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) (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...
an intricate network of regulation. Furthermore, pupylated target proteins can be recruited to a bacterial proteasome complex consisting of the 20S proteasome core and the AAA-ATPase ring Mpa (mycobacterial proteasomal ATPase), where recognition of Pup takes place (16–18). Pupylated substrates can escape this fate, however, when Pup is cleaved off by the depupylase Dop. The fate of a pupylation target is therefore tightly controlled by all four activities.

In agreement with its structural similarity to PafA, Dop also features an active site nucleotide (10). Yet, cleavage of the isopeptide bond does not require energy and it has been shown that Dop does not turn over ATP stoichiometrically with substrate deamidated or Pup cleaved off (5, 15). It is, therefore, an intriguing question what role the nucleotide plays in the catalytic mechanism of the enzyme.

To investigate the role of the nucleotide in the active site of Dop we used a combination of biochemical experiments and X-ray crystallographic analysis. Our results show that the active site of Dop requires ADP and inorganic phosphate to support both the deamidation and the depupylation activities. This has implications for the catalytic mechanism and suggests that Dop is mechanistically more closely related to the ligase PafA than previously thought.

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 marginal deamidase activity in the presence of non-hydrolyzable ATP analog ATPyS (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 (P_i) 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 P_i (Fig. 1). After 12 s ~40% of PanB-Pup are depupylated and complete turnover of PanB-Pup in the presence of ADP and P_i 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 P_i 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 (Ap_5A), a competitive inhibitor of adenylate kinase, was shown to suppress activity of the E. coli enzyme at a ratio of Ap_5A 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 Ap_5A

FIGURE 1. Depupylation of PanB-Pup is catalyzed by Dop in the presence of ADP and P_i. A, 3 μM Dop was incubated with 3 μM PanB-Pup, 0.5 mM nucleotides, and 10 mM phosphate at 30 °C. The adenylate kinase inhibitor Ap_5A, where present, was used at a concentration of 0.3 mM. A, SDS-polyacrylamide gels showing depupylation time courses of PanB-Pup in the presence of different nucleotides. B, densitometric analysis of the PanB band in relationship to the total amount of PanB used in the reaction. PupE is poorly stained by Coomassie Blue and was not expected to contribute to the density of the PanB-Pup band at the concentrations under which the assay was performed.
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 Pi, 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 Pi are bound in the active site with high occupancy as indicated by the density (Figs. 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-amino/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 Pi, 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 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-Fi (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-Fi 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 Pi also dissociate off during this time frame, and suggests that Pi 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 P1, we measured turnover time traces of Pup-Fi providing Dop with either ATP or ADP and P1 (Fig. 6). In agreement with the notion that P1 is required in the active site for catalysis, when ADP and P1 instead of ATP is provided, no lag phase is observed.

**Dop Activity Depends on ADP and Pi Concentrations**—To assess the ADP and phosphate concentration dependence of the Dop activity, we used Pup-Fi to record depupylation time traces at varying concentrations of ADP or P1, 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 P1 concentration featuring half-maximal activity at 73 ± 11 μM P1. When the P1 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.
The two enzymes involved in the pupylation and de pupylation of proteins in actinobacteria, the ligase PafA and the depupylase 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, PafA and Dop are structural homologs and the residues forming the active site are highly conserved (10, 13). A defining feature of the active site of carboxylate-amine ligase family members is the nucleotide binding site. The role of ATP in the ligation reaction is to activate the carboxylate for nucleophilic attack.

**Discussion**

The two enzymes involved in the pupylation and de pupylation of proteins in actinobacteria, the ligase PafA and the depupylase 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, PafA and Dop are structural homologs and the residues forming the active site are highly conserved (10, 13). A defining feature of the active site of carboxylate-amine ligase family members is the nucleotide binding site. The role of ATP in the ligation reaction is to activate the carboxylate for nucleophilic attack.

**FIGURE 2.** Crystal structure of Dop reveals ADP and P$_i$ in the active site. **A**, AcelDop active site with bound ADP, phosphate, magnesium, and sodium ions. The unbiased mF$_o$ − DF$_c$ Fourier-simulated annealing omit map was calculated with a model in which all but the protein atoms and waters were omitted and is contoured at 5σ levels. **B**, schematic representation of polar interactions between Dop active site residues, ADP, phosphate, magnesium ions, sodium ion, and water. Residues labeled in *blue* are located in the C-terminal domain of Dop. **C**, comparison of active sites of AcelDop (*green*) and *S. cerevisiae* glutamate cysteine ligase (PDB code 3lvv, *gray*, residue numbers in parentheses). The phosphate group of the transition state mimic buthionine sulfoximine phosphate overlays with the inorganic phosphate bound by Dop.
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-aminogroup 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 depupylation (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 [α-32P]ATP is turned over to [α-32P]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 depupylase/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 depupylation, 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-
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 \(^{15}N\)Dop and in vivo for Dop from \textit{Mycobacterium tuberculosis} (\(^{15}N\)Dop). 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\(_{\text{i}}\), with structures of other members of the carboxylate-amine/ammonia ligase family in complex with phosphorylated inhibitors results in excellent congruence of P\(_{\text{i}}\) 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\(_{\text{i}}\) formed efficiently. Dop in absence of Pup or in the presence of a Pup variant shortened by one residue hydrolyzes ATP only very slowly (Fig. 4). Pup thus supports the activation of water in the active site needed for the attack on the \(\gamma\)-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.

**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 \(\mu\text{M}^{32}\text{P}\)Dop were incubated with 100 \(\mu\text{M} \text{Pup-Fl and 100 } \mu\text{M ATP spiked with 200 mCi/mmol of } (\alpha^{32}\text{P})\text{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 } \sim 12 \text{ min, during which ATP gets hydrolyzed, the depupylation reaction reaches maximal speed. At 30 min } \sim 90\% \text{ of Pup-Fl is depupylated and } \sim 40\% \text{ of ATP is left. The depupylation reaction progress was monitored by fluorescence anisotropy under the same conditions as in A, omitting } (\alpha^{32}\text{P})\text{ATP. C, chemical structure of the model substrate Pup-Fl.}
An earlier study suggested that Asp-95 of \(^{\text{Mt}}\)Dop (corresponding to Asp-94 in \(^{\text{Ac}}\)Dop) 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 \(^{\text{Mt}}\)Dop. Pup-DON features a highly reactive aliphatic diazo group that, after addition of a proton to the \(\epsilon\)-carbon and elimination of \(N_2\), forms a carbenium ion 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 \(\epsilon\)-carbon 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 (Ce versus C8), 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 taking place first and creating a lag phase in the depupylation 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 (H\(_2\)^{18}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 (H\(_2\)^{18}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, 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 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. 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, 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 enzymes. Furthermore, it contributes to our understanding of the close evolutionary relationship between the two opposing players in the pupylation system, the Pup ligase PafA and the depupylase Dop.

**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. cellulosyaticus* (AcDop) 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. AcDop 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 (C$_{17}$Dop) with a N-terminal His$_6$-TEV protease cleavage site fusion was expressed from pET24 and purified similarly to AcDop. After size exclusion chromatography, C$_{17}$Dop 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 C$_{17}$Dop. 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 AcDop 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. AcDop 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 were 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 (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 was interpreted as a sodium ion (n4) based on coordination geometry and hydrogen bond...
Dop Catalytic Mechanism

Table 1: Data collection and refinement statistics

| Data collection and refinement statistics | Value |
|------------------------------------------|-------|
| PDB ID | SLRT |
| Crystal form | Space group: P 3 2 1 |
| | Unit cell: 72.398 |
| | Dimensions (Å): 215.25 |
| | Molecules/ASU: 1 |
| | Total reflections: 506,641 (23 851) |
| | Unique reflections: 56,899 (2.622) |
| | Multiplicity: 8.9 (9.1) |
| | Completeness (%): 1.00 (1.00) |
| | Mean I/σ(I): 25.52 (1.50) |
| | Wilson B-factor (Å²): 39.17 |
| | Rmerge: 0.044 (1.425) |
| | Rfree: 0.047 (1.51) |
| | CC1/2: 1.00 (0.607) |

Refinement

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

Root mean square deviations

| Value |
|-------|
| Bonds: 0.027 |
| Angles: 1.13 |
| Dihedrals: 14.97 |

Ramachandran plot

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

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