D-alanine–D-alanine ligase as a model for the activation of ATP-grasp enzymes by monovalent cations

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ABSTRACT

The ATP-grasp superfamily of enzymes shares an atypical nucleotide-binding site known as the ATP-grasp fold. These enzymes are involved in many biological pathways in all domains of life. One ATP-grasp enzyme, D-alanine–D-alanine ligase (Ddl), catalyzes the ATP-dependent formation of the D-alanyl–D-alanine dipeptide essential for bacterial cell-wall biosynthesis, and is therefore an important antibiotic drug target. Ddl is activated by the monovalent cation (MVC) K⁺, but despite its clinical relevance and decades of research, how this activation occurs has not been elucidated. We demonstrate here that activating MVCs bind adjacent to the active site of Ddl from Thermus thermophilus and used a combined biochemical and structural approach to characterize the MVC activation. We found that TtDdl is a Type II MVC-activated enzyme, retaining activity in the absence of MVCs. However, the efficiency of TtDdl increased ~20-fold in the presence of activating MVCs, and it was maximally activated by K⁺ and Rb⁺ ions. A strict dependence on ionic radius of the MVC was observed, with Li⁺ and Na⁺ providing little-to-no TtDdl activation. To understand the mechanism of MVC activation, we solved crystal structures of TtDdl in complex with K⁺, Rb⁺, or Cs⁺. Comparison of these structures with apo TtDdl revealed no evident conformational change on MVC binding. Of note, the identified MVC-binding site is structurally conserved within the ATP-grasp superfamily. We propose that MVCs activate Ddl by altering the charge distribution of its active site. These findings provide insight into the catalytic mechanism of ATP-grasp enzymes.

INTRODUCTION

The ATP-grasp superfamily includes more than 20 enzymes, with many having been structurally characterized (Table S1). The defining feature of this superfamily is the ATP-grasp fold, which binds ATP between two αβ domains. In addition to ATP, the catalytic mechanism of ATP-grasp enzymes generally utilizes separate carboxylate and nucleophilic substrates, with the reaction considered to proceed through the formation of an acylphosphate intermediate (Figure 1A). While the utilization of ATP is common between members of the superfamily, the composition of additional substrates varies, resulting in a wide range of biomolecular products. These enzymes are therefore involved in a diverse range of biological pathways in all domains of life, such as the oxidative stress...
response (Glutathione synthetase), gluconeogenesis, fatty acid synthesis, amino acid catabolism (Carboxylases), purine biosynthesis (Pur synthetases), antibiotic biosynthesis (L-amino acid ligases) and cell wall biosynthesis (D-alanine–D-alanine ligase) (Table S1). As such, the role of ATP-grasp enzymes in these various pathways has been extensively studied, with many investigated for their promise as drug targets and for molecular biosynthesis.

D-alanine–D-alanine ligase (Ddl), one of the first characterized ATP-grasp enzymes, catalyses the energy dependent conjugation of two D-alanine molecules to form the D-alanyl-D-alanine dipeptide and is essential for cell wall biosynthesis. Due to this important role, Ddl has been primarily investigated as an antibiotic drug target and has been identified as the primary target of the second investigated anti-tuberculosis agent D-cycloserine (DCS). Ddl has also been investigated for the in vitro biosynthesis of D-amino acid dipeptides (1, 2). The Ddl enzyme from Thermus thermophilus (TtDdl) was previously used as a model to investigate the reaction mechanism of these enzymes and was employed in this study (3). The enzymatic activity of TtDdl requires the formation of a dimer, with a single monomer consisting of an N-terminal domain (Met1-Gly104), a central domain, (Ala105-Leu192) and a C-terminal domain (Ser193-Thr319) (Figure 2). Each subunit possesses a single ATP binding site, formed by the ATP-grasp fold, and two D-alanine (D-ala) binding sites. Both ATP and the two D-ala substrates bind in adjacent sites at the center of the Ddl monomer to facilitate the formation of the D-alanyl-D-alanine dipeptide (D-ala-D-ala). Studies of Ddl from other bacterial species have identified that the first D-ala site has higher affinity for D-ala than the second (4–7). The catalytic mechanism of Ddl is also accompanied by cumulative conformational changes induced by substrate binding. For TtDdl this involves three loop regions, loop 1 (aa. 155-161: the P-loop), loop 2 (aa. 217-234: the Ω-loop) and loop 3 (aa. 289-293) (Figure 2).

The catalytic mechanism of Ddl is proposed to proceed with an ordered ter-ter mechanism through two half-reactions and involves the formation of two reaction intermediates: an acylphosphate intermediate, and a tetrahedral intermediate (Figure 1B). In the first half reaction, ATP and two Mg$^{2+}$ bind and facilitate closure of the P-loop (3, 8). Subsequently, the first D-ala (D-ala$_1$) binds adjacent to ATP inducing the closure of the Ω-loop. Here, the carboxylate of D-ala$_1$ attacks the γ-phosphate of ATP (1), leading to the formation of the acylphosphate intermediate, D-ala-phosphate (2). In the second half-reaction, a second D-ala (D-ala$_2$) then enters the active site. The primary amine of D-ala$_2$ must be in its deprotonated form (NH$_2$) to react with D-ala-phosphate. The mechanism by which this deprotonation occurs remains unclear for Ddl (8, 9). The deprotonated D-ala$_2$ then attacks the phosphorylated carbonyl carbon of D-ala$_1$ (3), resulting in the formation of a tetrahedral intermediate, D-ala-D-ala-phosphate (4). This coincides with the closure of loop 3 and the formation of a ‘fully closed’ complex (3, 10). The tetrahedral intermediate then collapses to form the D-ala-D-ala dipeptide and inorganic phosphate (P$_i$) (5). Following collapse, P$_i$ is released first from TtDdl, followed by D-ala-D-ala and ADP (8, 11).

It has also been determined that Ddl is activated by the monovalent cation (MVC) potassium (K$^+$) (12). For over 60 years K$^+$ has been included for the kinetic analysis of Ddl with little insight into how MVCs activate the enzyme. As a result, the mechanism by which this activation occurs remains unknown (Figure 1B). The activation of enzymes by MVCs is categorized into one of two general mechanisms, referred to as Type I activation or Type II activation (13, 14). Type I activation involves the direct interaction of the MVC with the substrate, and so the MVC is required for enzyme activity. For Type II activation the MVC is not essential for enzyme activity. In this case the MVC binds at an allosteric site, causing an increase in substrate binding affinity and/or enzyme activity. Ddl was previously determined to be a Type II activated enzyme. The pioneering kinetic study of Ddl by Neuhaus et al. revealed that the MVC K$^+$ was required for optimal activity of Enterococcus faecalis Ddl, characterized by a 1.4-fold increase in $k_{cat}$, and a 3-fold decrease in the $K_M$ for the D-ala substrate in the presence of 10 mM KCl (12). NH$_4^+$ was also shown to be a less potent activator than K$^+$, while Na$^+$ did not affect enzyme activity. Similar observations of K$^+$ activation were later reported by Prosser et al. for Mycobacterium tuberculosis Ddl (6). Kinetic studies of other ATP-grasp enzymes have also reported activation by MVCs (Table S1). Similar to Ddl, the mechanism for MVC activation of other ATP grasp enzymes is poorly understood,
largely due to the location of MVC binding remaining unknown. Therefore, it is yet to be determined if this superfamily shares the same mechanism for MVC activation, or if unique mechanisms exist.

Historically a kinetic approach has been used to identify activation of enzymes by MVCs, however this does not provide full details of the activation mechanism. Recent advances in structural and spectroscopic techniques have allowed a more complete understanding of these mechanisms (13, 15). To this end, X-ray crystallography has proven to be an especially powerful technique. Through exploiting the unique anomalous scattering properties of heavy metal MVCs, such as Rb\(^+\) and Cs\(^+\), the MVC binding sites can be identified through co-crystallization with the enzyme of interest. This also allows for direct comparison of enzyme structure in the absence and presence of MVCs, providing insight into the mechanism of activation. In this study we have investigated the MVC activation of TiDdl using a combination of steady-state kinetic analysis and X-ray crystallography. This combined approach revealed that TiDdl is activated by the larger MVCs K\(^+\), NH\(_4\)\(^+\), Rb\(^+\) and Cs\(^+\), and allowed the identification of an MVC binding site within the active site of TiDdl. Based on these findings we have proposed a mechanism for the MVC activation of Ddl. Comparison with members of the ATP-grasp superfamily revealed that this previously unidentified site is structurally conserved in many ATP-grasp enzymes. The broader significance of this discovery with respect to MVC activation of other enzymes in the ATP-grasp superfamily is also addressed.

RESULTS

**TiDdl is selectively activated by MVCs.**

The kinetic analysis of TiDdl was completed to determine the effect of K\(^+\) and other MVCs on catalysis. While K\(^+\) was previously shown to activate Ddl from other species, this had not been determined for TiDdl. For this purpose, a colorimetric antimony-phosphomolybdate assay was used to monitor the production of P\(_i\), from the enzymatic activity of TiDdl. The kinetic parameters \(k_{\text{cat}}\) and \(K_M\) were determined for the ATP and D-ala\(_2\)/D-ala\(_2\) substrates in a low MVC reaction buffer (Table 1, see Methods). These were determined as 16.2 \(\mu\)M, 1250 \(\mu\)M and 4020 \(\mu\)M respectively. To investigate the effect of K\(^+\) on TiDdl activity, the kinetic parameters were also determined in the presence of 10, 50 and 100 mM KCl (Table 1). For both \(K_M\) and \(k_{\text{cat}}\), a slight decrease was observed in the presence of 50 mM and 100 mM KCl. In contrast, \(K_M\) D-ala\(_2\) decreased 10-fold in the presence of 10 mM KCl, and up to 20-fold for 50 and 100 mM KCl. A similar decrease was also reflected in \(K_M\) D-ala\(_1\). This reduction in D-ala \(K_M\) is consistent with previous observations (5, 6). To compare the effect of these MVCs on TiDdl activity, \(k_{\text{cat}}/K_M\) D-ala\(_2\) was used as a measure of enzyme efficiency. As the addition of KCl showed little to no effect on \(k_{\text{cat}}\), this translated to a ~20-fold more efficient enzyme at 50 mM KCl. This indicates that TiDdl is a more efficient enzyme in the presence of K\(^+\) and is, therefore, activated by this MVC.

In the case of MVC activated enzymes, a preference for Na\(^+\) or K\(^+\) is commonly observed, with MVCs of similar ionic radius often able to substitute with a comparable effect. Therefore, the effects of LiCl, NaCl, NH\(_4\)Cl, RbCl and CsCl salts on TiDdl activity were also determined at an intermediate concentration of 50 mM (Figure 3 and Table S2). The smaller MVCs Li\(^+\) and Na\(^+\) produced a small effect, with an apparent 1.5-fold increase in TiDdl efficiency when compared to the low MVC condition. In contrast, larger MVCs showed a 10 to 20-fold increase in efficiency. This confirms that activation of TiDdl is not the result of an ionic strength effect. Interestingly, substrate inhibition by D-ala was observed in the presence of the activating MVCs K\(^+\), NH\(_4\)\(^+\), Rb\(^+\) and Cs\(^+\) (Figure S2). This has not been reported previously for Ddl. We predict this may be due to non-productive binding of D-ala, a result of the low \(K_M\) D-ala\(_2\) which was determined to be decreased ~10-fold compared to Ddl from other species. The order of MVC activation was thus determined as K\(^+\) > Rb\(^+\) > NH\(_4\)\(^+\) > Cs\(^+\) > Na\(^+\) ≈ Li\(^+\) ≈ low MVC. Therefore, this kinetic analysis demonstrated that K\(^+\), and the larger MVCs NH\(_4\)\(^+\), Rb\(^+\) and Cs\(^+\) activate TiDdl.

**Identification of a catalytic MVC binding site.**

After determining that TiDdl is activated by MVCs we aimed to identify catalytically relevant binding sites by X-ray crystallography. An approach to identifying such sites is by crystallising the target protein in the presence of MVCs (16). For this purpose, the larger electron density and anomalous scattering properties of Rb\(^+\) and Cs\(^+\) were exploited. Cocrysalts of TiDdl complexed with Mg\(^{2+}\)-ATP, D-ala-D-ala and Rb\(^+\) or Mg\(^{2+}\)-ATP, D-ala-D-ala and Cs\(^+\) were grown as described in Methods. Datasets for both conditions were collected at
13000 eV at the Australian Synchrotron and the structures solved to 1.90 Å and 2.30 Å (17). Additional datasets were collected at 15250 eV and 8500 eV for the Rb + and Cs + bound structures respectively, to obtain a strong anomalous signal. Anomalous difference Fourier maps were used to identify MVC binding sites. In total 2 separate MVC sites were identified per Tt Ddl monomer for both Rb + and Cs + (Figure 4A). Anomalous peak heights and atomic parameters for each site are detailed in Table S4. Site 1 is located at the edge of the dimerization interface, coordinated by Trp133 and solvent (Figure S3). This is distant from the active site and is unlikely to be responsible for the activating effect. This conclusion is supported by the low occupancies for Rb + modelled at this site, despite a high concentration of 150 mM RbCl in the crystallisation condition.

In contrast, Site 2, herein referred to as the K + cleft, is in close proximity to the active site (Figure 4B). The K + cleft is defined by the sidechains of Glu282, Asn284, Met114, Lys116, Glu87 and the backbone of Leu283 and Cys113. Both Rb + and Cs + share the same coordination sphere of 7 coordinating interactions (Figure 4C/D). This includes the carboxylate of Glu282, the amide of Asn284, the backbone carbonyl O of Cys113 and Leu283 and 3 ordered waters, arranged in a capped trigonal prismatic geometry. Notably Glu282 also coordinates to both Mg 2+ that bridge the triphosphate group of ATP. This suggests an important role for Glu282 in Ddl catalysis, supported by a previous site-directed mutagenesis study of E. coli Ddl that reported a ~500-fold decrease in activity for an equivalent Glu282→Gln mutant (9). Additionally, multiple sequence alignment and structural superposition of Tt Ddl to other Ddl enzymes also reveals that the residues forming the K + cleft are highly conserved (Figure S4). Together, this provides strong evidence that MVC binding at the K + cleft is responsible for activation of Tt Ddl.

**K + binding at the K + cleft does not induce conformational change.**

Having identified the MVC binding site of Tt Ddl, we further investigated the influence of K + at separate stages of the Ddl reaction mechanism. The mechanism of MVC activation for Type II activated enzymes commonly involves conformational change in the vicinity of the MVC binding site. As Tt Ddl is a Type II activated enzyme, K + induced conformational change was investigated by capturing Tt Ddl at different stages of the catalytic cycle. By completing crystallization experiments (see Methods), 5 structures of Tt Ddl, representing 4 different stages of Ddl catalysis, were solved; Apo Tt Ddl (PDB:6U1C), Tt Ddl in complex with ADP, P, and K + (PDB:6U1H), Tt Ddl in complex with ADP, the phosphorylated form of D-cycloserine (DCSP) and K + (PDB:6U1I), Tt Ddl in complex with ADP, P, D-ala-D-ala and K + (PDB:6U1J), and Tt Ddl in complex with ADP, CO 3−, D-ala-D-ala and K + (PDB:6U1K) (Figure S5). The high resolution (1.67 Å) of the latter structure allowed clear identification of K + bound at the K + cleft. The mean K +-O bond length of 2.91 Å across all of these structures is consistent with a reported value of 2.91 ± 0.179 Å for [ 17 K + (18). A complete summary of these atomic parameters for each modelled cation is reported in Table S5. In each of these structures K + shared the same coordination sphere described for Rb + and Cs + (Figure 5A/S6). Flexibility in coordinating residues of the K + cleft was also observed, being dependent on the ionic radius of the bound MVC. Accommodation of the bulkier Cs + produced the greatest effect, possibly explaining the reduced potency in comparison to activation by K + and Rb +. As the activating effect of these MVCs was associated with the D-ala substrate, the possibility of conformational change at the K + cleft or within the D-ala pocket was considered. To identify whether K + binding at the K + cleft induces conformational change, the captured states of K + bound Tt Ddl were superposed with apo Tt Ddl (Figure 5B). Overall, minimal conformational change was observed for the coordinating residues of the K + cleft. The only observable difference was the rotation of Glu282 from the apo to K + bound enzyme, which is consistent with ordering of the sidechain following the binding of ATP and Mg 2+. Therefore, this suggests that MVC activation of Tt Ddl is not elicited through conformational change at the K + cleft. As the activating effect of K + was associated with the D-ala substrates, the D-ala1 binding pocket was similarly compared (Figure 5C). Again, no conformational change was observed in the residues forming the D-ala1 binding pocket. Notably, the key residues Glu13 and Arg268, which facilitate hydrogen bond-mediated substrate recognition, remain in the same conformation in both the apo and ligand bound forms of Tt Ddl. The only considerable difference is in the conformation of the P-loop, however this is accounted for by the absence of a nucleotide substrate in the structure of apo Tt Ddl. Together, this
structural analysis shows that $K^+$ binding at the $K^+$ cleft does not result in rearrangement of active site residues and suggests that activation of $Tt$Ddl occurs through a mechanism that does not involve significant conformational change.

The $K^+$ cleft is structurally conserved between members of the ATP-grasp superfamily.

Enzymes of the ATP-grasp superfamily share high conservation in their nucleotide binding sites and in the first step of catalysis, which involves the formation of an acylphosphate intermediate (Figure 1A). As summarized in Table S1, some ATP-grasp enzymes have been determined to be activated by MVCs. Therefore, we predicted that MVCs may also activate other members of the ATP-grasp superfamily by a similar mechanism. Structures have been solved by X-ray crystallography for many of these enzymes, allowing direct comparison with the $K^+$ cleft of $Tt$Ddl. Initially, examples of ATP-grasp enzymes with an MVC bound at this position were considered. In all, 2 cases were identified, with the first of these being carbamoyl phosphate synthetase (CPS) (19). Superposition of the carboxyphosphate domain of E. coli CPS with $Tt$Ddl revealed that the $K^+$ cleft of $Tt$Ddl overlays with a bound $K^+$ ($K^+_3$) of CPS (Figure 6A/B). Closer inspection of this site reveals high conservation between the two enzymes, with the only major difference being substitution of Met114 in Ddl, which rely on the phosphorylation of a catalytic histidine residue rather than a carboxyrate substrate. Overall, the $K^+$ cleft of Ddl was observed to be structurally conserved in ATP-grasp enzymes, particularly for Glu282, with most of these enzymes catalysing reactions which proceed through the formation of an acylphosphate intermediate. This suggests that other enzymes of the ATP-grasp superfamily may share the same Type II mechanism for MVC activation as proposed for Ddl.

DISCUSSION

This revealed that the $K^+$ cleft is structurally conserved for most of these ATP-grasp enzymes, and for the D-amino acid ligases VanA and VanG, complete conservation of the $K^+$ cleft was observed. Overall, the Glu282 and Asn284 positions showed high conservation, being conserved in 80% and 56% of the ATP-grasp enzymes analysed, respectively. While the Met114 residue was poorly conserved, most substitutions at this position project away from the $K^+$ cleft and do not block the MVC binding site. Of all the enzymes analysed, kinase and thiokinase members showed the least conservation in both the primary and secondary structure of the $K^+$ cleft (Figure S6, Table S6). One plausible explanation for this is due to differences in the catalytic mechanism for these enzymes compared to Ddl, which rely on the phosphorylation of a catalytic histidine residue rather than a carboxylate substrate.
P. The carboxylate sidechain of Glu282 also acts as a bidentate ligand for Mg2, which coordinates the oxyanions of the β/γ Pi (Figure 7A). We propose that when an activating MVC is bound at the K+ cleft the charge distribution of this coordination network is altered (Figure 7B). This may occur through the MVC attenuating the negative charge of the Glu282 carboxylate anion balanced by bidentate coordination of D-ala1, increasing the binding affinity of D-ala1 and resulting in an increased rate of D-ala-phosphate formation.

The complex of TiDdl with DCSP shown in Figure 7C/E mimics the binding mode of D-ala-phosphate and demonstrates how MVC binding at the K+ cleft could also modulate the Lewis acidity of Mg1 and Mg2 prior to nucleophilic attack by D-ala. This model is consistent with the ordered nature of the Ddl catalytic mechanism. If the binding affinity of D-ala1 is increased, this would result in faster D-ala-phosphate formation. As D-ala-phosphate formation is increased, D-ala can more readily react with D-ala-phosphate to form the tetrahedral intermediate, and upon collapse, D-ala-D-ala. This explains the observed decrease in KM for both D-ala substrates. The fact that a similar effect is not observed in Kcat supports product release being the rate-limiting step, consistent with previous isotope exchange experiments utilizing D-ala-D-ala and D-ala-D-X ligases (11, 22). The high conservation observed in the K+ cleft suggests that this mechanism of MVC activation applies generally for Ddl enzymes (Figure S4). Furthermore, the structural conservation of the K+ cleft and the key coordinating Glu residue between the ATP-grasp enzymes suggests that this mechanism of activation may apply more broadly across the superfamily.

This discovery of structural conservation at the K+ cleft within the functionally diverse members of the ATP-grasp superfamily provides a basis for further investigation of MVC activation. Several ATP-grasp enzymes require MVCs for maximal activity, however for many the effect of MVCs has yet to be elucidated (Table S1). As the functions of ATP-grasp enzymes are so diverse, understanding the role of MVCs would provide insight into many catalytic schemes. This is particularly relevant for ATP-grasp enzymes that show promise as drug targets and for enzyme catalysed chemical synthesis. Members of the ATP-grasp superfamily that have been investigated as drug targets include Ddl, Biotin Carboxylase, Tubulin Tyrosine Ligase, and the purine biosynthesis enzyme PurK (23–27).

In this study we demonstrate that the presence of activating MVCs has a substantial effect on the efficiency of Ddl, likely through altering the charge distribution at the active site. If this is found to apply to other ATP-grasp enzymes, we predict that using K+ concentrations that more closely reflect physiological conditions will allow more accurate characterization of inhibitors in vitro. More recently, L-amino acid ligases (LALs) have also been investigated for the industrial production of a range of products, including antibiotics and peptides for use in healthcare and consumable products (28–31). Despite many ATP-grasp enzymes sharing high similarity to Ddl, to our knowledge the activation of LALs by MVCs has not been investigated. Investigating activation by MVCs may lead to improvements in activity and broaden substrate specificity of LALs. Such examples demonstrate the importance of further characterising MVC activation for the ATP-grasp superfamily.

CONCLUSION

Through the structural investigation of Thermus thermophilus Ddl we have identified the site responsible for MVC activation, the K+ cleft, providing the first mechanistic insight into K+ activation of Ddl. Type II MVC activation of TiDdl was also confirmed, with activation being dependent on the ionic radius. For the Group I MVCs Li+ - Cs+, the smaller Li+ and Na+ showed little to no activation. In contrast, maximal activation was observed for the larger K+ and Rb+. Crystal structures of TiDdl captured at distinct stages of the catalytic mechanism revealed minimal conformational change in the presence of K+, suggesting that a conformational change is not responsible for the activating effect. We propose that activation occurs through K+ altering the charge distribution within the active site, increasing the binding affinity for the carboxylate substrate D-ala1 and promoting the formation of the acylphosphate intermediate. Structural comparison of TiDdl with other ATP-grasp enzymes has revealed that the K+ cleft is structurally conserved, suggesting the same mechanism of activation may be applicable to other ATP-grasp enzymes. Therefore, we recommend that future studies of ATP-grasp enzymes consider activation by MVCs.
**EXPERIMENTAL SECTION**

**Materials.**

For the enzyme activity assay, potassium antimonyl tartrate trihydrate and ammonium molybdate tetrahydrate were purchased from Sigma Aldrich, St. Louis, MO. Ascorbate and all chloride salts were of analytical grade or higher. KH$_2$PO$_4$ standards were prepared from a 10 mM KH$_2$PO$_4$ stock solution. For crystallization experiments, 50% PEG 3350, magnesium formate dihydrate (1 M) and Bis-Tris pH 6.8 was prepared by dropwise addition of 2 M NaOH. ATP, ADP, D-ala, DCS and D-ala-D-ala were purchased from Sigma Aldrich.

**Protein expression and purification.**

The vector pET16b was purchased from Genscript, Piscataway, NJ for the overexpression of TtDdl with a C-terminal hexahistidine tag. The TtDdl-pET16b vector was transformed into *Escherichia coli* BL21(DE3). For protein expression, 2 flasks containing 1 L lysogeny broth (LB) supplemented with 100 µg/ml ampicillin were inoculated with 20 ml of an overnight culture. Cells were grown at 37°C to an OD$_{600}$ of 0.5 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). TtDdl was expressed for 16 hours at 16°C. Cells were pelleted by centrifugation at 5000 x g for 20 min. Cell pellets were resuspended in 15 ml Buffer A (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol (BME) and lysed by 5 rounds of cell disruption. The lysate was clarified by centrifugation at 40000 x g for 40 min. The supernatant was applied to a Zetasep NiNTA column (emp Biotech) and eluted with a gradient of Buffer B (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 250 mM imidazole and 5 mM BME).

**Measurement of TtDdl activity.**

To measure the activity of purified TtDdl in the presence and absence of various monovalent cations (Li$^+$, Na$^+$, K$^+$, NH$_4^+$, Rb$^+$ and Cs$^+$), the antimonyl-phosphomolybdate colorimetric endpoint assay described by Bartolommei et al. was used, with several modifications (32). For all experiments, a 2x colour reagent (CR) (255 mM sulfuric acid, 1.2 mM ammonium molybdate tetrahydrate, 18 mM ascorbic acid, 72 µM potassium antimonyl tartrate trihydrate) was prepared from stock solutions of each component and diluted in MilliQ H$_2$O. The assay was optimized for use in a 96-well microplate format. Validation of the assay is provided in Figure S1. Calibration curves of KH$_2$PO$_4$ standards in the absence and presence of ATP and D-ala showed linearity for the measured concentration range of 1-15 µM P, in a 150 µl sample. All experiments were performed at 30°C in a reaction buffer containing 50 mM Tris-HCl pH 7.3 and 10 mM MgCl$_2$, with the concentration of ATP, D-ala and MVC varied. Prior to starting the reaction, both TtDdl and the reaction were equilibrated to 30°C. To start the reaction, 75 µl TtDdl (50 mM Tris-HCl pH 7.3, 10 mM MgCl$_2$) was added to 75 µl of reaction buffer (50 mM Tris-HCl pH 7.3, 10 mM MgCl$_2$ and 2x ATP, D-ala and MVC) and mixed by pipetting. The reaction was allowed to proceed for exactly 10 min. To stop TtDdl activity and begin colour development, 150 µl of 2x CR was added to the 150 µl sample and mixed by pipetting. Colour was developed for 10 min. Colour development was quenched with 20 µl of a 30% citric acid solution to prevent interference from acid-catalysed ATP hydrolysis in the presence of CR, giving a final volume of 320 µl. Absorbance was measured at a wavelength of 890 nm (Pherastar FSX microplate reader, BMG Labtech, Ortenberg, Germany) after 5 min. To account for acid-catalysed ATP hydrolysis during colour development, the absorbance of a no enzyme blank was subtracted from each sample. A separate calibration curve for 1-15 µM KH$_2$PO$_4$ was measured for each individual experiment, with the slope used to calculate the concentration of P, released by TtDdl catalysed ATP hydrolysis.
**Determination of kinetic parameters.**

For determination of kinetic parameters of ATP ($K_{M}$, $k_{cat}$) reactions were assayed for 8 concentrations ranging from at least 0.5 – 10x $K_{M}$, with 50 mM D-ala and 6.25 nM TtDdl (Figure S2). To determine the effect of varying KCl concentrations, the experiment was completed in the presence of 0, 10, 50 and 100 mM KCl. Experiments were repeated at least twice, with $n=3$ technical replicates for each concentration assayed. For determination of kinetic parameters of D-ala ($K_{M}$ D-ala1, $K_{M}$ D-ala2, $k_{cat}$) reactions were assayed for ≥7 concentrations ranging from at least 0.3 – 5x $K_{M}$, with 300 μM ATP and 12.5 mM TtDdl (Figure S2). To determine the effect of various MVCs, the experiment was completed for 0, 10, 50 and 100 mM KCl, and for 50 mM LiCl, NaCl, NH₄Cl, RbCl and CsCl. Experiments were repeated at least twice, with $n=3$ technical replicates for each concentration assayed. To determine $K_{M}$ data was fit with the Michaelis – Menten Equation (1) for a single binding site by non-linear regression. To determine $k_{cat}$, $K_{M}$ D-ala1 and $K_{M}$ D-ala2 data was fit with Equation (2) by non-linear regression, as derived by Neuhaus et al for the steady state kinetics of two identical substrates with ordered binding (5). As $K_{M}$ D-ala1 was outside the range of measurable D-ala concentrations for the assay in the presence of >10mM KCl, $K_{M}$ D-ala1 was reported as less than the lowest concentration of D-ala assayed for these conditions (Table 1). The parameters $k_{cat}$ and $K_{M}$ D-ala2 and their standard deviations ($\Delta k_{cat}$ and $\Delta K_{M}$) were then used to calculate $k_{cat}/K_{M}$ D-ala2 values. The standard deviation for $k_{cat}/K_{M}$ D-ala2 values $\Delta (k_{cat}/K_{M}$ D-ala2) was calculated as: $\Delta (k_{cat}/K_{M}$ D-ala2) = $(k_{cat}/K_{M}$ D-ala2)[$(\Delta k_{cat}/ k_{cat})^2 + (\Delta K_{M}$ D-ala2/$K_{M}$ D-ala2)^2]^{1/2}$.

**Crystallization of TtDdl.**

For crystallization of TtDdl in complex with Rb⁺, 10 mg/ml TtDdl was incubated with 5 mM ATP, 5 mM MgCl₂, 50 mM D-ala and 150 mM RbCl for 20 min at 60°C. Crystals of TtDdl in complex with Cs⁺ were similarly obtained by incubating 10 mg/ml TtDdl with 5 mM ATP, 5 mM MgCl₂, 50 mM D-ala and 80 mM CsCl for 20 min at 60°C. Crystals of the product complex of TtDdl were obtained without addition of substrates or products, with 10 mg/ml TtDdl kept on ice prior to crystallization. To obtain apo crystals of TtDdl, 10 mg/ml TtDdl was heated at 60°C for 20 min prior to crystallization. Crystals of TtDdl in complex with ADP, DCSP and K⁺ were obtained by incubating 10 mg/ml TtDdl with 37 mM DCS for 30 min at 60°C. Crystals of TtDdl in complex with ADP and P₂ were obtained by incubation of 10 mg/ml TtDdl with 3 mM ATP, 8 mM MgCl₂ and 150 mM KCl for 20 min at 60°C. Crystals of TtDdl in complex with ADP and D-ala-D-ala were obtained by incubating TtDdl with 3 mM ADP, 8 mM MgCl₂, 40 mM D-ala and 150 mM KCl for 20 min at 60°C. A carbonate anion (CO₃²⁻) was modelled to clearly planar electron density at the expected position of the γ-P; of bound ATP. This was previously observed in structures E. coli Ddl (33). The unexpected presence of products in several of these structures suggests that the TtDdl used for crystallography copurified with substrates/products. The incubation step at 60°C was included as it appeared to promote crystal formation. Crystals were obtained by adding 1 μl of incubated TtDdl to 2 μl of well solution (0.5 ml) containing 15-18% PEG 3350, 100 mM Bis-Tris pH 6.8 and 100 mM magnesium formate, using the hanging drop vapour diffusion method in 24 well plates (Costar, Corning, NY) stored at 16°C.

**Data collection, structure determination, and refinement.**

Crystals were transferred to Paratone-N for cryoprotection and flash frozen in liquid nitrogen. Datasets were collected at the MX1 and MX2 beamlines of the Australian Synchrotron (17). Indexing and integration was completed using XDS (34). Aimless (CCP4) was used for scaling and merging datasets (35). The structures of TtDdl were solved by molecular replacement using Phaser with a previously solved structure of TtDdl (PDB: 2ZDH) as the search model (3, 36). For data collected at 15250 eV (RbCl, PDB:6U1E) and 8500 eV (CsCl, PDB:6U1D) the corresponding model...
solved at 13000 eV was used. Modelled ligands and waters were removed from all search models. Solutions from Phaser were refined in Phenix. These models were subjected to multiple rounds of rebuilding in Coot followed by B-factor and positional refinement in Phenix until R-factors converged (37, 38). The results, including data processing and refinement statistics, are presented in Table S3. For the final models, greater than 95% of modelled residues were favoured in the Ramachandran plot. The only outlier, Gly86, fits well in the 2Fo-Fc electron density map. For identification of MVC binding sites anomalous difference maps were generated to a resolution of 3.5 Å for TtDdl-ATP-D-ala-D-ala-Rb<sup>+</sup>anom and TtDdl-ATP-D-ala-D-ala-Cs<sup>+</sup>anom datasets using phenix.maps (38). K<sup>+</sup> were modelled using a combination of Fo-Fc difference maps, occupancies, B-factors and bond lengths for validation.

**Structure comparison and data analysis.**

Protein secondary structure was assigned using DSSP (39). Structural superposition was completed using secondary structure matching in Coot (37). Figures for visualising enzyme structure were generated using PyMOL (40). PDB2PQR and APBS were used for electrostatic surface calculations (41). Multiple sequence alignment was performed using Clustal Ω (42).

**Data availability**

All structures have been deposited to the RCSB Protein Data Bank (https://www.rcsb.org/) with the accession codes 6U1C, 6U1D, 6U1E, 6U1F, 6U1G, 6U1H, 6U1I, 6U1J and 6U1K. All remaining data are contained within the article.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. Catalytic mechanism of ATP-grasp enzymes. A) The general mechanism of ATP-grasp enzymes proceeds through two half reactions - (i) The carboxylate substrate reacts with ATP to form an acylphosphate intermediate, requiring a divalent metal (Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$). (ii) The nucleophile substrate attacks the electrophilic acylphosphate intermediate to form the product. B) The proposed catalytic mechanism of Ddl. This proceeds with an ordered ter-ter mechanism (three substrates and three products) through two half reactions. The first half reaction (i) involves the phosphorylation of D-ala$_1$ (1) to form the acylphosphate intermediate D-ala phosphate (2). In the second half reaction (ii), D-ala phosphate is attacked by D-ala$_2$ (3), resulting in the formation of a tetrahedral intermediate, D-ala-D-ala phosphate (4). This second intermediate then collapses within the active site to form D-ala-D-ala (5) and inorganic phosphate (6). It has been determined that K$^+$ is also required for optimal activity; however, the mechanism by which this occurs has been elusive.
Figure 2. Overall fold of TtDdl. A) Apo form of a single TtDdl monomer (PDB:6U1C). The N-terminal domain, central domain and C-terminal domain of the TtDdl monomer are coloured green, yellow and cyan, respectively. Loops 1-3 are labelled and shown in red. For apo TtDdl all loops exist in the open conformation, with the Ω-loop (Loop 2) disordered and not observed for aa. 217-234. B) The product complex (ADP, P\(_i\) and D-ala-D-ala bound) of a single TtDdl monomer (PDB: 6U1J). All loops are closed, with the Ω loop becoming ordered and covering the substrate binding pocket. ADP, P\(_i\) and D-ala-D-ala are shown in pink spheres.
Table 1. Effect of KCl on TriDl catalysis.

| Condition | $K_{M_{\text{ATP}}}$ (µM) | $K_{M_{\text{D-ala}}}$ (µM) | $K_{M_{\text{D-ala1}}}$ (µM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_{M_{\text{D-ala2}}}$ (s$^{-1}$ M$^{-1}$) |
|-----------|--------------------------|---------------------------|---------------------------|-----------------|----------------------------------|
| Low MVC$^b$ | 16.2 ± 1.1               | 4020 ± 630$^c$            | 1250 ± 490$^c$            | 1.26 ± 0.04$^c$ | 315 ± 50$^c$                      |
| 10 mM KCl  | 17.3 ± 1.0               | 315 ± 19                  | <55$^d$                   | 1.48 ± 0.02     | 4690 ± 290                        |
| 50 mM KCl  | 12.4 ± 0.5               | 150 ± 15                  | <50$^d$                   | 1.07 ± 0.02     | 7140 ± 710                        |
| 100 mM KCl | 12.1 ± 0.7               | 190 ± 24                  | <50$^d$                   | 1.04 ± 0.03     | 5460 ± 700                        |

$^a$All kinetic parameters reported were determined from n=2 experiments each with n=3 technical replicates unless stated otherwise. Error represents ± standard deviation. $^b$<1 mM Na$^+$ present with ATP. $^c$Determined from n=5 experiments each with n=3 technical replicates. $^d$Lowest concentration of D-ala assayed.
Figure 3. TrDdl is activated by MVCs. Addition of 50 mM KCl, NH₄Cl, RbCl and CsCl (blue) resulted in increased TrDdl efficiency, indicated by an increase in $k_{cat}/K_M$ for D-ala₂. This effect was in contrast to the Low MVC, 50 mM LiCl and 50 mM NaCl conditions (red), showing that this was not due to a change in Cl⁻ concentration or ionic strength. Data shows $k_{cat}/K_M$ for D-ala₂ calculated from n=2 experiments, except for the Low MVC condition (n=5 experiments). Error bars represent ± standard deviation; calculated as detailed in the Experimental Section.
Figure 4. MVC binding sites of TtDdl. A) Anomalous difference maps for TtDdl-Rb\textsuperscript{+} anom (15250 eV, blue mesh; Top) and TtDdl-Cs\textsuperscript{+} anom (8500 eV, purple mesh; Bottom) contoured to 6\(\sigma\) and 5\(\sigma\) respectively. The corresponding models are shown as yellow and green ribbons respectively, with ATP and D-ala-D-ala shown as sticks. B) Rb\textsuperscript{+} (blue sphere) and Cs\textsuperscript{+} (cyan sphere) binding at Site 2. Site 2 (the K\textsuperscript{+} cleft) is in close proximity to the active site. Electron density for simulated annealing composite omit maps (2F\textsubscript{o}-F\textsubscript{c}, 1.5\(\sigma\)) is shown for the ligands of Rb\textsuperscript{+} (blue mesh) and Cs\textsuperscript{+} (purple mesh) bound TtDdl. Bound Mg\textsuperscript{2+} are shown as green (Rb\textsuperscript{+}) and dark green (Cs\textsuperscript{+}) spheres. The three loop regions of TtDdl (red; P-loop, \(\Omega\)-loop and Loop 3) are in the closed conformation. C) The coordination network of Rb\textsuperscript{+} (blue spheres) and Cs\textsuperscript{+} (cyan spheres) at the K\textsuperscript{+} cleft extends to both Mg\textsuperscript{2+} and ATP. The coordination sphere of Rb\textsuperscript{+} and Cs\textsuperscript{+} is shown as blue and cyan dashes, respectively. Coordinating waters are shown as red (Rb\textsuperscript{+}) and pink (Cs\textsuperscript{+}) spheres, with the water bridging the MVC and Mg\textsuperscript{2+} labelled (H\textsubscript{2}O\textsubscript{B}). Coordinating residues and the residues forming the K\textsuperscript{+} cleft are shown as yellow (Rb\textsuperscript{+}) and green (Cs\textsuperscript{+}) sticks. The coordination spheres of bound Mg\textsuperscript{2+}; green spheres (Rb\textsuperscript{+}) and dark green spheres (Cs\textsuperscript{+}), are shown as green and dark green dashes, respectively. The P-loop and \(\Omega\)-loop are omitted for clarity. D) Electron density for feature enhanced maps (2F\textsubscript{o}-F\textsubscript{c}, 2\(\sigma\)) of TtDdl-Rb\textsuperscript{+} (left; blue mesh) and TtDdl-Cs\textsuperscript{+} (right; purple mesh) Site 2 coordination spheres.
Figure 5. K⁺ binding at the K⁺ cleft does not induce conformational change. A) Electron density for feature enhanced map (2Fo-Fc, 2σ) of TriDdl-K⁺ (PDB: 6U1K) at the K⁺ cleft. The coordination sphere of K⁺ (purple spheres; purple dashes) is analogous to that observed for Rb⁺ and Cs⁺ (Figure 4C/D). B) Superposition of apo (yellow) and K⁺ bound (cyan) TriDdl structures (Table S3). Minimal conformational change is evident on K⁺ binding at the K⁺ cleft throughout the catalytic cycle. Bound Mg²⁺ are shown as green spheres, with coordinating bonds shown as green dashes. Coordinating waters are shown as red spheres. Bound ligands were omitted for clarity. C) Superposition of apo (yellow) and K⁺ bound (cyan) TriDdl structures shows no change in conformation of residues forming the D-ala binding sites. ADP and DCSP (PDB:6U1I) are shown as cyan sticks, with the DCS moiety indicating the position of the D-ala₁ pocket. Mg²⁺ are shown as green spheres. The P-loop is seen to be in the open conformation for apo TriDdl as no nucleotide is bound. The Ω-loop is omitted for clarity.
Figure 6. MVC binding sites of ATP-grasp enzymes. A) Superposition of carboxyphosphate domain of E. coli carbamyol phosphate synthetase (EcCPS; blue, PDB: 1JDB) with TtDdl in complex with ADP and P_i (yellow, PDB:6U1H). In the ATP-grasp superfamily CPS is unique in the fact that it has a Type I mechanism of activation, with K^+ essential for its activity. The 3 separate K^+ binding sites (K^+_1, K^+_2 and K^+_3; grey spheres) of EcCPS carboxyphosphate domain are labelled. MVC activation of CPS has been attributed to K^+_1 and K^+_2, while the role of K^+_3 remains unknown. Superposition with TtDdl reveals that K^+_3 binds at the same position as the K^+ cleft. Bound Mg^{2+} (TtDdl) and Mn^{2+} (EcCPS) are shown as green and dark green spheres, respectively. K^+ bound to TtDdl is shown as a purple sphere. Bound ADP and P_i are shown as sticks. B) Comparison of K^+_3 of EcCPS (grey sphere, grey dashes) with K^+ at the K^+ cleft of TtDdl (purple sphere, purple dashes). The overall structure of the site is highly conserved between the two enzymes, with the key difference being an additional contact from Glu126 of CPS, at the equivalent position of Met114 of TtDdl. Residues forming the K^+ cleft are shown as sticks. C) Superposition of E. coli Biotin carboxylase (EcBC; light blue, PDB:3RV4) with TtDdl (PDB:6U1H). For EcBC, Cs^+ (cyan sphere, cyan dashes) was modelled at the same position as K^+ (purple sphere, purple dashes) bound at the K^+ cleft of TtDdl. Coordinating waters are shown as red spheres (K^+) and pink spheres (Cs^+). Residues forming the K^+ cleft are shown as sticks. Bound Mg^{2+} are shown as green (TtDdl) and dark green (EcBC) spheres. The P-loop is omitted for clarity.
Figure 7. Proposed mechanism of MVC activation for TtDdl. A) Proposed Mg\(^{2+}\)-ATP complex in the absence of an activating MVC. Mg\(_1\) and Mg\(_2\) (green spheres, green dashes) act to stabilize the negative charge of the triphosphate moiety of ATP, facilitating nucleophilic attack by D-ala\(_1\). Model was prepared by superimposing the structures of TtDdl in complex with RbCl (PDB:6U1D, yellow) and TtDdl in complex with ADP and D-ala (PDB:2ZDH, cyan). Coordinating atoms and waters are shown as red spheres. B) Mg\(^{2+}\)-ATP complex in the presence of an activating MVC. The bound MVC (purple sphere, purple dashes) balances the negative charge of the Glu282 carboxylate anion, allowing Mg\(_2\) to withdraw additional electron density from the \(\beta/\gamma\) P\(_i\). This reduces the electrostatic repulsion between the \(\gamma\) P\(_i\) of ATP and the nucleophilic carboxylate anion of D-ala\(_1\), increasing the binding affinity of the D-ala\(_1\) substrate. Model prepared as described in 6A. C) Reaction intermediate complex following phosphoryl transfer. TtDdl in complex with Mg\(^{2+}\)-ADP and DCSP mimics the acylphosphate intermediate. On transfer of the \(\gamma\)-P\(_i\) to DCS, Mg\(_1\) shifts to stabilize the negative charge of all 3 phosphate groups, replacing the interaction with Asp270 by coordination of an additional water. Model represents the structure of TtDdl in complex with DCSP (PDB:6U1I). D) Chemical representation of the Mg\(^{2+}\)-ATP complex in the presence of an activating MVC. Dashed lines represent coordinating bonds, with distances labelled in angstroms (PDB:6U1D). The black arrow adjacent to Glu282 represents the shift of charge for the carboxylate group on MVC binding. * Bond projects out of the page. E) Chemical representation of the Mg\(^{2+}\)-ADP-DCSP complex in the presence of an activating MVC. Dashed lines represent coordinating bonds, with distances labelled in angstroms (PDB:6U1I). * Bond projects out of the page.
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