Depupylase Dop Requires Inorganic Phosphate in the Active Site for Catalysis*

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Analogous to eukaryotic ubiquitination, proteins in actinobacteria can be post-translationally modified in a process referred to as pupylation, the covalent attachment of prokaryotic ubiquitin-like protein Pup to lysine side chains of the target protein via an isopeptide bond. As in eukaryotes, an opposing activity counteracts the modification by specific cleavage of the isopeptide bond formed with Pup. However, the enzymes involved in pupylation and depupylation have evolved independently of ubiquitination and are related to the family of ATP-binding and hydrolyzing carboxylate-amine ligases of the glutamine synthetase type. Furthermore, the Pup ligase PafA and the depupylase Dop share close structural and sequence homology and have a common evolutionary history despite catalyzing opposing reactions. Here, we investigate the role played by the nucleotide in the active site of the depupylase Dop using a combination of biochemical experiments and X-ray crystallographic studies. We show that, although Dop does not turn over ATP stoichiometrically with substrate, the active site nucleotide species in Dop is ADP and inorganic phosphate rather than ATP, and that non-hydrolyzable analogs of ATP cannot support the enzymatic reaction. This finding suggests that the catalytic mechanism is more similar to the mechanism of the ligase PafA than previously thought and likely involves the transient formation of a phosphorylated Pup-intermediate. Evidence is presented for a mechanism where the inorganic phosphate acts as the nucleophilic species in amide bond cleavage and implications for Dop function are discussed.

In pupylation, proteins are marked by the post-translational modification of lysine side chains with the small, monomeric prokaryotic ubiquitin-like protein (Pup)2 (1–4). Bacterial pupylation shows many functional parallels to eukaryotic ubiquitination, for example, the employment of a macromolecular tag, the nature of the generated covalent linkage, and the role played as an important recognition element in a protein degradation pathway involving a proteasome complex. However, bacteria have evolved this functionally analogous system independently, and the enzymes involved in pupylation and depupylation are not related to ubiquitination or deubiquitination systems but rather belong to the superfamily of carboxylate-amine/ammonia ligases (5–7).

Ligation of Pup to target proteins is catalyzed by the enzyme PafA (proteasome accessory factor A) and results in the formation of an isopeptide bond between the side chain carboxylate of the C-terminal glutamate of Pup and the ε-amino group of a lysine residue in the target protein (5, 8). In accordance with the energy requirement of isopeptide bond formation the ligation process requires the turnover of ATP. The structure of PafA shows that it has a similar active site arrangement as glutamine synthetase (GS), consisting of a curved anti-parallel β-sheet with ATP bound at one end of the β-sheet cradle and the triphosphate chain running along the strands toward the opposite side of the sheet, where the glutamate residue of Pup is bound (9, 10). The γ-carboxylate of the C-terminal glutamate of Pup binds in close proximity to the γ-phosphate, allowing an attack of the glutamyl γ-carboxylate oxygen on the γ-phosphate of ATP to cleave off ADP and generate the γ-glutamyl phosphate-mixed anhydride intermediate of Pup (11). This phospho-Pup intermediate is activated for the nucleophilic attack of the ε-amino group of the target lysine in the next step, which then leads to formation of the isopeptide bond. This reaction is chemically similar to the activation of the glutamate side chain for the attack of ammonia in GS. However, whereas bacterial GS is an oligomeric assembly (a double ring of hexamers) with the active sites buried in deep pockets at the intra-ring subunit interfaces (12), the Pup ligase PafA is active as a monomer and features a broad, easily accessible active site (9, 10).

In mycobacteria and several other actinobacteria, Pup is encoded with a C-terminal glutamine instead of glutamate, necessitating deamidation of the C-terminal glutamine to glutamate before ligation to a target is possible. This activity is carried out by Dop (deamidase of Pup), which is structurally highly similar to the ligase PafA and is also encoded in the pupylation gene locus (5, 10, 13). Intriguingly, Dop also opposes the ligase activity by catalyzing the specific cleavage of the isopeptide bond formed between Pup and the protein (14, 15).

Thus, at least in mycobacteria, Dop is involved in both the pupylation and depupylation of protein substrates, suggesting...
Results

Non-hydrolyzable ATP Analogs Cannot Support Dop Activity—It was shown earlier that Dop does not hydrolyze ATP stoichiometrically during the reaction progress of deamidation (5) and depupylation (15). In addition it was found that Dop shows only minimal deamidase activity in the presence of the non-hydrolyzable ATP analog AMP-PCP (5). To better understand the role of the nucleotide for the catalytic activity of Dop we analyzed the depupylation reaction of the known pupylation substrate PanB (ketopantoate hydroxymethyltransferase) in the presence of different nucleotides. The enzymatic removal of Pup from PanB-Pup was probed by pulling aliquots along the depupylation time course and quenching them with SDS-loading dye to stop the reaction. Aliquots from the respective time points were subjected to SDS-PAGE followed by Coomassie staining (Fig. 1A). In addition, the gels were densitometrically evaluated for a more quantitative assessment (Fig. 1B). In the presence of ATP, all PanB-Pup is converted to PanB within 3 min under the conditions used, whereas in the presence of the non-hydrolyzable analog AMP-PCP even after 30 min no PanB-Pup has been depupylated. This is a curious observation, considering that ATP is not turned over stoichiometrically during the reaction.

One possible explanation could be that ATP cleavage, although not accompanying substrate turnover, is nevertheless required to produce the correct arrangement of the active site. A consequence of this would be that hydrolyzed ATP, i.e. ADP and phosphate (Pi) in the active site should be able to support the reaction, although no stoichiometric turnover is taking place during the reaction. Indeed, testing this hypothesis, we found that depupylation can be catalyzed in the presence of ADP and Pi (Fig. 1). After 12 s ~40% of PanB-Pup are depupylated and complete turnover of PanB-Pup in the presence of ADP and Pi is reached after 2 min compared with 3 min with ATP. Interestingly, ADP alone shows no activity on the time scale where in the presence of ADP and Pi or ATP complete conversion occurs. However, at later time points depupylation activity slowly starts up. This activity of Dop in the presence of ADP alone can be traced to minute amounts of adenylate kinase impurities in the recombinantly produced proteins, which results in very slow turnover of ADP to ATP and AMP. It was shown that due to the high catalytic rate of the enzyme, contaminating Escherichia coli adenylate kinase present at 0.01% in highly pure protein preparations can perturb experiments under ADP-only conditions (19). Bisadenosine pentaphosphate ([Ap5A], a competitive inhibitor of adenylate kinase, was shown to suppress activity of the E. coli enzyme at a ratio of Ap5A to nucleotide of 2:1. Unfortunately, the inhibitor carries about 1% ATP impurity, preventing us from using it in the ADP-only time trace at that ratio. However, the addition of 0.3 mM Ap5A

![Image](https://example.com/image1)

**FIGURE 1. Depupylation of PanB-Pup is catalyzed by Dop in the presence of ADP and Pi.** 3 μM [32P]Dop was incubated with 3 μM PanB-Pup, 0.5 mM ATP + Ap5A, ATP + Ap5A, AMP-PCP, ADP + Ap5A, ADP + Pi, ADP, ATP, and with no added nucleotide, for 15 min at 30 °C. The reaction was stopped with SDS-loading dye to suppress activity of the E. coli enzyme at a ratio of Ap5A to nucleotide of 2:1.
to the ADP time trace results in a decrease of the observed activity, whereas activity in the presence of ATP remains unchanged, indicating that activity in the presence of ADP stems from the adenylate kinase impurity and not from Dop.

Co-crystal Structure of Dop and ATP Reveals ADP and Phosphate as the Active Site Species—One of our previously solved crystal structures of Dop contained ATP (10). However, as the nucleotide was not co-crystallized but soaked into the crystal, the occupancy was poor. To investigate whether the active site species in Dop might be ADP and P_i rather than ATP, we now co-crystallized ATP and Dop to obtain full occupancy.

The co-crystallization attempts with Dop from Acidothermus cellulolyticus (AcelDop) and ATP yielded well diffracting crystals. The structure was solved by molecular replacement using the previously solved Dop structure (Protein Data Bank code 4b0r (10)) and was refined to 1.9 Å. With the exception of a disordered region between residues 42 and 79 in the so-called Dop loop, the electron density was continuous. The structure clearly shows that ADP and P_i are bound in the active site with high occupancy as indicated by the density (Figs. 2A and 3). In contrast to the earlier, ATP-soaked structure, all magnesium binding sites characteristic for members of the carboxylate-amine/ammonia ligase superfamily (11) were occupied and their importance for mediating contacts between the amino acid residues and the phosphate groups becomes obvious (Fig. 2A and B). An additional Mg^{2+} binding site (n5) was observed that contributes to the binding of the β-phosphate. All Mg^{2+} ions are coordinated in almost perfect octahedral symmetry. Notably, Asp-94, a residue previously shown to be important for activity (10, 13) and proposed to form a mixed anhydride intermediate during catalysis (20), is coordinating Mg^{2+} at the n1 position.

A comparison of the active site of Dop with the one of Saccharomyces cerevisiae glutamate cysteine ligase (ScGCL) in complex with ADP and the transition state analog (TSA) inhibitor buthionine sulfoximine phosphate (PDB 3lvv (21)) (Fig. 2C) shows that the phosphate complexed by Dop superimposes almost perfectly with the phosphate group of the TSA, indicating the general relevance of the bound phosphate and in particular the conservation of the phosphate location within the carboxylate-amine/ammonia ligase superfamily. Arg-472 of ScGCL is positioned to form hydrogen bonds with the sulfoximine oxygen and an oxygen of the phosphate group. The equivalent residue in Dop (Arg-227), while offset from Arg-472 in the alignment by 3.4 Å, nevertheless, is kept at the same distance to the phosphate. Although in the absence of an additional hydrogen bond partner the guanidyl group only coordinates the P_i, the conformational freedom of Arg-227 would allow forming a similar stabilizing interaction with Pup as Arg-472 of ScGCL with the TSA. Judging by the position of the carboxylate end of the TSA it is likely that Arg-205 in Dop coordinates the free C-terminus of Pup.

ATP Hydrolysis Depends on the C-terminal Residue of Pup—Our structural analysis suggests that ATP is cleaved into ADP and phosphate in the active site of Dop. In contrast to the ligase, this reaction is not coupled to the turnover of substrate. Nevertheless, it is possible that the presence of Pup in the active site might influence the cleavage. To investigate a potential role of Pup in the hydrolysis of ATP we therefore followed the conversion of [α-32P]ATP to [α-32P]ADP in the active site of Dop by TLC and phosphorimaging in the absence and presence of Pup. As the ATP cleavage presumably occurs to configure the active site rather than to turn over the substrate, a high concentration of Dop (30 μM) had to be employed to detect the cleavage. When Pup was absent, Dop showed only a very low basal ATPase activity (Fig. 4, upper left panel). However, when either PupE or PupQ were included in the reaction mixture, significant amounts of ADP were produced in the measured time frame of 50 min (Fig. 4, two upper middle panels). This increased Dop activity is due to a stimulation of the ATP cleavage by Pup and not due to any possible impurity in the Pup preparations, because PupQ or PupE alone did not show turnover in the same time period (Fig. 4, two lower middle panels). To test whether hydrolysis of ATP is stimulated by a conformational change of Dop upon binding of Pup or rather by the presence of the very C-terminal residue in the active site, a Pup variant lacking the last residue (PupGG) was used. Strikingly, PupGG was not able to accelerate ATP hydrolysis over the background level that Dop exhibits on its own, indicating that indeed the C-terminal residue of Pup in the active site stimulates the hydrolysis of ATP.

ATP Hydrolysis Is Necessary for Dop Activity—we next analyzed whether ATP hydrolysis is required for the active site to carry out catalysis of the depupylation reaction. To obtain a continuous record of the depupylation reaction we used the fluorescent model substrate Pup-Fl (22), which can be monitored by fluorescence anisotropy. In parallel, we followed the ATP hydrolysis radiochemically (Fig. 5A). The depupylation reaction shows an initial lag phase, during which Pup-Fl is turned over only very slowly (Fig. 5B). Maximal Dop activity is reached after ~12 min under the used conditions, at which point an ATP amount has been cleaved that is equivalent to 1.4 times the Dop active sites. This is rather close to stoichiometric with active sites, considering that ADP and P_i also dissociate off during this time frame, and suggests that P_i is indeed required for catalysis. To further demonstrate that the lag phase in the presence of ATP is due to ATP first needing to be cleaved to ADP and P_i, we measured turnover time traces of Pup-Fl providing Dop with either ATP or ADP and P_i (Fig. 6). In agreement with the notion that P_i is required in the active site for catalysis, when ADP and P_i instead of ATP is provided, no lag phase is observed.

Dop Activity Depends on ADP and P_i Concentrations—To assess the ADP and phosphate concentration dependence of the Dop activity, we used Pup-Fl to record depupylation time traces at varying concentrations of ADP or P_i with the other component held constant, respectively (Fig. 7). In the presence of 0.5 mM ADP, the rate of depupylation under steady-state conditions follows a saturation curve with respect to P_i, concentration featuring half-maximal activity at 73 ± 11 μM P_i. When the P_i concentration is held constant at 5 mM, the depupylation activity increases with the concentration of ADP, showing a half-maximal rate at 31 ± 6 μM ADP. Measuring the depupylation rate as a function of ATP concentration, we obtained a half-maximal rate at 2.3 ± 0.3 μM ATP.
Discussion

The two enzymes involved in the pupylation and de pupyla-

tion of proteins in actinobacteria, the ligase PafA and the depu-

pylase Dop, are evolutionarily related. Both belong to the large

family of carboxylate-amine ligases, enzymes that catalyze the

formation of an amide linkage between a carboxylate and an

amine via an acylphosphate intermediate (6, 7). Accordingly,

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attack by the amine and to drive the otherwise thermodynamically unfavorable ligation reaction. For the Pup ligase PafA, the activation of Pup occurs by formation of a γ-glutamylphosphate mixed anhydride intermediate at the C-terminal glutamate of Pup (11). This intermediate is poised in the active site, protected from hydrolysis, for reaction with a lysine ε-amino group of an incoming pupylation substrate. As a close homolog of PafA, Dop features a nearly identical ATP-binding site (10), however, thermodynamically, ATP turnover is not required, since breakage of an amide linkage is entropically favorable. In accordance with that, amide bond cleavage catalyzed by Dop is not accompanied by stoichiometric turnover of ATP, neither during deamidation nor during depyupylation (5, 15). Nevertheless, our structural analysis clearly identified ADP and Pi in the active site of Dop, indicating that ATP does not merely play a structural role to maintain active site configuration. Although it was previously shown that the ATP analog ATPγS is able to support a very low level of deamidase activity (5), this might be due to the tendency of this analog to exhibit some cleavage of the bond to the γ-phosphate. In contrast, the ATP analog AMP-PCP employed in this study is not known to undergo hydrolysis (23). The fact that Dop does not exhibit any activity with this nucleotide analog (Fig. 1) indicates that cleavage of the bond to the γ-phosphate of ATP and, therefore, the presence of ADP and phosphate in the active site of Dop is crucial for catalysis. This is further supported by our finding that radiolabeled \(^{32}\)P-ATP is turned over to \(^{32}\)P-ADP independent of substrate turnover (Fig. 4, upper panels). Another mechanistically revealing observation is that ADP and phosphate but not ADP alone can support Dop activity (Figs. 1 and 6). Together, these lines of evidence strongly point to a mechanistic role of inorganic phosphate in the depyupylase/deamidase reaction. We propose that the role of the phosphate in the active site of Dop is the formation of a transient phospho-Pup intermediate (Fig. 8), a scenario also supported by the evolutionary relationship with the carboxylate-amine ligase family, where exactly such an intermediate is formed in the forward reaction. An inorganic phosphate oxygen attacks the side chain carbonyl carbon of the Pup C-terminal glutamine or, in the case of depyupylation, the carbonyl carbon of the isopeptide bond between Pup and substrate, thereby bringing about the cleavage of the amide bond. The ammonium/amine leaving group dissociates from the enzyme, and in the next step water, likely activated for nucleo-

FIGURE 3. ADP and Pi are bound in the active site of Dop. A, unbiased mF_o - DF_c Fourier map at 4.2 σ contour level (gray) and 8 σ contour level (green) after molecular replacement with PDB code 4b0r (which did not include any ligands) and one round of refinement in PHENIX without any further model building. The final model is shown for orientation. B, final model of Dop with ADP and Pi, in the active site. The Pi is separated 4.2 Å from the ADP β-phosphate. The anomalous difference Fourier map (gray, 3.5 σ contour level) shows the location of the phosphorus and sulfur atoms.

FIGURE 4. ATP hydrolysis mediated by Dop is dependent on the C-terminal residue of Pup. The C-terminal residues glutamine or glutamate of Pup accelerate the ATP hydrolysis mediated by Dop. A Pup variant lacking the C-terminal residue (PupGG) shows the same ATPase activity as Dop alone. ATP and the Pup variants alone show negligible ATPase activity over the assay time. 30 μM CysDop was incubated with 190 μM PupE, 250 μM PupQ, or 250 μM PupGG and 100 μM ATP (100 mCi/mmol of [α-\(^{32}\)P]ATP) at 23 °C.
philic attack by Asp-94 in the active site, hydrolyzes the phospho-Pup intermediate, thereby releasing Pup from the enzyme. The importance of this aspartate during catalysis has been demonstrated previously, as mutation to either alanine (10) or asparagine (13) abolishes Dop activity in vitro for AcelDop and in vivo for Dop from Mycobacterium tuberculosis (MtbDop). In agreement with the lack of a thermodynamic requirement for ATP hydrolysis, inorganic phosphate remains in the active site and is ready for another round of catalysis. This mechanism is similar to the reaction where GS catalyzes the conversion of glutamine to glutamate in the presence of ADP and arsenate (12).

Superposition of the Dop active site containing ADP and P_i with structures of other members of the carboxylate-amine/ammonia ligase family in complex with phosphorylated inhibitors results in excellent congruence of P_i with the phosphate group of the inhibitors (Fig. 2C). This lends strong support to the existence of a phosphorylated Pup intermediate during the depupylation reaction catalyzed by Dop, which resembles the well characterized intermediates from the other family members (21, 24–26), including the phosphorylated Pup from PafA (11).

Intriguingly, only in the presence of Pup, and more specifically in the presence of the Pup C-terminal residue in the active site of Dop, are ADP and P_i formed efficiently. Dop in absence

### FIGURE 5. Initial ATP hydrolysis is necessary for depupylation of model substrate Pup-Fl. A, ATP hydrolysis catalyzed by Dop in the absence and presence of model substrate Pup-Fl. 25 µM [γ-32P]ATP was incubated with 100 µM Pup-Fl and 100 µM ATP spiked with 200 mCi/mmol of [γ-32P]ATP. The time point at 2 min was not taken into account because of the different running behavior. B, after an initial lag phase of ~12 min, during which ATP gets hydrolyzed, the depupylation reaction reaches maximal speed. At 30 min ~90% of Pup-Fl is depupylated and ~40% of ATP is left. The depupylation reaction progress was monitored by fluorescence anisotropy under the same conditions as in A, omitting [γ-32P]ATP. C, chemical structure of the model substrate Pup-Fl.

### FIGURE 6. Depupylation lag phase disappears in the presence of ADP and P_i. 1.25 µM [γ-32P]Dop was incubated with 5 µM Pup-Fl and 100 µM ATP, ADP, or ADP + 10 mM P_i. The depupylation time course in the presence of ADP and P_i does not exhibit the lag phase observed in the absence of ATP. ADP in the absence of P_i does not enable depupylation of Pup-Fl.

### FIGURE 7. The depupylation activity of Dop is dependent on the concentrations of ADP and P_i. The steady-state rates of Dop-catalyzed Pup-Fl cleavage were measured as a function of P_i (A), ADP (B), or ATP (C) concentrations. Averages of three experiments are presented with the corresponding standard deviations (error bars). Data are fitted to \( r(x) = \frac{r_{\text{max}}}{K_{\text{app}} + [x]} \), where \( x \) indicates the P_i, ADP, or ATP concentration. \( K_{\text{app}} \) is given with its fitting standard error. 0.25 µM Dop and 5 µM Pup-Fl were used.

of Pup or in the presence of a Pup variant shortened by one residue hydrolyzes ATP only very slowly (Fig. 4). Dop thus supports the activation of water in the active site needed for the attack on the γ-phosphate of ATP. This occurs either directly or indirectly by slight rearrangements of active site residues, such as, for example, Asp-94. The requirement of Pup binding for efficient ATP cleavage might serve as a protection mechanism against uncontrolled ATP hydrolysis by free Dop.
An earlier study suggested that Asp-95 of MtbDop (corresponding to Asp-94 in AcelDop) participates in catalysis by forming a covalent mixed anhydride intermediate with Pup (20). This hypothesis was based on the observation that a nucleophilic Pup derivative acting as an irreversible trap, Pup-DON (6-diazo-5-oxo-L-norleucine), forms a covalent bond with Asp-95 in the active site of MtbDop. Pup-DON features a highly reactive aliphatic diazo group that, after addition of a proton to the \( \epsilon \)-carbon and elimination of \( \text{N}_2 \), forms a carbocation that readily reacts with any nucleophiles in the vicinity, such as, for example, carboxylates. This is in perfect agreement with a mechanism in which this aspartate acts as catalytic base to activate water. It should also be noted in this context that the carbocation in the Pup-DON trap, which is attacked by the nucleophile, is positioned one bond length deeper in the active site than the side chain carbonyl carbon of Pup \( (\text{Asp versus } \text{Glu}) \), a position that is never occupied by the isopeptide bond or the carbamoyl group of glutamine. Pup with a C-terminal asparagine was found to resist deamidation, indicating the significance of the length of the side chain and the precise position of the carbonyl carbon in the active site (13).

Further support for our proposed mechanism is given by the sequence of events with ATP hydrolysis taken place first and creating a lag phase in the deacetylation reaction. Depupylation reaches maximal velocity only after the ADP and phosphate have been generated in the active site (Figs. 5 and 6). In agreement with this interpretation, in the presence of ADP and P, the lag phase is absent.

In an earlier study it was shown that, during the cycle of catalysis, \( ^{18}\text{O} \)-water \( (\text{H}_2^{18}\text{O}) \) is incorporated only into Pup and not into Dop (20). Furthermore, the authors showed that hydroxylamine can act as a nucleophile to form Pup-hydroxamate, indicating that during the Dop-catalyzed reaction an activated carbonyl must exist. These findings are in agreement with our proposed mechanism, as the resolution of the phosphorylated Pup intermediate by water \( (\text{H}_2^{18}\text{O}) \) or hydroxylamine directly leads to the experimentally identified Pup species, \( ^{18}\text{O} \)-labeled Pup or Pup-hydroxamate, respectively.

Taken together, our findings on the fate of ATP in the Dop active site, previous mutational and biochemical studies of Dop, and the high degree of structural conservation between members of the carboxylate-amine ligase family, in particular the
nearly identical active site configuration of Dop and PafA, strongly indicate that Dop catalyzes the cleavage of the isopeptide bond by a mechanism representative of the reverse reaction of PafA (Fig. 8). In a first step, Dop hydrolyzes ATP to produce ADP and P$_i$, which remain bound in the active site. This at the same time prevents Dop from acting as a ligase. In the next step, the active site-bound P$_i$ attacks the isopeptide bond and forms the transient phosphorylated Pup intermediate. During this step Arg-227 plays a critical role, likely by stabilizing the transition state with its guanidyl group. In the last step, the resolution of the phosphorylated Pup intermediate is mediated by nucleophilic attack of water, activated by the active site catalytic residue Asp-94, on the carbonyl carbon of Pup. The two tetrahedral transition states that flank the transiently formed phospho-Pup intermediate are further stabilized by the Mg$^{2+}$ ion coordinated by residues Glu-10, Asp-94, and Glu-99 in the active site.

Our experimental results demonstrate that Dop activity can be supported by binding ATP that is then cleaved in the active site or by direct binding of ADP and P$_i$. Bacterial cytosolic concentrations in an exponentially growing culture lie at around 0.5 mM for ADP and around 8–10 mM for ATP (27). However, whereas ADP changes only moderately, ATP decreases significantly in stationary phase, leading to a variation in the ATP/ADP ratio from 10 to 3 depending on the growth conditions (28). Single-cell measurements in continuously growing bacterial cultures showed a distribution of ATP concentrations with a mean value of 1.5 mM (29). The concentration of inorganic phosphate in the bacterial cytosol lies at about 10 mM (30). Taking into account this information, both binding of ATP followed by cleavage or binding of ADP and P$_i$ can occur depending on the conditions. As both can support the activity, should the ratio of ATP to ADP change significantly, this would not result in a significant change of Dop activity.

Our biochemical analysis supported by X-ray crystallographic data provides the framework for understanding the mechanism of the depupylase/deamidase enzyme Dop and thus provides insight that might be exploited for the rational design of antituberculosis drugs aimed at interfering with the pupylation system. The close evolutionary relationship between the two opposing players in the pupylation system, the Pup ligase PafA and the depupylase Dop, provides insight that might be exploited for the rational design of antituberculosis drugs aimed at interfering with the pupylation system.

**Experimental Procedures**

**Chemicals and Reagents**—Chemicals were obtained from Sigma unless otherwise noted. 5-FAM Lys was provided by AnaSpec. [α-32P]ATP was obtained from Hartmann Analytic (Braunschweig, Germany) at a specific activity of 15 TBq (400 Ci)/mmol. Polyethyleneimine TLC plates were provided by VWR International. ADP was further purified by ion-exchange chromatography (6-ml Resource Q column, GE Healthcare Life Sciences) and desalted by size exclusion chromatography (100 ml Superose 12 column, GE Healthcare Life Sciences).

**Protein Expression and Purification**—Dop from *A. cellulolyticus* (AcelDop) was expressed from isopropyl β-D-1-thiogalactopyranoside-inducible pET21 vector in *E. coli* Rosetta (DE3) cells (Invitrogen) with a C-terminal tobacco etch virus (TEV) protease cleavage site-His$_6$ fusion and purified by affinity chromatography using a 5-ml Hi Trap IMAC HP column (GE Healthcare Life Sciences) charged with Ni$^{2+}$. After washing the protein-charged column with 50 ml of buffer W (50 mM HEPES-NaOH, pH 8, at 23 °C; 500 mM NaCl, 40 mM imidazole), protein was eluted with buffer W containing 300 mM imidazole. Protein-containing fractions were pooled and dialyzed for 1 h at 4 °C against buffer D (50 mM HEPES-NaOH, pH 8, at 4 °C, 150 mM NaCl). The C-terminal histidine tag was cleaved at the TEV protease cleavage site by addition of His-tagged TEV protease and further dialysis for 15 h. TEV protease was removed via affinity chromatography. AcelDop was further purified by size exclusion chromatography using a Superdex 75 column in 20 mM HEPES-NaOH, pH 8, at 20 °C and 50 mM NaCl. *Corynebacterium glutamicum* Dop (CgluDop) with an N-terminal His$_6$-TEV protease cleavage site fusion was expressed from pET24 and purified similarly to AcelDop. After size exclusion chromatography, CgluDop was further purified by anion exchange chromatography with a Mono Q HR 10/10 column (GE Healthcare Life Sciences) to reduce the amount of *E. coli* adenylate kinase impurity. A linear gradient from 0 to 1 M NaCl in 20 mM Tris-HCl, pH 7, at 23 °C was used to elute CgluDop. Buffer was changed to buffer R (50 mM HEPES-NaOH, pH 8, at 23 °C, 150 mM NaCl) either by PD-10 desalting columns (GE Healthcare Life Sciences) or by Amicon Ultra centrifugal filters (Merck Millipore).

**Crystallization of Dop**—Crystallization of AcelDop was carried out in sitting drop vapor diffusion plates at a protein concentration of 8–12 mg/ml at 20 °C by mixing 2 μl of protein solution with 1 μl of reservoir solution. AcelDop formed crystals in reservoir solutions consisting of 18–23% (w/v) PEG 3350, 100 mM Bistris propane, pH 8.25–9.0, at 20 °C, 200 mM KSCN, 20 mM MgCl$_2$, and 5 mM ATP (pH 8). Before flash cooling the crystals with liquid nitrogen, PEG 400 was added in 5% (v/v) steps to a final concentration of 30% (v/v) by using reservoir solution supplemented with PEG 400.

**Data Collection, Processing, Structure Determination, and Refinement**—A data set was collected at beamline X06SA of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland) at 100 K and data were indexed and integrated using XDS (31). Initial analysis of data was performed using POINTLESS (32) and PHENIX.xtriage (33). Scaling was subsequently done by AIMLESS (34). The structure was solved by molecular replacement in PHASER-MR (35) using the previously solved Dop structure (PDB code 4b0r (10)) as a search model. After an initial refinement step in PHENIX (33), well defined density was obtained in the active site indicating that ADP and P$_i$ are bound rather than ATP (Fig. 3A). Attempts to refine the structure with ATP resulted in strong difference peaks. Additionally, the anomalous difference Fourier map was calculated and supports the location of the phosphorus atoms with a distance of 4.2 Å between the ADP β-phosphate and the P$_i$ (Fig. 3B). The model was further improved by iterative model building in COOT (36) and refinement in PHENIX. Statistics are summarized in Table 1. An additional strong electron density peak near the P$_i$ was interpreted as a sodium ion (n4) based on coordination geometry and hydrogen bond interactions.
TABLE 1
Data collection and refinement statistics

Values in parentheses are for highest-resolution shell, \( R_{merge} = \sum |I_{h,i} - \langle I_{h,i} \rangle|/\sum I_{h,i} \), where \( \langle I_{h,i} \rangle \) is the mean intensity of the reflections.

<table>
<thead>
<tr>
<th>Crystal form</th>
<th>P 3 2 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 3 2 1</td>
</tr>
<tr>
<td>Unit cell</td>
<td>72,398</td>
</tr>
<tr>
<td>Dimensions (Å)</td>
<td>72,398</td>
</tr>
<tr>
<td>215.25</td>
<td></td>
</tr>
<tr>
<td>90.90 120</td>
<td></td>
</tr>
<tr>
<td>Molecules/ASU</td>
<td>1</td>
</tr>
</tbody>
</table>

Data collection

<table>
<thead>
<tr>
<th>Wavelength (Å)</th>
<th>0.99998</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>38.88–1.85</td>
</tr>
<tr>
<td>Total reflections</td>
<td>506,641 (23 851)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>56,899 (2,622)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>8.9 (9.1)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100 (1.00)</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>25.52 (1.50)</td>
</tr>
<tr>
<td>Wilson B-factor (Å²)</td>
<td>39.17</td>
</tr>
<tr>
<td>( R_{merge} )</td>
<td>0.044 (1.425)</td>
</tr>
<tr>
<td>( R_{free} )</td>
<td>0.047 (1.51)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>1 (0.607)</td>
</tr>
</tbody>
</table>

Refinement

| Non-hydrogen atoms | 3,996 |
| Macromolecules | 3,689 |
| Ligands | 83 |
| Water | 224 |
| Protein residues | 464 |

Root mean square deviations

| Bonds | 0.027 |
| Angles | 1.13 |
| Dihedrals | 14.97 |

Ramachandran plot

| Favored (%) | 97 |
| Allowed (%) | 2.6 |
| Outliers (%) | 0.21 |
| Rotamer outliers (%) | 0.52 |
| Clashescore | 4.54 |
| Average B-factor | 49.53 |
| Macromolecules | 49.25 |
| Ligands | 52.69 |
| Water | 53.07 |
| Number of TLS groups | 11 |

Acknowledgments—We thank B. Blattmann and C. Stutz-Ducom for support with data collection.

References


Dop Catalytic Mechanism

Gel-based Depupylation Assays—3 μM MtbPanB CgluPup (produced as described in Ref. 15 and further purified to reduce with 20 mM MgCl₂, 0.5 mM nucleotides and additional 10 mM Na/K-phosphate, pH 7, at 23 °C in the case of ADP. Still present minute amounts of adenylate kinase activity could be reduced by addition of 0.3 mM AP₅A. The formation of PanB was monitored by SDS-PAGE and Coomassie staining and analyzed using GelAnalyzer software version 2010a (38). The fraction of PanB is expressed in relationship to total PanB (sum of PanB and PanB-Pup). Pup was not taken into account because of poor staining.

Fluorescence Anisotropy-based Depupylation Assay of Pup-Fl—Pupylation of 5-FAM Lys and the depupylation assay were carried out as described (22). For the generation of Pup-Fl, 1 mM 5-FAM Lys and 250 μM CgluPupE were incubated in the presence of 10 μM CgluPafA with 5 mM ATP. Depupylation was carried out in buffer R for data in Fig. 5 or in buffer A (100 mM Bistris propane-HCl, pH 7.5, at 23 °C, 150 mM NaCl, and 0.05% Tween 20) for the data in Figs. 6 and 7. The reactions were supplemented with 10 or 20 mM MgCl₂ and varying concentrations of ATP, ADP, and P₃. Single time courses are shown in Fig. 6. The trace in Fig. 5 represents the average of three time courses. All traces for the depupylation rate dependence on P₃, ADP, and ATP were set up in triplicate and the initial velocities were determined by a linear fit for the linear part of the individual time traces. The depupylation rate dependence on the concentration \( r(t) \) was fitted with OriginPro 9 (OriginLab Corporation) to the function,

\[
\text{Eq. 1}
\]

where \( x \) indicates the P₃, ADP, or ATP concentration.

Radioactive Assay to Monitor ATP Hydrolysis—Assays were performed as described (11). In brief, 30 μM CgluDop was incubated with 190 μM CgluPupE, 250 μM CgluPupQ, or 250 μM CgluPupGG and 100 μM ATP (100 mCi/mmol of \( \alpha^{-32}P \) ATP) at 23 °C in reaction buffer R. A molecular dynamics phosphor-imaging screen (GE Healthcare) was exposed to the dried TLC plates for 1–2 h and subsequently scanned using a Typhoon Trio phosphorimaging system (GE Healthcare). Images were analyzed using ImageJ software version 1.48 (39) to determine the ratio, \( r \), of non-hydrolyzed ATP to total nucleotide (ATP and ADP) for each reaction time point, \( t \). To correct for the background hydrolysis of Dop without Pup-Fl (−Pup-Fl), the fraction of non-hydrolyzed ATP, \( f \), at each time point, \( t \), during the depupylation of Pup-Fl was normalized to the corresponding background \( f(t) = r(t, + Pup-Fl)/r(t, − Pup-Fl) \).
Dop Catalytic Mechanism


