets for the development of more-effective anti-M. tuberculosis (Mtbd) drugs. PafA, as the sole Prokaryotic Ubiquitin-like Protein ligase in the Pup-proteasome System (PPS) of Mtb, is an attractive drug target. Here, we show that the activity of purified Mtb PafA is significantly inhibited upon the association of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) to PafA residue Serine 119 (S119). Mutation of S119 to amino acids that resemble AEBSF has similar inhibitory effects on the activity of purified Mtb PafA. Structural analysis reveals that although S119 is distant from the PafA catalytic site, it is located at a critical position in the groove where PafA binds the C-terminal region of Pup. Phenotypic studies demonstrate that S119 plays critical roles in the function of Mtb PafA when tested in M. smegmatis. Our study suggests that targeting S119 is a promising direction for developing an inhibitor of M. tuberculosis PafA.

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1. Introduction

Tuberculosis (TB), caused by M. tuberculosis (Mtbd), is one of the most common causes of death worldwide (WHO, 2016). At present, this global health problem is exacerbated by the emergence and spread of M. tuberculosis strains that are resistant to antibiotics including multidrug resistant (MDR) and extensively drug resistant (XDR) strains. According to the latest statistics, only 52% of patients with MDR-TB and 28% with XDR-TB can be treated effectively (WHO, 2016). In the past 50 years, only two new drugs, bedaquiline (Goel, 2014) and delamanid (Hoagland et al., 2016), have been successfully developed to address MDR-TB (Zumla et al., 2013, Mduli et al., 2015). To obtain more effective treatment options for MDR-TB, there is an urgent need to develop new drugs with different mechanisms of action.

Ubiquitin-dependent protein degradation in eukaryotes plays a central role in many cellular functions, such as post-translational quality control, cell proliferation, differentiation and development (Grabe et al., 2011, Yau and Rape, 2016). Ubiquitin is covalently attached to specific lysine residues of target proteins through a complicated multi-step ligation reaction and eventually delivers doomed proteins for proteasomal degradation (Hershko et al., 2000). Similar to this process in eukaryotic cells, proteins are targeted to the proteasome via a prokaryotic ubiquitin-like protein modifier termed Pup in Mtb (Pearce et al., 2008, Striebel et al., 2009). The inactive form of Pup has a C-terminal glutamine: conversion of this residue to glutamate (Pup E) by

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the enzyme Dop (Striebel et al., 2009) activates Pup for ligation. Activated Pup is then attached to target proteins by PafA, the sole ligase in the Pup-proteasome System (PPS) (Pearce et al., 2006, Pearce et al., 2008, Striebel et al., 2009, Sutter et al., 2010, Guth et al., 2011). Pupylated proteins are then directed into the proteasome via recognition of Pup by Mycobacterium proteasomal ATPase (Mpa) (Sutter et al., 2009, Wang et al., 2009, Striebel et al., 2010, Wang et al., 2010). Analogous to deubiquitination, depupylation also occurs in Mtb and is catalyzed by Dop (Burns et al., 2010, Imkamp et al., 2010b) and PafA (Zhang et al., 2017). Previous studies showed that the Pup-proteasome System (PPS) of Mtb is required for resistance to nitric oxide and is essential for Mtb to cause lethality in mice (Darwin et al., 2003, Darwin et al., 2005, Lamichhane et al., 2006, Gandotra et al., 2007, Sumanovic et al., 2015). To our knowledge, the PPS is only present in the Nitrospira and Actinobacteria (Imkamp et al., 2015) and is not present in most other bacteria, including gut microbiota. These unusual properties of the Mtb PPS make it an attractive target for drug development. Previous strategies for inhibiting the Mtb PPS focused on the 20S proteasome (Lin et al., 2009, Cheng and Pieters, 2010, Lin et al., 2010, Clements et al., 2013, Lin et al., 2013, Zheng et al., 2014, Totaro et al., 2017), however, owing to the high degree of mechanistic and structural conservation of mammalian and mycobacterial proteasomes, inherent toxicity is inevitable (Cheng and Pieters, 2010). On the other hand, PafA shares no homology with ubiquitin ligases in eukaryotes (Festa et al., 2007, Burns et al., 2009, Bode and Darwin, 2014), suggesting that there may be no or few side effects for drugs that target PafA. Unfortunately, to date, effective inhibitors of PafA have not been identified.

Here, we show that the serine protease inhibitor, AEBSF (4-(2-aminoethyl) benzene sulfonfyl fluoride), is a potent inhibitor of purified Mtb PafA. We further show that this compound binds to PafA via S119. Biochemical analysis demonstrated that substitution of S119 with aromatic amino acid residues, mimicking the binding of AEBSF, almost completely abolishes the pupylase and depupylase activity of Mtb PafA. Further structural analysis showed that this inhibition of PafA activity is a consequence of defective Pup binding to PafA even though this residue is far from the PafA catalytic site. Finally, phenotypic studies demonstrated that S119 is critical for the function of Mtb PafA and essential for Msm ΔPafA survival under nitrogen limitation and in macrophages. Overall, our work shows that S119 is situated within a critical, small-molecule accessible region of PafA whose modification inhibits PafA activity and is thus a highly promising target for the development of inhibitors of M. tuberculosis PafA.

2. Materials and Methods

2.1. Protein Cloning, Expression, and Purification

All genes were cloned from the Mtb reference strain H37Rv. PafA was cloned into pTrc99a as described previously (Striebel et al., 2009). All PafA variants were constructed using a QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies). C-terminal Flag-His6-tagged Mpa and PanB were cloned into pET28a. Sequences of primers used in this study are given in Table S1. All recombinant proteins were expressed in E. coli BL21 by growing recombinant E. coli BL21 cells in 1 L LB medium to an A600 of 0.6 at 37 °C. Protein expression was induced by the addition of 0.2 mM isopropyl-D-thiogalactoside (IPTG) before incubating cells overnight at 16 °C. Proteins were purified on Ni-NTA affinity columns and stored at −80 °C. Pup5 and N-terminal 5-Carboxyfluorescein-cys-Pup5 were synthesized by GL Biochem, Shanghai, China.

2.2. Inhibitor Profile of PafA Pupylation Activity

All chemicals or inhibitors were purchased from Sigma-Aldrich. PafA was incubated with different inhibitors at 25 °C for 0.5 h in a pupylation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl2 and 10% (v/v) glycerol). Pupylation reactions included PanB-Flag (8 μM), Pup5 (10 μM) and PafA (0.5 μM) pre-incubated with inhibitors and were incubated at 25 °C for 6 h with 5 mM ATP in pupylation buffer. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining and western blotting with an anti-Pup monoclonal antibody (Abmart) and an anti-Flag antibody (Sigma-Aldrich Cat# P2983, RRID: AB_439685).

2.3. Pupylation Assays

PanB pupylation assay reactions were carried out in pupylation buffer containing PanB-Flag (8 μM), Pup5 (10 μM) and PafA (0.5 μM) at 25 °C for 6 h, while Mpa pupylation assay reactions were carried out in pupylation buffer containing Mpa-Flag (6 μM), Pup5 (10 μM) and PafA (0.5 μM) at 25 °C for 2 h with 5 mM ATP. For pupylation assays using lysate as the substrate, reactions included Msm ΔPafA lysates (10 μg), Pup5 (10 μM) and PafA (0.5 μM) and were incubated at 25 °C for 20 min with 5 mM ATP in pupylation buffer. In AEBSF inhibitory assays, reactions were carried out as described above, except that PafA (0.5 μM) was pre-incubated in AEBSF for 30 min at 25 °C. Unbound AEBSF was removed by dialysis using a Spectra/Por (R) Dialysis Membrane (Sangon Biotech, Shanghai, China). Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining and western blotting.

2.4. Identification of the AEBSF Binding Site on PafA by LC-MS/MS

PafA (0.5 μM) was incubated with AEBSF (0.25 mM or 0.5 mM) at 25 °C for 0.5 h in pupylation buffer. After SDS-PAGE and Coomassie staining, PafA bands were cut out and in-gel digested with trypsin. The tryptic peptide digests of the proteins were analyzed using an LC system (Nano Pump, Ultimate 3000, Dionex, Thermostof) coupled with an ESI-Q-TOF mass spectrometer (MaXis, Impact, Bruker Daltonik, Germany). The peptide sequences were determined by searching MS/MS spectra against the Protein database using the Mascot (version 2.4, Matrix Science) software suite with a precursor ion mass tolerance of 20 ppm and fragment ion mass tolerance of 0.05 Da. Carbamidomethyl (C) was set as the fixed modification, and oxidation (M) was set as the variable modification. For modification of AEBSF, +183.0354 Da was set as a variable modification on serine, and a maximum of two missed cleavage of trypsin was chosen. MS data are available (“ProteomeXchange: PXD007045 and PXD006930”).

2.5. Quantitative Pupylation Assays

Quantitative pupylation assays were performed according to a previously described procedure (Ofer et al., 2013), except that N-terminal 5-Carboxyfluorescein-cys-Pup5 was used instead of N-terminal 5-iodoacetamidofluorescein-cys-Pup5. The fluorescence intensity of each sample was measured with a Synergy 2 microplate reader (Biotek Instruments) at 494 nm (excitation) and 522 nm (emission).

2.6. Determination of PafA Activity

We cloned PafA and its variants with a C-terminal Flag-tag into the mycobacterial expression vector pMV261 or pSMT3 via BamHI and HindIII restriction sites. These plasmids were then transformed into WT M. smegmatis (Msm) or Msm ΔPafA. Msm cells were grown at 37 °C in 100 ml 7H9 medium containing 30 μg/ml Kan or 50 μg/ml Hyg with 0.5% glycerol, 0.05% Tween-80 to an OD600 of 2.0. Cells were collected by centrifuging at 5000 rpm for 10 min and were then lysed in pupylation buffer with a high-pressure cracker (Union-Biotech, Shanghai, China). These lysates were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining and western blotting.
2.7. Equilibrium MD Simulations

The initial structure used for equilibrium simulations was the crystal structure of the C. glutamicum PafA/Pup complex (pdb: 4BJR) (Barandun et al., 2013). Alanine and phenylalanine mutants at S126 were generated using VMD (Humphrey et al., 1996). Force-field parameters for covalent attachment of AEBSF to the hydroxyl group of serine were generated using CGenFF (Vanommeslaeghe et al., 2010, Yu et al., 2012) and the initial structure of the modified protein was generated using VMD. All proteins were solvated in TIP3 water in 0.15 M NaCl and minimized and equilibrated using VMD/NAMD and the CHARMM 27 force field (MacKerell et al., 1998, Phillips et al., 2005). The particle mesh Ewald algorithm was employed to treat electrostatic interactions, and van der Waal interactions were treated with a cut-off of 12 Å. Langevin dynamics were employed to maintain a constant temperature of 310 K and a Nose-Hoover Langevin piston was used to maintain a constant pressure of 1 atm. The integration step was set to 2 fs. Extended simulations (35 ns) were performed for each complex. In the movies of the simulations, adjacent frames of the 80 ps/frame simulations were smoothed to facilitate understanding of the conformational changes.

2.8. Lysine Pupylation Assays

Pupylation reactions were carried out in pupylation buffer containing free lysine (80 mM), PupE (10 μM) and PafA variants (0.5 μM).
each) to which 5 mM ATP was added, and were incubated at 25 °C for 15 min. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining. The reactions in the AEBSF inhibitory assay were carried out as above, except that serially diluted PafA (0.5 μM) that had been pre-incubated in AEBSF in pupylation buffer at 25 °C for 0.5 h was used instead of untreated PafA (0.5 μM). Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining.

2.9. Bio-layer Interferometry of Pup and PafA Interaction Kinetics

The kinetics of Pup and PafA binding was measured using a ForteBio 70 Octet system. PupMED was biotinylated using an EZLink Sulfo-NHS-LC-Biotin protein biotinylation kit (Thermo Scientific, Bremen, Germany) according to the manufacturer’s instructions. Biotinylated PupMED was tethered on the tip surface of a streptavidin-coated sensor, and then exposed to its binding partners in sample dilution (SD) buffer (1 × PBS, pH 7.4 with 0.02% Tween-20 and 0.1% BSA). Binding was measured by the coincident change in the interference pattern. Super strepavidin (SSA) tips (Pall ForteBio, Menlo Park, USA) were prewet in SD buffer, the coincident change in the interference pattern. Super strepavidin (SSA) tips (Pall ForteBio, Menlo Park, USA) were prewet in SD buffer, and the binding process involved establishing a stable baseline (60 s), loading of 100 μg/ml biotinylated PupMED (300 s), balancing tips with SD buffer (60 s), association with 500 nM PafA variants or PafA pre-incubated with 2 mM AEBSF (dissolved in SD buffer) (300 s) and then eluting non-specific binding with SD buffer (300 s). BLI data were analyzed using ForteBio Data Analysis Software 7. Data were fit to a 1:1 binding model to calculate an association and dissociation rate, and the Kd was calculated using the ratio Kd = Kd/Koff.

2.10. Protein Depupylation Assays

In PanB depupylation assays, PanB-Flag-PupMED was enriched using an anti-FLAG M2 Affinity gel (Sigma-Aldrich, St. Louis, USA). PanB-PupMED (0.75 μM) was depupylated by PafA variants (1.5 μM each) with 5 mM ADP in phosphate buffer (50 mM Na2HPO4 pH 8, 100 mM NaCl, 20 mM MgCl2, and 10% (v/v) glycerol) for 6 h at 25 °C. In the AEBSF inhibitory assay, the reactions were carried out as above, except that PafA (0.5 μM) was pre-incubated with AEBSF in pupylation buffer at 25 °C for 0.5 h. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining and western blotting.

2.11. Msm Survival Under Nitrogen Limitation

Nitrogen limitation assays were performed according to a previously described procedure (Elharar et al., 2014) with slight modification. Briefly, the Msm MC3155 strain was grown at 37 °C in a defined minimal nitrogen medium containing 40 mM KH2PO4, 22 mM KH2PO4, 1.7 mM sodium citrate, 0.4 mM MgSO4, 0.4% glycerol (v/v), and 0.05% Tween-80 (v/v).

2.12. Msm Survival in THP-1 Cell

Human THP-1 cell lines were grown in RPMI 1640 medium (Invitrogen, California, USA) with 10% fetal calf serum (Invitrogen, California, USA). Cultures of THP-1 cells were maintained at final gross of 4 × 106 cells and were treated with 10 ng/ml polymethyl acrylate (PMA) for 12 h to induce a macrophage-like state. After removing the suspension, adherent cells grew for 8 h, and then infected with Msm expressing PafAmab with different mutations at OD560 = 0.7 for 2 h at a multiplicity of infection (MOI) of 10. The culture plate wells were then washed with incomplete RPMI 1640 medium and the cells were incubated in fresh complete RPMI 1640 medium with 10 ng/ml gentamicin for 12, 24, or 48 h. Cells were harvested for CFU counting and then washed three times with 1× PBS. Cells used for CFU counting were lysed in 7H9 broth containing 0.05% SDS for 10 min. Three sets of tenfold serial dilutions of the lysates were prepared at each time point in 0.05% Tween-80, and portions were plated on 7H10 agar plates. Plates were placed at 37 °C for 3–5 days before counting.

3. Results

3.1. AEBSF Inhibits Mtb PafA Pupylase Activity

During initial preliminary experiments on the function of PafA, we noted the presence of background proteolytic activity and so examined a range of well-known protease inhibitors to block this activity. Unexpectedly, we discovered that one of these compounds, AEBSF (4-((2-aminoethyl) benzenesulfonyl fluoride), an irreversible inhibitor of serine proteases, strongly inhibited Mtb PafA pupylation of PanB (Fig. S1), a well-studied substrate of PafA pupylation activity (Pearce et al., 2006, Striebel et al., 2009). To confirm this result, pupylation assays were carried out with serially diluted AEBSF, and we found that the loss of PafA-mediated pupylation of PanB is indeed proportional to the concentration of AEBSF (Fig. 1a). To rule out the possibility that this reduction is due to the binding of AEBSF to PanB, we pre-incubated PafA with AEBSF, then used dialysis to remove unbound AEBSF. As AEBSF inhibits serine proteases by irreversible covalent binding, we reasoned that its inhibition of PafA might be through a similar mechanism and thus that the inhibition would persist even after extensive dialysis. Indeed, we found significant and dose-dependent reduction of pupylation even after dialysis (Fig. S2a). These results clearly demonstrate that the reduced pupylation of PanB was due to covalent attachment of AEBSF to PafA. We also observed a similar reduction in the pupylation of Mpa, another well-studied PafA substrate (Pearce et al., 2006, Delley et al., 2012) in the presence of AEBSF (Fig. 1b), further supporting a PafA-specific effect.

Having confirmed the inhibitory effect of AEBSF on PafA using affinity-purified proteins (PanB and Mpa), we then tested the global effect of AEBSF on pupylation using total proteins (cell lysates) as the substrate. As many proteins in Mtb and M. smegmatis (Msm), a fast-growing non-pathogenic relative of Mtb used as a model organism for Mtb, are highly homologous, and cell lysates of Msm are more convenient to prepare, we performed this experiment using Msm cell lysates. We first constructed a Msm PafA knock-out strain (Msm ΔPafA) to knockout endogenous protein pupylation in Msm, as Msm PafA exhibits high overall sequence similarity to Mtb PafA (94% identity, 100% similarity) and is also active in pupylation. The total lysate of the Msm ΔPafA strain was then used to test the inhibitory effect of AEBSF on exogenously added proteins (Fig. 2). S119 was critical for Mtb PafA pupylation activity. (a) PafA S119 was identified as an AEBSF binding site by LC-MS/MS analysis. PafA (0.5 μM) was incubated with AEBSF (0.5 μM) for 0.5 h in pupylation buffer at 25 °C, then trypsin-digested and subjected to MS/MS analysis. A 183 Da molecular weight increase is predicted on AEBSF-mediated sulfonation. (b) Pupylation reactions included PanB-Flag (8 μM), PupMED (10 μM) and PafA variants (0.5 μM each) and were incubated at 25 °C for 6 h with 5 mM ATP in pupylation buffer. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue (CB) staining and western blotting with an anti-FLAG antibody. Quantification of the pupylation level of PanB was based on CB staining and is shown in the lower panel. Data are representative of one experiment three independent biological replicates (mean and s.e.m. of n = 3 samples), *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test). (c) Pupylation reactions included Mpa-Flag (6 μM), PupMED (10 μM) and PafA variants (0.5 μM each) and were incubated at 25 °C for 2 h with 5 mM ATP in pupylation buffer. Samples were analyzed by SDS-PAGE, followed by CB staining and western blotting with an anti-FLAG antibody. (d) Pupylation reactions included Msm ΔΔPafA lysates (10 μg), PupMED (10 μM) and PafA variants (0.5 μM each) and were incubated at 25 °C for 20 min with 5 mM ATP in pupylation buffer. Samples were analyzed by SDS-PAGE, followed by CB staining to serve as a loading control and western blotting with an anti-Pup of anti-PafA antibody. The asterisk denotes a cross-reactive band of the rabbit polyclonal anti-pup antibody. (e) Steady-state rates of PanB-Flag pupylation by WT PafA (black), S119A (blue) and S119F (red). Data are representative of three independent biological replicates (mean and s.e.m. of n = 3 samples). Data were fitted to the Michaelis-Menten equation.
Mod: 9, AEBSF[S]  Charge: 3+  MW: 2467.0484  Expect: 8e-005  Retention time: 47.4min

![Mass Spectrometry Graph]

**b**

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**IB:**

- **Flag:**
  - PanB-Pup
  - PanB

- **CBB:**
  - PanB-Pup
  - PanB

**Relative abundance**

![Graph showing relative abundance]

**c**

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**IB:**

- **Flag:**
  - Mpa-Pup
  - Mpa

- **CBB:**
  - Mpa-Pup
  - Mpa
  - PafA

**d**

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**IB:**

- **Pup:**
  - PanB-Pup
  - PanB

- **CBB:**
  - PanB-Pup
  - PanB

**e**

**Purification rate**

- WT PafA
- PafA S119F
- PafA S119A

- $V_{max} = 0.81 \pm 0.06 \mu M/min/MU$
- $K_M = 57.07 \pm 11.91 \mu M$
- $V_{max} = 0.31 \pm 0.02 \mu M/min/MU$
- $K_M = 53.89 \pm 19.19 \mu M$

![Graph showing purification rate]

Mtb PafA. As expected, no pupylation was observed in the lysate of Msm ∆PafA (Fig. 1c). Following incubation with Mtb PafA, we found that Mtb PafA efficiently pupylated many proteins in Msm ∆PafA cell lysates. Furthermore, PafA activity was inhibited by AEBSF in a dose dependent manner (Fig. 1c). In addition, we prepared purified Msm PafA and found that its pupylase activity could also be inhibited by AEBSF when the Msm ∆PafA cell lysates were used as the substrate (Fig. S2b).

Overall, these findings indicate that AEBSF is an effective inhibitor of the pupylase activity of purified Mtb PafA.

3.2. AEBSF Covalently Binds to S119 of Mtb PafA

To identify the binding site of AEBSF in purified Mtb PafA, we incubated PafA with AEBSF and then performed liquid chromatography–mass spectrometry (LC-MS/MS) analysis. We found that S119 is the predominant residue covalently bound by AEBSF (Fig. 2a).

We next investigated if S119 is critical for Mtb PafA pupylase activity. As AEBSF has a benzene ring, we speculated that the significant reduction in activity upon AEBSF binding may due to steric hindrance from the relatively large benzene ring. We suspected that mutation of serine to other aromatic amino acids, such as phenylalanine (F), tyrosine (Y), or tryptophan (W), that also have a benzene ring, might mimic the steric hindrance of AEBSF, and so constructed mutants S119F, S119Y and S119W. To determine the absolute requirement of this S119 in the pupylation reaction, we also investigated a mutation of S119 to alanine. Mutants of S115 (that does not interact with AEBSF) were used as controls. Strikingly, the S119F, S119Y and S119 W PafA mutants, similar to the well-known D57N PafA mutant that causes inactivation of PafA (Cerda-Maira et al., 2010), almost entirely lost their pupylase activity using PanB as the substrate (Fig. 2b; Fig. S3). Interestingly, PafA S119A exhibited approximately half of the pupylase activity of that of WT PafA or PafA S115 variants, which all exhibited similar activity as that of WT PafA (Fig. S3). We next examined the kinetic activity of the S119F, S119A and WT PafA using Michaelis-Menten assays and found that WT PafA exhibits a higher Vₘₐₓ than that of the S119A and S119F mutants (Fig. 2e). It is noteworthy that the Kₘ of WT PafA and S119A were similar, indicating that S119 is not directly involved in PanB binding.

We also found that PafA S119F was completely inactive in Mpa pupylation (Fig. 2c). To further confirm the effect of the S119F and S119A mutants, Msm ∆PafA total lysates were also used as substrates (mutant D57N being used as a control). Results indicated that the S119A mutant exhibited lower pupylase activity than WT PafA while the S119F mutant exhibited almost no pupylase activity in this assay (Fig. 2d).

Thus, taken together, these results indicate that AEBSF covalently attaches to S119, and that changes in the chemical nature of this residue, whether by AEBSF binding or by mutation to aromatic acids, can almost completely abolish the pupylase activity of purified Mtb PafA.

3.3. S119 is Critical for Mtb PafA Pupylase Activity in Bacterial Cells

To investigate whether PafA S119 contributes to Mtb PafA pupylase activity in bacterial cells, we performed experiments in model organism Msm which also has the Pup-proteasome system (Knipfer and Shrader, 1997, Imkamp et al., 2010a). Previous studies have shown that PafA is the only enzyme in mycobacteria that catalyzes protein pupylation (Pearce et al., 2008, Striebel et al., 2009). Consistent with this, we did not detect any bands in the Msm ∆PafA strain using an anti-pup antibody (Fig. 3). We transformed Msm ∆PafA with plasmids encoding...
119
M. tuberculosis_PafA
NNTDAGNSYGCHENYLVR
111-130
C. glutamicum_PafA
NNVDAGNSYGCHENYLVR
118-137

b  \text{PafA}_{C_{glu}}

\begin{figure}
\centering
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\caption{Structural comparison of PafA proteins. (a) Alignment of M. tuberculosis_PafA and C. glutamicum_PafA showing the regions of similarity. (b) Structural comparison of WT PafA and \text{PafA}_{C_{glu}}. (c) Close-up view of the Pup C-terminal region. (d) Molecular dynamics simulation of PafA and \text{PafA}_{C_{glu}}. (e) Kinetic analysis of PafA variants. (f) Western blot analysis of PafA variants with ATP treatment. (g) AEBSF inhibition of PafA activity.}
\end{figure}

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<th>(K_0) (nM)</th>
<th>(K_{on}) (1/Ms)</th>
<th>(k_{off}) (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PafA</td>
<td>53.1±0.85</td>
<td>1.16x10^4</td>
<td>6.7x10^-4</td>
</tr>
<tr>
<td>\text{PafA}_{AEBSF} 2 mM</td>
<td>99.2±2.00</td>
<td>8.46x10^4</td>
<td>8.4x10^-4</td>
</tr>
<tr>
<td>\text{PafA S119A}</td>
<td>68.1±1.24</td>
<td>8.74x10^4</td>
<td>5.9x10^-4</td>
</tr>
<tr>
<td>\text{PafA S119F}</td>
<td>122.1±3.05</td>
<td>9.92x10^4</td>
<td>1.21x10^-3</td>
</tr>
</tbody>
</table>
different Flag-tagged Paf<sub>Amo</sub> variants (Table S2) under the control of a mycobacterial hsp60 promoter. Paf<sub>Amo</sub> expression in Msm ∆PafA almost completely restored the levels of pupylation to that of WT Msm. We found that Msm ∆PafA/pMV·Paf<sub>Amo</sub> S119F exhibited a very low level of pupylation while Msm ∆PafA/pMV·Paf<sub>Amo</sub> S119A exhibited similar pupylation level to that of the Msm ∆PafA/pMV·Paf<sub>Amo</sub> strain. These data are consistent with our observations on Paf<sub>Amo</sub> puplyase activity in the above biochemical experiments. Thus, we conclude that S119 contributes to Mt<sub>b</sub> PafA pupylase activity in bacterial cells.

3.4. Structural Basis for the Involvement of S119 on Mt<sub>b</sub> PafA Activity

To determine the mechanistic role of S119 in pupylation, we aligned the Mt<sub>b</sub> PafA amino acid sequence with PafA from representative actinomycetes and <i>C. glutamicum</i> (Fig. S4a). We found that the sequences surrounding S119, including S119 itself, are highly conserved (Fig. 4a; Fig. S4a). Since there is no available crystal structure of Paf<sub>Amo</sub>, we examined the known structure of PafA from Cglu that exhibits high overall sequence similarity to Paf<sub>Amo</sub> (53% identity, 92% similarity).

Previous studies reported that the binding site for the C-terminal region of Pup is close to the N-terminal end of PafA<sub>Cglu</sub> (Ozcelik et al., 2012, Barandun et al., 2013), which is close to the location of S126 (the homologous residue of Paf<sub>Amo</sub> S119). More specifically, the PafA-binding region of Pup consists of two small α-helices and a short C-terminal tail (Fig. 4b). Pup wraps around PafA<sub>Cglu</sub> with Serine 126 located within a loop that defines one side of a groove on PafA<sub>Cglu</sub> that interacts with the Pup C-terminal tail (Fig. 4b). Interestingly, the S126 side-chain does not face into this groove but is instead buried within a pocket in the interior of PafA<sub>Cglu</sub>, where it makes close contact with Asn119, Val120, Arg128, and His188 (which, with S126, we will refer to as the “S126-pocket residues”) (Fig. 4c).

To determine how modification of S126 might perturb PafA<sub>Cglu</sub> pupylation, we examined its effects on the binding of Pup to PafA<sub>Cglu</sub> by performing all-atom molecular dynamics (MD) simulations. To this end, we studied equilibrium simulations of the structures of WT PafA<sub>Cglu</sub>, S126F and S126A mutants, and PafA<sub>Cglu</sub> to which EABSF is covalently attached at S126 (S126-EABSF), using the aforementioned Pup-PafA<sub>Cglu</sub>atomic model as the starting structure for the simulations. Strikingly, even before the simulations began, simply changing the S126 side-chain to that of phenylalanine or attaching EABSF to the S126 side-chain hydroxyl group results in a significant steric overlap with S126-pocket residues, particularly Arg185 (Fig. 4c). Thus, this observation suggests that these modifications might disrupt the close-packed organization of the S126-pocket, and with it, the Pup-binding groove in PafA<sub>Cglu</sub>, which might then lead to weakened binding of Pup.

Indeed, extended simulations of these structures showed that, while the S126-pocket in WT PafA<sub>Cglu</sub> is highly stable, it collapses and is significantly more unstable in S126F and S126-EABSF, S126F exhibiting similar significant fluctuation as in S126-EABSF, and to a lesser extent, in S126A (Fig. 5b, Movie S1) As a result, both the C-terminal region and the helices of Pup exhibit significant fluctuations in the S126F and S126-EABSF structures, with smaller changes in S126A, while the PafA<sub>Cglu</sub>/Pup complex is quite stable (Fig. 5b, Movie S2). Thus, these results suggest that modifications to S126 perturb pupylation as a result of destabilization of this Pup-binding groove in PafA, thereby weakening the binding strength of Pup to PafA.

To test this suggestion directly, the strength of the interactions between Mt<sub>b</sub> PafA and Pup was examined using Bio-Layer Interferometry (BLI). We found that Paf A S119A exhibits a slightly lower affinity for Pup than WT PafA, and that PafA S119F or PafA co-incubated with 2 mM EABSF exhibited equilibrium dissociation constants (K<sub>D</sub>) that are twice as high as that of WT PafA (Fig. 4e). Therefore, these results support the notion that the inhibitory effect of EABSF is due to defective Pup binding.

To obtain a better understanding of defective Pup binding in Mt<sub>b</sub> PafA S119 mutants and to exclude possible effects on protein-substrate binding, we examined the ability of Mt<sub>b</sub> PafA to pupylate free lysine under different conditions. Clearly, the docking of free lysine in the PafA active site does not require most of the residues involved in the interaction of PafA with protein targets (Regew et al., 2016). We found that PafA S119A shows a weakened pupylation activity on free lysine compared to that of the WT enzyme, while lysine pupylation by PafA S119F, S119Y and S119 W was undetectable (Fig. 4f). Pre-incubation of PafA with increasing concentrations of AEBSF resulted in a gradual loss of pupylation on free lysine (Fig. 4g).

Taken together, these results suggest that S119 plays a critical role in the Pup C-terminal binding pocket in Mt<sub>b</sub> PafA and that it can function as an effective inhibitor binding site.

3.5. S119 is Critical for Mt<sub>b</sub> PafA Depupylase Activity

Recently, Zhang et al. found that Mt<sub>b</sub> PafA also exhibits depupylase activity on pupylated proteins (Zhang et al., 2017). This depupylase activity is also expected to require a Pup-binding pocket to bind the pupylated protein. Thus, we speculated that S119 may also be critical for Mt<sub>b</sub> PafA depupylase activity.

To differentiate depupylation from pupylation, we performed pupylation and depupylation reactions in two different buffer systems (Zhang et al., 2017). The biggest difference between the two reactions is the concentration of PO<sub>4</sub><sup>−</sup> (see Materials and Methods). We examined the depupylation activity of purified Mt<sub>b</sub> PafA variants for pupylated PanB. Consistent with this earlier work, we also found that PafA S119F exhibits almost no depupylation activity while the depupylation activity of PafA S119A is significantly reduced. Next, we examined the depupylation activity of PafA pre-incubated with AEBSF on pupylated PanB. As with pupylation, we observed that AEBSF inhibited depupylation in a dose-dependent manner (Fig. 5b). Thus, S119 also contributes to the depupylation activity of purified Mt<sub>b</sub> PafA.

3.6. S119 is Critical for Function of Mt<sub>b</sub> PafA that Essential for Msm ∆PafA Survival Under Nitrogen Limitation and in Macrophages

To determine whether the defective activity of Mt<sub>b</sub> PafA S119 variants could cause phenotypic changes, Msm ∆PafA strains with different Paf<sub>Amo</sub> variants (Table S2) were tested under nitrogen limitation and also in macrophages. As shown above, the level of pupylation in the Msm ∆PafA strains is due to the introduced pupylase activity of the Paf<sub>Amo</sub> variants (Fig. 3). In addition, it is also known that the PPS plays an important role under nitrogen limitation (Elharar et al., 2014, Fasellaro et al., 2016). Thus, we expected the growth of these strains to be proportional to their pupylase activity under nitrogen limitation. Results demonstrated that this is indeed the case (Fig. 6a). Further, when we complemented WT Paf<sub>Amo</sub> in the Paf<sub>Amo</sub> S119F strain, bacterial colony-forming unit (CFU) numbers recovered to a level similar to that of the WT Paf<sub>Amo</sub> strain. These findings indicate that S119 is critical for the function of Mt<sub>b</sub> PafA, which itself plays an essential role in Msm ∆PafA survival under nitrogen limitation.

Considering that PPS is required for Mt<sub>b</sub> resistance to nitric oxide and is essential for Mt<sub>b</sub> survival in mice, we reasoned that the PPS pathway of Msm may also be required for Msm survival in macrophage cells (Darwin et al., 2003, Lamichehane et al., 2006, Gandotra et al., 2007, Sanamovic et al., 2015). To test this, THP-1 macrophage cells were infected with Msm ∆PafA strains bearing different Paf<sub>Amo</sub> variants (Fig. 6b, Table S2). The survival of Msm strains in these cells was monitored at three time points, namely 12 h, 24 h, and 48 h. Markedly lower survival rates were observed in the Msm ∆PafA/pMV261 and Msm ∆PafA/pMV·Paf<sub>Amo</sub> S119F strains, but not in the Msm ∆PafA/pMV·Paf<sub>Amo</sub> and Msm ∆PafA/pMV·Paf<sub>Amo</sub> S119A strains, compared to the WT Msm strain at all the three time points. In addition, when Paf<sub>Amo</sub> was re-introduced into the Msm ∆PafA and Msm ∆PafA/
**Fig. 5.** S119 is critical for *Mtbc* PaFA depyrophosphorylase activity. (a) PanB-Pup<sup>2</sup> (0.75 μM) were depyrophosphorylased by PaFA variants (1.5 μM each) with 5 mM ADP in phosphate buffer for 6 h at 25 °C. Samples were analyzed by SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining or western blotting with an anti-flag antibody. Quantitation of PanB-Flag based on CBB staining is shown in the lower panel. Data are representative of three independent biological replicates (mean and s.e.m. of 3 samples), *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test). (b) As in Fig. 5a, except that the PaFA (1.5 μM) pre-incubated with AEBSF at 25 °C for 0.5 h was used instead of PaFA variants.

**Fig. 6.** S119 is critical for the function of *Mtbc* PaFA that is essential for *Msm* ΔPaFA survival under nitrogen limitation and in macrophages. (a) Exponentially growing cultures (OD600 = 0.5) of Msm expressing PaFA<sub>Mtb</sub> with different mutations were centrifuged and resuspended in media lacking nitrogen sources. Following resuspension, aliquots were collected at the indicated time points for determination of live cell concentration (n = 3). (b) Survival of Msm expressing PaFA<sub>Mtb</sub> with different mutations in primary human monocyte-derived macrophages infected for 0–48 h. Following macrophage disruption, aliquots were collected at the indicated time points for determination of live Msm concentration (n = 3). *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test).
pMV- PafA<sub>Mtb</sub> S119F strains, these strains recovered their survival ability to a level similar to that of WT Msm.

We noted that under “Nitrogen limitation” conditions, the bacteria grew ~2 logs more after 3 days, while in THP-1 cells, most bacterial strains died by 48 h. We also noted that Msm grew much slower under the “Nitrogen limitation” condition than under normal conditions, and our results were consistent with others (Elharar et al., 2014, Fancellaro et al., 2016). We used a “Nitrogen limitation” condition to partially mimic the stress environment that Mtb faces in macrophage cells. However, actual pressures inside macrophages are much more complicated, and may arise from reactive nitrogen intermediates, reactive oxygen intermediates, nutrient restriction and other factors. The difference in Msm survival and growth between the “Nitrogen limitation” condition and macrophage cells may be due to the different stresses that the bacteria are facing.

Thus, we conclude that S119 is critical for the function of Mtb PafA which is itself essential for Msm ∆PafA survival in macrophages.

4. Discussion

In this study, we identified Mtb PafA S119 as an effective inhibitor binding site using the serine protease inhibitor, AEBSF. We found that purified PafA variants of S119 mutated to aromatic amino acids, mimicking AEBSF-binding, present no or only very low catalytic activity in papulysin and depapulysin reactions. Furthermore, Mtb PafA S119 variants with defective enzymatic activity could not compensate for the significant growth defects of Msm ∆PafA under nitrogen limitation and in macrophages.

Guided by the PafA<sub>Mtb</sub> crystal structure (Özcelik et al., 2012, Barandun et al., 2013), we found that Serine 126 of PafA<sub>gst</sub>, the homologous residue of PafA<sub>Mtb</sub> S119, is a highly conserved residue that is located close to the groove that binds the Pup C-terminal. MD simulations of PafA mutants or with AEBSF covalently attached to PafA clearly revealed disruption of this binding groove and a weakening of the interaction with both the C-terminal tail and α-helices of Pup. These predictions were verified using BLI, which showed that Pup-binding of PafA S119 variants or of PafA co-incubated with AEBSF was significantly reduced compared to that of WT PafA. These results are also consistent with the clearly defective papulysin of lysine, whose binding does not involve substantial contact with PafA (Regev et al., 2016), by PafA S119 variants or PafA co-incubated with AEBSF, as compared to that of WT PafA. Thus, although we cannot completely rule out that the reduced papulysin may result from a catalytic defect, our data strongly support the proposal that the inhibitory effect of the interference of the PafA S119-pocket, a non-active site location, is due to a reduced binding of Pup. There are a number of advantages for non-active site-based inhibitor design. One main advantage is avoidance of direct binding competition with substrates of high affinity which often result in off-target effects (Nussinov and Tsai, 2013). In this respect, we suggest that attempts to interfere with the PafA S119-pocket should be an attractive approach for acquiring a highly efficient inhibitor for PafA.

Recently, Darwin et al. found that PafA<sub>Mtb</sub> possesses depapulysin activity in vitro in addition to its papulysin activity (Zhang et al., 2017). Here, we propose that the Pup binding pocket participates in bifunctional catalytic activities. Considering our findings and the structure base of PafA S119, we speculate that S119 is also critical for the depapulysin activity of Mtb PafA. Indeed, PafA S119F almost completely lost depapulysin activity and PafA S119A lost the majority of its depapulysin activity. As the crystal structure of PafA<sub>Mtb</sub> is not yet available, our findings may pave the way to reveal the depapulysin mechanism of PafA<sub>Mtb</sub>.

MS analysis showed that AEBSF reacts with the hydroxy group of the Mtb PafA S119 residue to form a sulfonyl derivative. However, as AEBSF is a general serine protease inhibitor and may bind to many proteins, it can not be treated as an anti-Mtb lead compound and tested in vivo directly. Potential strategies could be applied to enhance the selectivity and sensitivity of AEBSF for PafA, for example, by modifying the reactive side chain but retaining the sulfonyl fluoride of AEBSF. There are many successful cases of other inhibitor/enzyme complexes that have followed this strategy, for example, the enhancement of the specificity of the inhibitor for an intracellular lipid binding protein (Chen et al., 2016). Alternatively, since PafA does not have a canonical serine protease active site, it should be possible to screen or rationally design molecules that would interact with S119 but not conventional serine proteases. Such site-specific inhibitor design has been proven to be a successful strategy (Ostrem et al., 2013, Ostrem and Shokat, 2016, Dawidowski et al., 2017), including in the development of mutant-specific RAS inhibitors (Stephen et al., 2014, Ostrem and Shokat, 2016, Janes et al., 2018). In this case, to discriminate the inhibition of both the WT and the mutant KRAS, Shokat et al. developed a series of site-based inhibitors that covalently attach to the mutant cysteine residue of KARS (Ostrem et al., 2013). The cysteine acting as a nucleophile to form a covalent bond with the inhibitors is reminiscent of the interaction of AEBSF with serine observed here in PafA.

Most of the assays performed in this study were at the biochemical or bacteria cell levels. To strengthen the clinical significance of the major finding that S119 is critical for Mtb PafA, it will be important in future to carry out some in vivo assays, especially with the Mtb PafA mutants, using pathogenic Mtb strains. Since PafA (especially PafA S119) is highly conserved between Mtb and Msm, it is reasonable to argue that what we observed using Msm will be similar in Mtb. The role of PafA in Mtb virulence has already been demonstrated in vitro and in vivo (Darwin et al., 2003, Festa et al., 2007, Samanovic et al., 2015). When Mtb PafA activity is significantly disrupted by the interruption targeting S119 location, it is anticipated that the virulence of Mtb will be reduced dramatically in vivo. In addition, in an on-going study based on the findings of this study, we have already identified several compounds using a computer assisted drug screening strategy that targets the S119 location, which show ~20 µM efficacy on BCG and H37Ra (data not shown). To further strengthen the clinical potential of our findings, the best way is to screen compounds targeting PafA S119 and validate the compounds that we have already identified in vivo using a pathogenic Mtb strain, or even MDR/XDR strains. However, performing such studies are not currently feasible and out of the scope of the present study.

Taken together, our results demonstrate that S119 could serve as a promising precise target for developing inhibitors of M. tuberculosis PafA. Both papulysin and depapulysin activities of purified PafA can be significantly inhibited upon modification of S119, and these modifications profoundly affect the growth of the bacteria under conditions important for its survival. Our results provide clear direction for developing inhibitors of Mtb PafA, thus holding promise for developing new compounds for combating Mtb.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

S.C.T. conceived the idea. H.W.J. performed enzyme activity assays and functional analysis. X.D.W., H.N.Z. and T.W. performed protein...

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References


