**Mycobacterium tuberculosis** Prokaryotic Ubiquitin-like Protein-deconjugating Enzyme Is an Unusual Aspartate Amidase*"‡"§

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**Background:** Dop is critical for the full virulence of Mycobacterium tuberculosis; however, its mechanism is not understood.

**Results:** Asp-95 was identified as a catalytically significant residue.

**Conclusion:** This work suggests that Asp-95 functions either as a direct nucleophile forming a unique anhydride intermediate or is part of a catalytic center that includes polarized water as the nucleophile.

**Significance:** Understanding the mechanism of Dop can help guide the design and selection of inhibitors.

Deamidase of Pup (Dop), the prokaryotic ubiquitin-like protein (Pup)-deconjugating enzyme, is critical for the full virulence of Mycobacterium tuberculosis and is unique to bacteria, providing an ideal target for the development of selective chemotherapies. We used a combination of genetics and chemical biology to characterize the mechanism of depylation. We identified an aspartate as a potential nucleophile in the active site of Dop, suggesting a novel protease activity to target for inhibitor development.

Proteasomes are protein complexes that degrade proteins that the cell has marked for destruction. The prokaryotic ubiquitin-like protein (Pup)-deconjugating enzyme, present in Actinobacteria and Nitrospira, is critical for the virulence of Mycobacterium tuberculosis, the causative agent of tuberculosis and one of the world’s deadliest pathogens. In this pathway, the protein Pup post-translationally modifies proteins for proteolysis by a bacterial proteasome complex in a manner analogous to the ubiquitin-proteasome system in eukaryotes (see Fig. 1A) (1). Pup is activated by the enzyme deamidase of Pup (Dop), which deamidates the C-terminal glutamine of Pup (PupGln) to form glutamate (PupGlu). Proteasome accessory factor A (PafA), the Pup ligase, subsequently ligates the newly formed side chain carboxylate to a lysine residue of the target protein (2). Pupylated proteins are guided into the proteasome through the binding of Pup to Mycobacterium proteasomal ATPase, which unfolds proteins prior to delivery into the proteasome core composed of 14 α- and 14 β-subunits (3–6). Dop also functions as a depyrophosphatase to remove Pup from substrate proteins prior to proteasomal destruction (7–9). These six proteins are the minimal requirement for a functional Pup-proteasome pathway in mycobacteria (5, 10).

Despite the functional homology between the ubiquitin-proteasome system and the Pup-proteasome pathway, similarity at the protein level is limited. Most notably, Dop and PafA, which are similar to each other, have no sequence or structural homologues within the ubiquitin-proteasome system. Reports have highlighted the similarity of Dop and PafA to proteins of the glutamine synthetase-fold superfamily (9, 11), including Escherichia coli YbdK, a γ-glutamyl-cysteine synthetase. Mutagenesis and biochemical analyses demonstrated that PafA follows this γ-glutamyl-cysteine synthetase model, where the C-terminal γ-carboxylate of PupGlu is activated through phosphorylation by ATP and subsequently ligated to the ε-amino group of lysine residues on target proteins (12). Despite the predicted structural homology to the glutamine synthetase/γ-glutamyl-cysteine synthetase-fold superfamily of proteins and to PafA, the mechanism of the Dop amidase activity remains unclear. Unlike PafA and other glutamine synthetase-fold proteins, Dop requires ATP binding, but not hydrolysis, suggesting that ATP is a co-factor (2, 7, 8). Additionally, protease inhibitors such as PMSF or iodoacetamide did not inhibit Dop (supplemental Fig. S1). Based on a structural model of Dop, we identified several residues that are critical for Dop activity (9). Although the model provided some insight into the active site of Dop, no obvious catalytic motif emerged.

Because Dop is critical for the full virulence of M. tuberculosis in vivo, and it is unique to bacteria, it provides a potentially...
ideal target for the development of selective chemotherapies against *M. tuberculosis*. Understanding how Dop cleaves the amide bond at the C terminus of Pup is necessary for the development and optimization of inhibitors to be used as drug candidates. Here, we have elucidated the mechanism of Dop activity using a substrate analogue trap along with genetic and chemical biology approaches.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Bacterial strains and plasmids used in this study are listed in supplemental Table S1. E. coli strains used for cloning and expression were grown in LB broth (Difco) or LB agar at 37 °C. *Mycobacterium smegmatis* strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80. Cultures were grown at 37 °C with aeration on an orbital shaker. *M. tuberculosis* cultures were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, 0.5% bovine serum albumin, 0.2% dextrose, and 0.085% sodium chloride. *M. tuberculosis* cultures were grown without shaking in 75-cm² vented flasks (Corning, Tewksbury MA) at 37 °C. Mycobacteria were transformed as described elsewhere (13). Antibiotics were used at the following concentrations: hygromycin, 150 μg/ml (*E. coli*), 50 μg/ml (*M. smegmatis* and *M. tuberculosis*); kanamycin, 25 μg/ml (*M. tuberculosis*).

Plasmids and primers used in this study are listed in supplemental Table S1. *M. tuberculosis* dop-his₆ was used for *in vitro* depupylylase assays and trapping assay. Pup⁻Ino⁻1 was purified as described previously (14). pMV-dopE10A and pMV-dopD95N complementation plasmids were described previously (9), and the mutations were constructed similarly by sewing overlap extension primers and using pET24b(+) as a template to form pET24b(+)his₆-pup. Dop was amplified using primers Nde-HA-Pup-f and pup-DGQ-r. pTYB2-pupDGln was generated by ligation of the fragment with pTYB2. pOLYG-Ms mdop-hispup was used as described previously (8). All plasmids were sequenced by GENEWIZ, Inc. (South Plainfield, NJ).

**Immunoblotting**—Total cell lysates were prepared as described elsewhere in detail (8). Samples were separated by SDS-PAGE. HA antibodies (Sigma-Aldrich), Pup monoclonal (mouse) and polyclonal (rabbit) antibodies, and Dop antibodies are described elsewhere (1, 8). Horseradish peroxidase-coupled anti-rabbit or anti-mouse secondary antibodies were used according to the manufacturer’s instructions (GE Healthcare). Detection of horseradish peroxidase was performed using either SuperSignal West Pico or West Femto Chemiluminescent Substrate (Thermo Scientific).

**Dop and Pup—Ino1 Purification—Dop-His₆ and Pup—Ino1-His₆ were purified essentially as described previously (8), except we used a *M. smegmatis* strain with a C-terminal deletion in GroEL1 (15). This deletion removes a polyhistidine sequence in GroEL1, which eliminates co-purification of GroEL1 with target proteins.

*M. tuberculosis* Lysate Preparation for Trapping Reactions—*M. tuberculosis* was grown to an *A₅₈₀* of 1 after which 50 *A₅₈₀* equivalent cell numbers were harvested and washed with 25 ml of 0.05% Tween 80 in PBS. The cells were resuspended in 1 ml of 50 mM Tris, pH 8, 50 mM NaCl and transferred to bead-beating tubes with 250 μl of zirconia silica beads. Cells were lysed by bead beating three times for 30 s. Lysates were filtered through 0.45-μm filters, glycerol was added to 12% final, and the samples were stored at −20 °C until further use.

**HA-Pup-6-diazo-5-oxo-L-norleucine (DON) Formation, Trapping Reactions, and Mass Spectrometry Analysis**—To produce HA-Pup-intein, 6 liters of LB was inoculated with 20 ml of an overnight culture of EHD853 and was grown at 37 °C until *A₅₆₄* = 0.49. The temperature was reduced to 18 °C before inducing with 0.5 mM isopropyl-β-D-galactopyranoside and was grown overnight at 18 °C. The cells were harvested and resuspended in 350 ml of lysis buffer (50 mM MOPS, pH 6.5, 500 mM NaCl, 100 mM sodium acetate, 1 mM PMSF, and 2.5 μg/ml DNase), homogenized (15 min), and microfluidized three times. The soluble fraction was collected by centrifugation at 4 °C, and 0.1% Triton X-100 was added to the clarified lysate, followed by stirring for 60 min at 4 °C. The sample was centrifuged at 4 °C for 30 min and filtered through a 0.45-μm filter. 50-ml chitin resin (New England Biolabs, Ipswich, MA) was equilibrated in binding buffer (50 mM MOPS, pH 6.5, 500 mM NaCl, 100 mM sodium acetate, and 0.1% Triton X-100), and the soluble lysate was applied slowly and allowed to stir for 60 min at 4 °C. The resin-lysate mixture was applied to a gravity-flow column. The column was washed with 4 column volumes of binding buffer, 2 column volumes of wash buffer II (50 mM MOPS, pH 6.5, 150 mM NaCl, and 100 mM sodium acetate), and 2 column volumes of wash buffer III (50 mM MOPS, pH 6.5, and 100 mM sodium acetate). Following the washes, 75 ml of MeSNa buffer (50 mM MOPS, pH 6.5, 100 mM sodium acetate, and 100 mM 2-mercaptoethane sulfonate) (Fluka) was added, sealed with Parafilm, and incubated overnight at 37 °C, after which 100 ml was eluted from the column. Wash buffer III (50 ml) was applied to the column and collected, and this was repeated two more times for a total elution of 250 ml. The sample was concentrated to 10 ml using a 3,000-molecular weight cut-off membrane. Eluate was centrifuged, and supernatant was filtered through a 0.45-μm filter. The yield was about 1.8 mg/liter, and ~80% of intact HA-Pup-MeSNa resulted, with the major contaminant being hydrolyzed HA-Pup.

Eluted HA-Pup-MeSNa (75 μM in 50 mM MOPS, pH 6.5, 100 mM sodium acetate, and 150 mM NaCl) was combined with 25 mM DON (Sigma) and 25 mM *N*-hydroxysuccinimide (Fluka) in 225 mM HEPES, pH 8.5, at room temperature for 18 h. The sample was extensively diazylated by 50 mM MOPS, pH 6.5, 100 mM sodium acetate, and 150 mM NaCl. The product was diluted, and mass spectrometry analysis revealed labeled product HA-Pup-DON formed at ~20% yield (~15 μM HA-Pup-DON per reaction).

For trapping reactions with *M. tuberculosis* lysates, 42 μl of lysate, 6 μl of HA-Pup-DON (~15 μM), 4.8 mM ATP, 5 mM MgCl₂, 1.2 mM DTT, and 50 mM NaCl in 50 mM Tris, pH 8 were mixed in a final volume of 50 μl at room temperature. At 2 h, SDS loading buffer was added, and samples were analyzed by 9% SDS-PAGE. For purified Dop trapping assays, 2.1 μg of *M. tuberculosis* Dop-His₆ or 7 μg of *M. smegmatis* Dop-His₆-
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Pup, 3 μl of HA-Pup-DON (~15 μm), 2 mM ATP, 3 mM MgCl₂, 1 mM DTT, and 50 mM NaCl in 50 mM Tris, pH 8, were mixed in a final volume of 25 μl at room temperature. At 2 h, SDS loading buffer was added, and samples were analyzed by 12% SDS-PAGE.

For immunoprecipitation of the HA-Pup-DON-Dop complex, 75 μg of M. smegmatis Dop-His₆-Pup, 60 μg of HA-Pup-DON (~15 μm), 2 mM ATP, and 10 mM MgCl₂ in 50 mM Tris, pH 8, were added in a final volume of 500 μl at room temperature. At 2 h, 1 ml of HA-agarose (Sigma-Aldrich), prewashed in NET buffer (5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40 in 50 mM Tris, pH 8), was added, and the sample was brought to 10 ml with NET buffer. The sample was incubated overnight at 4°C, washed three times with 10 ml of NET buffer, and eluted with the addition of SDS loading buffer and boiling. The sample was reduced with DTT (5 mM, 56°C, 30 min), and the resulting free sulfhydryl groups alkylated with iodoacetamide (25 mM, 25°C, 45 min) in the dark, quenched with DTT (10 mM), and then run on 4–12% SDS-PAGE gels, excised, in-gel digested with trypsin, and analyzed using LC-MS/MS using higher energy C-trap dissociation (HCD) fragmentation. To investigate the presence of possible PTMs, a large precursor mass tolerance search window of 300 Da (compared with the standard 50-ppm search window) was used to include hits from unknown PTMs. Possible PTM masses were identified by comparing the theoretical mass of high scoring peptides with the observed mass. Following identification of possible PTM masses, searches with a smaller precursor mass tolerance (50 ppm) were performed for the PTMs on the amino acids aspartate, glutamate, serine, threonine, and tyrosine that could act as nucleophiles. High resolution MS/MS spectra were additionally inspected by eye to assess the quality and correct assignment of the possible PTMs.

H₂¹⁸O Reactions and Mass Spectrometry Analysis—For ¹⁸O-water assays, reactions contained 4 μg of M. smegmatis Dop-His₆-Pup, 22.5 μg of Pup~Ino₁, 2.5 mM ATP, 20 mM MgCl₂, 1 mM DTT, and 50 mM NaCl in 50 mM Tris, pH 8, in a final volume of 250 μl, containing (or not for control) 50% ¹⁸O-water (Cambridge Isotope Laboratories, Andover, MA). After 3 h at 37°C, 1 ml of cold acetone was added, and the sample was allowed to precipitate overnight at ~20°C. Protein was collected by centrifugation for 15 min in the cold, the pellet was air dried, SDS loading buffer was added, and the samples were analyzed by 15% SDS-PAGE.

For mass spectrometric analysis, Pup and Dop bands were excised from the gel and cut into cubes of ~1 mm and transferred into 1.5-ml Eppendorf tubes. In-gel digestion was done as described previously except 100 ng of endoproteinase Asp-N was used for the digestion of the proteins in each band instead of trypsin. After digestion overnight, peptides were purified using Stage Tips (17), dried by vacuum centrifugation, and resuspended in 8 μl of 5% formic acid in glass inserts. 4 μl of each reaction was shot on an LTQ Orbitrap Elite mass spectrometer coupled to an Agilent 1200 series binary pump (Thermo Fisher Scientific). The peptides were loaded onto a hand-pulled fused silica microcapillary (125 μm × 15 cm, packed with Magic C18AQ, Michrom Bioreources, Auburn, CA) using a Famos autosampler (LC Packings, San Francisco, CA). Once loaded, the peptides were separated across a 23-min linear gradient of 6–33% solvent B (0.15% formic acid and 100% acetonitrile). Solvent A comprised 0.15% formic acid and 5% acetonitrile. Data were collected in a data-dependent mode using the TOP20 strategy (17). In each cycle, one full high resolution MS scan (resolution: 60,000) in the Orbitrap was followed by up to 20 MS/MS scans in the LTQ for the most intense ions at a 10⁶ Automatic Gain Control target for full MS and a 2 × 10⁶ AGC target for MS/MS, with a 500-ppm minimum signal threshold and an isolation width of 2 Da. Dynamic exclusion of selected ions was set to 20 s (±10 ppm relative to the precursor ion m/z), and ions assigned a charge state of 1⁺ or those of unassigned charge were rejected. Full MS spectra were collected from 300 to 1,500 m/z. The maximum ion accumulation times were set to 1,000 ms and 150 ms for full MS and MS/MS scans, respectively. The normalized collision energy was set to 35% and activation time to 10 ms. Collision-induced dissociation was used for fragmentation. The data were collected in centroid mode.

RAW files were converted to mzXML files using the program ReAdW. MS/MS spectra were searched using the SEQUEST search algorithm (version 28) from the TB Database project in September 2010 using a mass tolerance of 2 Da. The search parameters for post-translational modifications included dynamic modifications of 15.99 Da on methionine (oxidation) and glutamine as well as deamidation on glutamine of 0.98 Da. Protein hits were filtered at the peptide and protein level to contain <1% false positives, estimated by the number of decoy hits using in-house software using linear discriminate analysis based on Xcorr, ΔCn, precursor mass error and charge state, as described previously (18).
RESULTS

Pup-DON Trap Reveals an Aspartate as a Potential Dop Nucleophile—In the absence of a conserved catalytic motif in Dop, we used an approach similar to that used in the ubiquitin field to identify deubiquitinases (19, 20). Previous studies used intein-based chemistry to add electrophilic moieties such as vinyl methyl ester to the C terminus of ubiquitin to covalently trap nucleophilic cysteines in deubiquitinases. Because Dop has glutamine deamidase activity, we modified Pup with DON, a glutamine mimic produced by *Streptomyces* (21) that has been used to identify nucleophilic residues in glutamine-hydrolyzing enzymes (22, 23). DON did not inhibit Dop-dependent amido-hydrolysis in vitro (supplemental Fig. S2A); however, because Dop binds to Pup tightly (8), we attached DON to the C terminus of Pup to help deliver it to the active site. HA-tagged Pup, lacking its C-terminal glutamine, was produced as an intein-chitin-binding domain fusion protein, purified with chitin resin, and eluted with MeSNa to form HA-Pup-DON. C, Dop immunoblot (IB) of the reaction with HA-Pup-DON and lysates of *M. tuberculosis* WT, dop, and dop-complemented strains. All strains contain the integrative plasmid pMV306 (empty vector) or pMV306 with WT dop or dopE10A at the attB site of the chromosome. In C and F, the asterisk indicates a species only seen in the dop:*Mycobacterium MarT7* mutant and is predicted to be truncated Dop. Samples were separated on a 9% SDS-PAGE gel. D, HA-Pup-DON reaction with purified Dop. Slowed migration of *Mycobacterium smegmatis* Dop (left) and *Mycobacterium tuberculosis* Dop (right) only occurred in the presence of HA-Pup-DON and ATP. Samples were separated on a 12% SDS-PAGE gel. Arrowhead indicates HA-Pup-DON—Dop. The faster migrating species is specific to the Pup-DON trap as it was recognized alone by antibodies to the HA epitope (center lane). E, high resolution tandem mass spectrometry analysis of the HA-immunoprecipitated HA-Pup-DON—Dop species indicating a modification of mass 257.1 Da on Asp-95 of Dop. F, Dop immunoblot of the reaction with HA-Pup-DON trap and lysates of *M. tuberculosis* WT, dop, and dop-complemented strains. Samples were separated on a 10% SDS-PAGE gel. G, proposed mechanism of HA-Pup-DON and Dop conjugation reaction. HA-Pup-DON is protonated in the active site of Dop, followed by a nucleophilic attack to displace nitrogen, and this results in the covalent HA-Pup-DON—Dop adduct.
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To identify the residue that was labeled with HA-Pup-DON, we purified the HA-Pup-DON-Dop complex for analysis by MS. A PTM with the mass of 257.1 Da (corresponding to C$_{10}$H$_{15}$N$_{3}$O$_{5}$) was identified attached to Asp-95 (Fig. 1F). Among several residues in the proposed active site of Dop, Asp-95 is needed for Dop function in vivo (9), and mutation of this residue to asparagine abolished labeling by HA-Pup-DON (Fig. 1F). Following Dop-catalyzed protonation of the diazoketone moiety, we propose that Asp-95 acts as a nucleophile and displaces nitrogen, resulting in the HA-Pup-DON-Dop complex (Fig. 1G).

Site-directed Mutagenesis Eliminates Other Potential Nucleophiles—To gain further insight into the mechanism and potential role of Asp-95 as a nucleophile, we revisited the Dop active site model, which was reported previously (9). This model is based on limited structural homology to YbdK, a glutamine synthetase-fold enzyme, and accounts for residues 5–273 of M. tuberculosis Dop, including several important catalytic residues (9, 24). In the model, lysine is conjugated to the C-terminal glutamate of Pup via an isopeptide bond, reminiscent of the native Pup—protein conjugate (Fig. 2A). Pup is anchored in the active site by Arg-206, and an analogous arginine is present in YbdK and in the PafA model for anchoring of the substrate carboxylate (9). According to the model, Asp-95 is well positioned for nucleophilic attack on the Pup—Lys conjugate; however, to rule out the possibility that Dop uses another nucleophile for catalysis and a side reaction with HA-Pup-DON caused labeling of Asp-95, we mutated all conserved cysteines, serines, and threonines residues in Dop. When we compared M. tuberculosis Dop with PafA and Dop homologues from nine bacterial species, we found no conserved cysteines, four conserved serines (Ser-27, Ser-102, Ser-204, and Ser-295), and two conserved threonines (Thr-218 and Thr-262) (supplemental Fig. S3). Mutagenesis of the serines to alanines did not affect pupylation in M. tuberculosis (Fig. 2B). Mutagenesis of Dop Thr-262 reduced, but did not abolish, pupylation in vivo (Fig. 2B, compare first and last lanes). Although DopT$_{262A}$ stability was not affected, it is possible that Dop binding to certain substrates or Pup was impacted. Mutagenesis of Thr-218 resulted in normal pupylation (Fig. 2B). Collectively, these data suggest that neither serine nor threonine is critical for catalysis (Fig. 2B).

Evidence of an Anhydride Intermediate—Unable to pinpoint an alternative explanation for the labeling of Asp-95, we sought to gather more evidence of Asp-95 as a nucleophile. If Dop uses Asp-95 as a nucleophile, an anhydride intermediate would form; resolution of this intermediate with water hydrolysis yields the products. In $^{18}$O-water, an anhydride intermediate allows for a 50% chance that $^{18}$O-label will incorporate into Dop when the reaction proceeds. When Dop was incubated with the model pupylated substrate Pup—Ino1 (inositol-1-phosphate synthetase) in the presence of $^{18}$O-water, we observed $^{18}$O-water incorporation into the C terminus of Pup and not Dop (Fig. 3, A and B, and supplemental Fig. S4). Additionally, we did not observe double $^{18}$O-incorporation into Pup, suggesting that Dop does not perform futile rounds of hydrolysis. The $^{18}$O-labeling results neither supported nor refuted the carboxylate nucleophile mechanism.

Another method used to identify an anhydride intermediate is to trap it with a potent nucleophile. Hydroxylamine (NH$_{2}$OH) has been used successfully to trap acyl-enzyme intermediates (26–30), thus we decided to monitor the Dop-catalyzed depupylation reaction in the presence of hydroxylamine. Upon addition of hydroxylamine to the depupylation reaction with the substrate Pup—Ino1, we observed the formation of two species of Pup by Coomassie Brilliant Blue staining and immu-
nblotting with antibodies specific to Pup (Fig. 3C, center lanes). The slower migrating species depended on ATP, Dop, and Pup−Ino1, as preincubation of hydroxylamine with Dop or Pup−Ino1, followed by removal of hydroxylamine, did not produce this species (supplemental Fig. S5). Additionally, the slower migrating Pup species did not form upon addition of hydroxylamine to the gel above. Mow, Pup IB of the two species formed upon addition of hydroxylamine is shown. D, Pup after incubation with hydroxylamine. Chitin-binding domain is stained with 16% SDS-PAGE of the Pup−Ino1 depupylation reaction: (left) in the absence of hydroxylamine; (center) in the presence of hydroxylamine; and (right) in the presence of hydroxylamine without ATP. Middle panel, enhanced image of the 10–12-kDa region of the gel above. Bottom, Pup IB of the two species formed upon addition of hydroxylamine is shown. D, Dop reaction with hydroxylamine. Chitin-binding domain is stained with 16% SDS-PAGE of Pup reaction with Dop: (left) in the absence of hydroxylamine; (center) in the presence of hydroxylamine; and (right) in the presence of hydroxylamine without Dop. Note that this panel was enhanced to view all possible Pup species. E, Pup-hydroxymate (Pup-NHOH). LC chromatogram of the Pup 1 and Pup 2 species (labeled in C) is shown. Inset, presumed structure of Pup-NHOH. F, proposed mechanism of the Dop-catalyzed deconjugation reactions. The aspartate nucleophile attacks the Pup−substrate amide, forming an anhydride intermediate. Base-catalyzed deprotonation of a water molecule activates it to attack at the Pup side of the anhydride to form the products.

**DISCUSSION**

Collectively, our data suggest that Dop uses Asp-95 as a nucleophile. Whereas aspartate and glutamate are nucleophiles for glycosidases, dehalogenases, and phosphatases (31–33), to our knowledge, no known amidase uses aspartate as a nucleophile. There was controversy over whether an anhydride intermediate was formed with carboxypeptidase (34–39); at low temperatures and with various substrate analogues, an anhydride was detected (34, 36, 39). However, this intermediate has not been trapped, and other evidence suggests carboxypeptidase acts as a metalloprotease. For Dop, we used an electrophilic trap that reacted with Asp-95, we used site-directed mutagenesis to eliminate other potential nucleophiles, and we
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provided support for a nucleophilic mechanism by resolving the proposed anhydride intermediate using hydroxylamine and visualizing Pup-hydroxamate.

Although it is possible that hydroxylamine acted as an alternate nucleophile in place of water for the direct attack of substrate and because we have yet to detect directly the proposed anhydride intermediate, we cannot rule out that Dop catalysis proceeds by a different mechanism. For example, it is possible that His-96, which is critical for Dop activity in vivo (9), is conserved in all Dop orthologues and is absent from PafA and YbdK, could be important for positioning Asp-95 in Dop for base-catalyzed deprotonation of water (Fig. 2A). This could also explain the labeling of Asp-95 with the HA-Pup-DON trap (Fig. 1). Furthermore, although Dop does not have characteristics typical of aspartate proteases, which use two aspartates to deprotonate water and typically have acidic pH optimum, the Dop model suggests a resemblance to aspartate proteases. According to the model, we could envision Asp-95 and Glu-10 acting similarly to an aspartate dyad to activate water for attack of the Pup-lysine conjugate. Glu-10 is critical for the activity of Dop and its counterparts (nonproteolytic PafA and glutamine synthetase/γ-glutamyl-cysteine synthetase proteins) in vivo and in vitro (8, 9, 25); however, Glu-10 is predicted to coordinate Mg$^{2+}$ and ATP and not play a direct role in catalysis (11). Finally, we cannot rule out the possibility that Dop uses a different residue as a nucleophile, one that we did not test. These include lysine or the terminal amino group.

Based on our data, however, we propose that Asp-95 attacks the γ-carbonyl side chain amide bond at the C terminus of Pup$_{Glu}$ or Pup attached to a substrate to form an anhydride intermediate (Fig. 3F). In the second step of the reaction, a base in Dop would activate water for attack at the Pup side of the anhydride, resolving the intermediate to form Pup$_{Glu}$ and, in the case of the depupylation reaction, the unmodified substrate.

Dop has at least two roles in the Pup-proteasome pathway: (a) activator of Pup by deamidation and (b) recycler of Pup by depupylation. The function of Dop may not be limited to the Pup-proteasome pathway; as is the case with several deubiquitinasers of the ubiquitin-proteasome system, Dop could serve a regulatory function within the cell, dictating protein localization or activity (40). Therefore, targeting Dop with small molecule inhibitors could interfere with a wide range of biochemical pathways in M. tuberculosis, and it is our hope that such inhibitors will provide a basis for the development of novel antituberculosis compounds. To this end, we have developed a highly specific assay reagent to monitor Dop activity in a high throughput manner (41), and the data presented here will serve as a guide for the screening of inhibitors using this assay reagent.

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