Check for updates

RESEARCH ARTICLE

Thiolation-enhanced substrate recognition by D-alanyl carrier protein ligase DltA from *Bacillus cereus* [version 1; peer review: 2 approved]

Liqin Du, Yu Luo

Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E5, Canada

V1 First published: 13 May 2014, 3:106
<https://doi.org/10.12688/f1000research.4097.1>
Latest published: 13 May 2014, 3:106
<https://doi.org/10.12688/f1000research.4097.1>

Abstract

D-alanylation of the lipoteichoic acid on Gram-positive cell wall is dependent on *dlt* gene-encoded proteins DltA, DltB, DltC and DltD. The D-alanyl carrier protein ligase DltA, as a remote homolog of acyl-(coenzyme A) (CoA) synthetase, cycles through two active conformations for the catalysis of adenylation and subsequent thiolation of D-alanine (D-Ala). The crystal structure of DltA in the absence of any substrate was observed to have a noticeably more disordered pocket for ATP which would explain why DltA has relatively low affinity for ATP in the absence of any D-alanyl carrier. We have previously enabled the thiolation of D-alanine in the presence of CoA as the mimic of D-alanyl carrier protein DltC which carries a 4'-phosphopantetheine group on a serine residue. Here we show that the resulting Michaelis constants in the presence of saturating CoA for both ATP and D-alanine were reduced more than 10 fold as compared to the values obtained in the absence of CoA. The presence of CoA also made DltA ~100-fold more selective on D-alanine over L-alanine. The CoA-enhanced substrate recognition further implies that the ATP and D-alanine substrates of the adenylation reaction are incorporated when the DltA enzyme cycles through its thiolation conformation.

Open Peer Review

Approval Status

	1	2
version 1
13 May 2014	view	view
1. Ting Fang Wang , Academia Sinica, Taipei, Taiwan		
2. Charles Bell , Ohio State University, Columbus, OH, USA		

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Yu Luo (yu.luo@usask.ca)

Competing interests: No competing interests were disclosed.

Grant information: This work is supported by a Saskatchewan Health Research Foundation Phase 3 Team Grant to the Molecular Design Research Group at University of Saskatchewan, and by a Natural Sciences and Engineering Research Council Discovery Grant 261981-2010 to YL.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2014 Du L and Luo Y. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Du L and Luo Y. **Thiolation-enhanced substrate recognition by D-alanyl carrier protein ligase DltA from *Bacillus cereus* [version 1; peer review: 2 approved]** F1000Research 2014, **3**:106 <https://doi.org/10.12688/f1000research.4097.1>

First published: 13 May 2014, **3**:106 <https://doi.org/10.12688/f1000research.4097.1>

Introduction

The cell surface of most Gram-positive bacteria contains wall teichoic acid and lipoteichoic acid with a poly-alditol phosphate backbone. The remaining hydroxyls of the alditol moiety are ubiquitously modified by D-alanyl esterification or glycosylation^{1–3}. A *dlt* operon, which typically codes for DltA, DltB, DltC and DltD proteins, is required for the D-alanylation of lipoteichoic acids⁴. D-alanylation brings in positively charged amino-groups and partially neutralizes the net negative charges of the phosphate groups on the lipoteichoic acid backbone. The reduction of D-alanyl content on the cell surface has been found to be associated with increased autolysis^{3,5} and susceptibility to host defense peptides and other antibiotics^{6,7}. Impaired D-alanylation of lipoteichoic acid also reduces the ability of bacteria to colonize any surface⁸ and form antibiotics-resistant biofilms⁹. Therefore, the *dlt*-encoded proteins required for the lipoteichoic acid D-alanylation pathway could serve as novel targets for fighting emerging infectious diseases caused by Gram-positive pathogens¹⁰. The DltA inhibitor 5'-O-[N-(D-alanyl)-sulfamoyl] adenosine for example, designed by analogy to D-alanyl adenylate (D-Ala-AMP), has been shown to significantly suppress the growth of *B. subtilis* when used in combination with vancomycin¹¹.

The D-alanyl carrier protein ligase DltA (formed by ~500 amino acid residues)⁴ closely resembles (~30% sequence identity) the adenylation domains (also called adenosine monophosphate (AMP)-forming domains) found in bacterial non-ribosomal peptide synthetases¹². Its remote homologs include the acyl- and aryl-(coenzyme A) CoA synthetases and firefly luciferases¹³. As shown in Figure 1, DltA catalyzes the ATP-driven adenylation of D-alanine

and the subsequent transfer of the activated D-alanyl group to the thiol group of 4'-phosphopantetheine, which is covalently bound to a serine side chain of D-alanyl carrier protein DltC (~80 amino acid residues)⁴. As previously shown for such one-protein-two-enzymes homologs^{14,15}, crystal structures of two DltA proteins have also been observed in adenylation and thiolation conformations, respectively^{16,17} (Figure 1). The thiolation conformation of *B. subtilis* DltA (BsDltA)¹⁸ resembles previously determined structures of a bacterial acetate-CoA synthetase (ACS) and 4-chlorobenzoate-CoA ligase crystallized in their respective thiolation state^{15,19}. On the other hand, the adenylation conformation of *B. cereus* DltA (BcDltA)¹⁷ resembles those observed in the crystal structures of the firefly luciferase PheA (phenylalanine-activating domain of the first module of the *Bacillus brevis* gramicidin S synthetase I), DhbE (2,3-dihydroxybenzoate activation domain from *B. subtilis*), a yeast acetyl-CoA synthetase and 4-chlorobenzoate-CoA ligase^{13,14,20–22}. The two conformations captured in the crystal structures of the two closely related DltA proteins (56% sequence identity) are related by a ~146° rotation around a hinge residue Asp-399 in BcDltA (Asp-398 in BsDltA), which is an invariable residue equivalent to Asp-517 in *Salmonella typhimurium* acetyl-CoA synthetase²³. The region surrounding this hinge residue (residues Arg-397 to Glu-413 of BcDltA green in Figure 1B) contains an important triphosphate-interacting residue Arg-397²⁴ and a β-hairpin which serves as part of the pantetheine channel as observed in the bacterial acetyl-CoA synthetase²³.

We previously studied the adenylation reaction of BcDltA in the absence of any D-alanyl carrier to enable the thiolation reaction¹⁷.

Figure 1. Structure and function of DltA. **A.** A two-step reaction catalyzed by DltA. **B.** The adenylation and thiolation conformations of DltA. In the first adenylation reaction, D-alanine is converted into DltA-bound D-alanine adenylate (D-Ala-AMP). This reaction is catalyzed by the adenylation conformation of DltA shown as DltA/AMP complex. In the subsequent thiolation reaction, the activated alanyl group in the intermediate is transferred to the thiol group of DltC-linked 4'-phosphopantetheine (DltC-SH). The second reaction is catalyzed by the thiolation conformation of DltA shown also as DltA/AMP complex. Most part of the major N-terminal domain of DltA (residues 2 - 399) is shown in gray. The majority of the minor C-terminal domain of DltA (residues 400–504) is shown in salmon. Residues between Arg-397 and Glu-413 are highlighted in green.

The resulting specificity constants (k_{cat}/K_M) for D- and L-alanine differed by merely ~3-fold, which could not explain the fact that teichoic acid is overwhelmingly modified by D- but not L-alanine²⁵. As suggested by a study on a spectrum of DltA homologs which have residual aminoacyl-CoA synthetase activity⁴², CoA has been confirmed to be a substitute for the D-alanyl carrier protein DltC as the thiolation substrate of BcDltA²⁴. Here we report further biochemical analysis of BcDltA. Noticeable differences were observed in Michaelis constants K_M and turnover rates k_{cat} . The presence of CoA, hence the enabled adenylation and thiolation cycle, enhanced the enzyme's apparent affinities to its cognitive substrate ATP and D-alanine by approximately an order of magnitude. Since BcDltA is a slow enzyme with turnover rate less than 1 s^{-1} , much slower than bacterial Acyl-CoA synthetases ($\sim 10^2 \text{ s}^{-1}$)²³ and 4-chlorobenzoate-CoA ligase ($\sim 10 \text{ s}^{-1}$)²⁶, the observed Michaelis constants should closely approximate the corresponding dissociation constants, and therefore provide some insight into the stability of short-lived enzyme-substrate complexes. We also determined the structure of BcDltA in the absence of any substrate. This structure is noticeably more disordered than previously reported DltA structures^{16,17,24}, which may explain the enzyme's lower affinity to ATP in the absence of the other two substrates. Interestingly, CoA-enhanced affinities to ATP and D-alanine imply that the thiolation substrate CoA-bound BcDltA has higher affinity to both adenylation substrates as compared to CoA-free BcDltA.

Methods

Cloning, protein preparation and crystallization

All reagents were from VWR unless specified otherwise. The wild-type as well as the C269A mutant of DltA from *B. cereus* was cloned for over-expression of BcDltA as described previously¹⁷. The pET28-BcDltA construct carries an Ala-1 mutation at the N-terminus and eight extra residues at the C-terminus (LEHHHHHH). The soluble fraction of BcDltA was purified by nickel-affinity chromatography followed by gel filtration. BcDltA was concentrated to ~20 mg/mL by ultra-filtration.

Crystallization and structure determination

The concentrated BcDltA protein was crystallized using the hanging drop crystallization method at a room temperature of 21°C. The optimal well solution for crystallization contained 0.1 M MgCl_2 , 0.5 M KCl, 16% polyethylene glycol (PEG) 3,350 (Sigma-Aldrich) and 0.05 M Hepes-NaOH buffer at pH 7.2. Each drop was composed of 1 μL of protein and 1 μL of well solution. The plate-shaped crystals grew to a maximal size of 0.4 mm $\times$ 0.3 mm $\times$ 0.05 mm in three days. Crystals were gradually transferred to stabilizing solutions composed of the crystallization well solution supplemented with 8%, 16% and 24% glycerol, soaked for 1 minute, then flash-cooled to -173.15°C in a nitrogen stream generated by an Oxford CryoSystems device. A total of 400 0.4-degree oscillation images were acquired and processed using a Bruker Proteum-R system as already described²⁷. The previously solved BcDltA model (PDB code 3DHV)¹⁷ was used as the starting model for two-domain rigid body refinement followed by positional refinement using **Crystallography & NMR System (CNS)**²⁸. This resulting model was subjected to ten cycles of rebuilding and refinement using Arp/Warp²⁹. The rebuilt model was iteratively rebuilt using XtalView³⁰ and then refined using CNS²⁸. The final model had 90.8% of the residues

in the most favored regions on a Ramachandran plot. Val-301 with clear electron density and Asp-336 with blurry electron density were the only two residues found in the disfavored region. Statistics of the diffraction data, refinement and geometry are listed in **Table 1**. The molecular figures were generated using Molscript³¹ and rendered using Raster3D³². The coordinates and structure factors have been deposited in the Protein Data Bank^{33–35} (entry code 4PZP).

Tryptophan fluorescence measurement

The intrinsic tryptophan fluorescence of 1.0 ml 0.4 μM BcDltA solution with 0 to 2 mM ATP was acquired at a room temperature of 21°C using a PerkinElmer LS-55 fluorescence spectrometer. The excitation wavelength was 305 nm and a fluorescent emission in the

Table 1. X-ray crystallographic data collection and structure refinement statistics.

Substrate-free BcDltA	
Data collection	
Space group	P2 ₁
Cell dimensions	
a, b, c (Å)	52.9, 81.9, 59.3
α , β , γ (°)	90.0, 108.3, 90.0
Resolution (Å)	33.1–1.9 (2.00–1.90) ^a
^b R _{sym}	0.077 (0.321)
I/ σ I	7.5 (1.8)
No. reflections	32733 (1982)
Completeness (%)	86.3 (37.7)
Redundancy	4.3 (3.2)
Refinement	
Resolution (Å)	30.0–1.9
No. reflections	32658
^c R _{work} /R _{free}	0.218/0.261
No. atoms:	
Protein	3643
Water	279
Average B-factors:	
Protein	22.9
Water	27.7
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.47

Notes:

^a Values in parentheses refer to values in the highest resolution shell.

^b $R_{\text{sym}} = \sum |I_h - \langle I \rangle_h| / \sum I_h$, where $\langle I \rangle_h$ is average intensity over symmetry equivalents, h is reflection index. The summation is over all measured reflections.

^c $R_{\text{work}} = \sum |F_{\text{calc}} - F_{\text{obs}}| / \sum F_{\text{obs}}$. The summation is over all reflections used in refinement. R_{free} is calculated using a randomly selected 5% of the reflections set aside throughout the refinement.

310 nm to 390 nm range was recorded. The relative fluorescence increase at 345 nm was used to quantify the ATP-bound fraction of BcDltA. Assuming that the fluorescence gain is proportional to $[ATP]/(K_D + [ATP])$, Prism software (GraphPad Software) was used to derive the dissociation constant of ATP.

Pyrophosphate quantification assay

As previously described, pyrophosphate released from the adenylation reaction is broken down into phosphate by pyrophosphatase¹⁷. The resulting phosphate was quantified by a dye solution containing 0.033% w/v Malachite Green, 1.3% w/v ammonium molybdate and 1.0 M HCl³⁶. The 200 μ L reaction solutions contained 5 μ M BcDltA, 0.1 M KCl, 0.01 M MgCl₂, 0.05 M Tris-Hepes buffer at pH 7.2, 5 unit/mL of inorganic pyrophosphatase from baker's yeast (Sigma-Aldrich) and specified concentrations of D-alanine, ATP and CoA (Sigma-Aldrich). A volume of 25 μ L of reaction solution was retrieved every 3 or 5 minutes and mixed thoroughly with 475 μ L of the dye solution. The absorption at a wavelength of 620 nm was recorded after 90 seconds. The initial rates (1/2 of the phosphate concentration increase per minute) of the adenylation reaction were derived from the time courses of phosphate accumulation. The correlation between initial reaction rate and substrate concentration was fitted with Michaelis-Menten equation using the Prism software (GraphPad Software).

Thiol quantification assay

The free thiol group of CoA was quantified as described previously²⁴ by a dye solution composed of 1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTBN), or Ellman's reagent (Sigma-Aldrich) and 50 mM Tris-EDTA solution at pH 8.0. Absorption at a wavelength of 412 nm was used to quantify the concentration of free thiol group. The reaction rate was derived by the rate of thiol depletion. The correlation between initial reaction rate and substrate

concentration was also fitted with Michaelis-Menten equation using the Prism software.

Results

Crystal structure of substrate-free BcDltA

The same DltA protein from *B. cereus* with a C-terminal hexahistidyl fusion tag used in our previous crystallographic studies on BcDltA^{17,24} was crystallized in the absence of ATP, D-alanine or CoA. One crystal diffracted to 1.9 Å resolution and belonged to space group P2₁ (Table 1), the same space group as in previously reported crystals of BcDltA in complex with D-alanine adenylate¹⁷ and with ATP²⁴. Despite having 8 Å shorter crystallographic a axis, 5 Å shorter b axis and 5° smaller β angle than the previously reported crystal of DltA/D-alanine adenylate complex, the structure was successfully solved by rigid-body refinement using the previously determined BcDltA structure¹⁷ (PDB code 3DHV) as the starting model. The 504-residue BcDltA structure can be divided into two domains (Figure 2): an N-terminal major domain from the N-terminus to Asp-399, and a C-terminal minor domain from residue 400 to the C-terminus. The disposition of the two domains in the substrate-free BcDltA structure remains similar to that of the starting model (Figure 2A). The electron density map indicated several disordered regions (Ser-153 to Pro-159, Pro-363 to Glu-367, Arg-397 to Glu-413, Lys-433 to Tyr-440) with the corresponding regions in the starting model highlighted in magenta in Figure 2B. The first disordered region is part of a highly conserved P-loop (Thr-152 to Lys-160) found in homologous AMP-forming proteins^{37–39}. Due to its similar amino acid composition (glycine, serine, threonine and lysine) to that of P-loop or Walker A motif found in ATPases and GTPases⁴⁰, this loop has long been thought to catalyze the adenylation reaction. In the crystal structure of human medium-chain acyl-CoA synthetase in complex with ATP, this loop intimately interacts with the β - and γ -phosphates of the ATP substrate⁴¹. Functional

Figure 2. Structure of DltA in the absence of substrate. The ribbons representation of previously reported BcDltA structure (PDB entry: 3DHV) is shown with D-alanine-adenylate and surrounding side-chains in ball-and-stick model. The N-terminal domain is shown in gray, and the C-terminal domain in gold. **A.** The substrate-free structure of BcDltA is superimposed on the BcDltA/D-Ala-AMP complex. The N- and C-terminal domains are colored in cyan and magenta, respectively. **B.** The four corresponding regions of the BcDltA/D-Ala-AMP complex, which are disordered in the substrate-free form of BcDltA, are highlighted in magenta.

relevance of regions 363–367 and 433–440 are unknown. The former is located at a 2-turn helix at the surface of the N-terminal domain (Figure 2B). The latter is interacting with the longest disordered inter-domain region in this structure (397–413) which contains several key elements of DltA. Arg-397 has been observed to interact with the β -phosphate of ATP and to play an important role in catalysis²⁴. Asp-399, equivalent to Asp-398 in BsDltA, serves as the hinge residue for domain rotation. As observed for the equivalent Asp-402 of 4-Chlorobenzoate-CoA ligase¹⁵, the rotation around main-chain single bonds in this hinge residue could account for a 146° swing of the C-terminal domain as we compared the crystallized adenylation conformation of BcDltA and thiolation domain of BsDltA (Figure 1, bottom with the disordered inter-domain linker in green). The equivalent main-chain atoms in the N-terminal domains of BcDltA in adenylation conformation (PDB entry 3DHV) and BsDltA in thiolation conformation (PDB entry 3E7W) are superposed with a root-mean-deviation of 0.97 Å, and the deviation for the C-terminal domain was 1.00 Å. These values indicate that there is no dramatic conformational change within each domain in addition to the 146° rotation around the hinge aspartate residue. The C-terminal part of this flexible inter-domain region also contains a β -hairpin which has been observed to interact with CoA in homologous acetyl-CoA synthetase²³.

K_M and k_{cat} of BcDltA in the presence and absence of D-alanyl carrier CoA

In our previous study, we have verified that CoA can mimic D-alanyl carrier protein DltC²⁴, as also discovered for DltA homologs⁴². In that study, we have observed that the reaction rate is increased by nearly an order of magnitude by the presence of saturating concentration of CoA, which is explained by the faster release of the thiolation product rather than by release of the adenylation intermediate. In order to get a comprehensive understanding of the effects by CoA as the DltC mimic, we further studied the enzymatic properties of BcDltA in the presence of a saturating concentration (5 mM) of ATP or D-alanine, and in the absence of CoA or in the presence of a saturating concentration (5 mM) of CoA. The reaction rates derived from the pyrophosphate accumulation assay and the thiol depletion assay were similar (Figure 4). The thiolation assay was noticeably noisier than the pyrophosphate assay and we therefore limit the discussion to K_M and k_{cat} values derived from the pyrophosphate assay. Somewhat unexpectedly, BcDltA showed much higher apparent affinity, or decreased K_M value, towards ATP (0.46 mM to 0.01 mM) and D-alanine (1.1 mM to 0.03 mM) in the presence of 5 mM CoA (Figure 3 and Figure 4, Table 2). On the contrary, the apparent affinity towards L-alanine decreased in the presence of CoA, with K_M increased from 14.4 mM to 109 mM (Figure 4 and Table 2).

Relaxed D-alanine preference by the C269A BcDltA mutant protein

The side-chain of Cys-269 sits at the bottom of the D-alanine-binding pocket which may make VDW clash with the methyl side-chain of L-Alanine^{16,17}. We also studied the effect of CoA on D- and L-alanine preference of the C269A mutant of BcDltA (Figure 4 and Table 2). In the presence and absence of CoA, the C269A protein showed relaxed preference for D-alanine over L-alanine. As observed for the wild-type protein, CoA also enhances the D-alanine preference

Figure 3. Rate of pyrophosphate release and ATP-induced fluorescence gain. The reaction solutions contained 0.005 mM wild-type BcDltA, specified concentrations of ATP and CoA, 5 mM D-alanine, 0.1 M KCl, 0.01 M MgCl₂, 0.05 M Hepes-NaOH buffer at pH 7.2, 5 unit/mL of yeast inorganic pyrophosphatase. **A.** The initial rates of pyrophosphate accumulation divided by the BcDltA concentration are shown. The reaction rates in the presence of CoA are taken from a previous study²⁴. **B.** Relative fluorescence gains as a fraction of the fluorescence intensity in the absence of ATP are shown.

of this BcDltA mutant protein. However, the CoA-induced changes in Michaelis constant K_M were less dramatic (3.1 mM to 0.50 mM for D-alanine, 6.6 mM to 8.8 mM for L-alanine) than those observed for the wild-type protein.

ATP binding by BcDltA

Change in tryptophan fluorescence of BcDltA was minimal in the presence of ATP in the 10 micromolar range. We then found that at an excitation wavelength of 305 nm, the absorption of up to 2 mM of ATP was negligible and there was fluorescence gain associated with increasing concentration of ATP. The fluorescence gain-derived dissociation constant K_D for ATP (0.43 mM) was similar to the Michaelis constant K_M (0.46 mM) derived in the absence of CoA (Figure 3). There was no detectable fluorescence change

Figure 4. Rate of pyrophosphate release and thiol depletion. The reaction solutions contained 0.005 mM wild-type BcDltA, 5 mM D-alanine, 0.1 M KCl, 0.01 M MgCl_2 , 0.05 M Hepes-NaOH buffer at pH 7.2, 5 unit/mL of yeast inorganic pyrophosphatase and specified concentrations of ATP, alanine and CoA. Reaction rates for wild-type BcDltA are shown on the left, and those for the C269A mutant protein are shown on the right. The reaction rates in the absence of CoA are taken from a previous study¹⁷.

Table 2. Kinetic data for DltA protein from *B. cereus*.

BcDltA (substrate)	K_M ($\times 10^{-3}$ M)	k_{cat} (min^{-1})	k_{cat}/K_M ($\times 10^3 \text{ M}^{-1}\cdot\text{min}^{-1}$)
Pyrophosphate assay (5 mM ATP, - CoA) (previous work¹⁷)			
Wild-type (D-Ala)	1.1 ± 0.2	1.5 ± 0.1	1.4 ± 0.2
Wild-type (L-Ala)	14.4 ± 1.6	6.7 ± 0.4	0.47 ± 0.05
C269A (D-Ala)	3.1 ± 0.3	6.2 ± 0.2	2.0 ± 0.2
C269A (L-Ala)	6.6 ± 0.5	7.6 ± 0.3	1.2 ± 0.1
Pyrophosphate assay (5 mM ATP, 5 mM CoA) (this work)			
Wild-type (D-Ala)	0.03 ± 0.01	10.9 ± 0.2	363 ± 100
Wild-type (L-Ala)	109 ± 7	19.3 ± 0.7	0.18 ± 0.02
C269A (D-Ala)	0.50 ± 0.05	15.8 ± 0.3	32 ± 3
C269A (L-Ala)	8.8 ± 0.5	16.5 ± 0.4	1.9 ± 0.2
Thiol assay (5 mM ATP, 5 mM CoA) (this work)			
Wild-type (D-Ala)	0.02 ± 0.02	11.0 ± 0.3	550 ± 200
Wild-type (L-Ala)	48 ± 12	8.4 ± 1.0	0.18 ± 0.02
C269A (D-Ala)	1.0 ± 0.2	19.8 ± 0.8	19.8 ± 0.9
C269A (L-Ala)	20 ± 3	24.3 ± 1.6	1.2 ± 0.2
Pyrophosphate assay (5 mM D-Ala, 5 mM CoA) (previous work²⁴)			
Wild-type (ATP)	0.01 ± 0.02	12.9 ± 0.6	1290 ± 2000
Pyrophosphate assay (5 mM D-Ala, - CoA) (this work)			
Wild-type (ATP)	0.46 ± 0.05	1.46 ± 0.04	3.2 ± 0.04

to derive K_D values for D-alanine. For CoA, we were not able to isolate fluorescence change from absorption by CoA in millimolar concentration.

Data set widget

8 Data Files

<http://dx.doi.org/10.6084/m9.figshare.1018489>

Discussion

DltA strongly prefers D- over L-alanine

Bacteria selectively incorporate D- over L-alanine in cell wall components. There appears to be no exception in ubiquitous esterification of lipoteichoic acids by alanine²⁵. The enantiomer selectivity of BcDltA observed in the absence of any D-alanyl carrier¹⁷, however, has been intriguingly mediocre. The newly acquired kinetic data in the presence of saturating CoA, a DltC mimic, shows that the k_{cat} values are less than 2-fold different for D- and L-alanine (10.9 and 19.3 min^{-1}) while the K_M values are more than 1000-fold different favouring D-alanine over L-alanine (0.03 and 109 mM). Adding the fact that the intracellular concentration of D-alanine (in the order of $10^2 \mu\text{M}$) is approximately 10-fold more abundant than the L-enantiomer (in the order of $10^1 \mu\text{M}$)⁴³, DltA functioning at a saturating concentration of the D-alanyl carrier protein DltC would favor the ligation of the D-enantiomer by approximately 4 orders of magnitude. Such striking enantiomer selectivity is consistent with the much lower L-alanine content found in lipoteichoic acid²⁵. A recent study has shown that the *dlt* operon is induced by cell envelope

stress such as acidic pH and antibiotics⁴⁴. It is possible that stress-induced expression of DltC may reach a saturating concentration for interacting with DltA and therefore ensure the almost exclusive enantiomer selectivity as observed in the presence of the DltC mimic. The comparison between the kinetic properties of the wild-type and the C269A mutant proteins also supports the notion that Cys-269, and its equivalence in other DltA proteins, contributes to the enantiomer selectivity of DltA^{16,17}.

Thiolation conformation of DltA is compatible with adenylation substrates

It is satisfying to observe the stringent D-alanine preference. At the same time, it is also puzzling to appreciate that such selectivity on the chirality of alanine, a substrate of the adenylation reaction, can only be achieved in the presence of CoA, a substrate of the thiolation reaction. The intrinsic fluorescence of tryptophan in BcDltA enabled us to derive the dissociation constant K_D for ATP in the absence of other substrates. The new form of crystal structure in the absence of any substrate is noticeably more disordered than the previously observed adenylation conformation of BcDltA¹⁷ and thiolation conformation of BsDltA¹⁶. The longest such disordered region is between Arg-397 and Glu-413, which contains the inter-domain hinge residue Asp-399, interacts with β -phosphate of ATP, and forms the pantetheine channel. Possibly, this important region remains disordered in the presence of saturating D-alanine, therefore providing an explanation for the similar values between the above mentioned K_D (0.43 mM) and the K_M (0.46 mM) for ATP, in the presence of saturating D-alanine but in absence of CoA. The more disordered nature of the substrate-free conformation of BcDltA

also implies that the previously observed adenylation and thiolation conformations are intrinsically unstable unless stabilized by the interaction with one or more substrates. This structural feature of BcDltA likely explains the relatively low sub-millimolar affinity for ATP, since part of the stabilizing BcDltA-ATP interactions would be used to compensate the cost of establishing the adenylation conformation of the protein.

BcDltA is a very slow enzyme. Unless the substrate dissociation step happens to be extremely slow as well, we could approximate the observed K_M values to the K_D values of corresponding BcDltA-substrate intermediates, and therefore enable reasoning in the context of structural stability of such intermediates. As such, we reasoned that the approximately one order of magnitude difference in K_M values for the adenylation substrates ATP and D-alanine observed in the presence and absence of the thiolation substrate may imply the existence of a quadruple intermediate of the DltA enzyme in complex with all three substrates, which may be markedly different from a ternary intermediate of BcDltA with the two adenylation substrates.

We then resorted to three-dimensional model building so as to answer the question on which of the adenylation and thiolation conformations may be compatible with binding to all three substrates. The BcDltA/D-alanine-adenylate complex (PDB entry 3DHV)¹⁷ was chosen as the adenylation conformation and as the reference set of atomic coordinates. The adenylation intermediate was dissected to generate the D-alanine model. The N-terminal domain of the BcDltA/ATP complex (PDB entry 3FCE)²⁴, which is also in the adenylation conformation, was superposed on the reference set to orient the ATP substrate. The N-terminal domain of the BsDltA/AMP complex (PDB entry 3E7W) was also superposed on the reference set to derive the re-oriented thiolation conformation. Main-chain atoms equivalent to those interacting with AMP in BcDltA (270–272, 292–299) in the quadruple complex of acetyl-CoA synthetase in its thiolation conformation (PDB entry 2P2F)²³ were superimposed on the reference set to orient the CoA model. In the adenylation conformation (Figure 5A, the pantetheine channel is apparently blocked by the main-chain atoms immediately preceding the catalytic Lys-492 of BcDltA (Lys-491 of BsDltA). Although we could not completely rule out the possibility that an allosteric

Figure 5. Structural model of DltA in complex with ATP, D-alanine and CoA. α traces of the two modelled quadruple complexes are shown in stereo. Except for the green-colored region between Arg-397 and Glu-413, the N- and C-terminal domains are colored in gray and salmon, respectively. The three substrates and selected side-chains are shown in ball-and-stick model. **A.** The quadruple complex in adenylation conformation. Residue numbers in BcDltA are shown. **B.** The quadruple complex in thiolation conformation. Residue numbers in BsDltA are shown.

site for CoA exists, no such site has ever been observed for this superfamily of enzymes. Therefore, a quadruple complex in the adenylation conformation is unlikely. The thiolation conformation (Figure 5B), on the other hand, appears to be compatible with binding ATP so long as Arg-408 of BcDltA (Arg-407 of BsDltA) adopts another rotamer. It has been previously observed that the homologous acetyl-CoA synthetase in its thiolation conformation binds AMP, acetate and CoA²³, and that BsDltA in its thiolation conformation binds AMP and appears to have a well-formed D-alanine-binding pocket¹⁶. These structural evidences seem to suggest that a quadruple intermediate may form with the enzyme in its thiolation conformation but not in the adenylation conformation. Since the thiolation conformation has an AMP-binding site and its N-terminal domain, which provides most of the ATP-interacting residues such as the P-loop and Arg-397, remains essentially identical to that in the adenylation conformation, it is not surprising that only one arginine side-chain is required to adopt another rotamer to accommodate ATP. This arginine residue (Arg-408 of BcDltA) forms a salt bridge with the divalent cation-anchoring side-chain of Glu-298 in BcDltA (Glu-297 in BsDltA)²⁴, which appears to be an important structural feature to modulate the conformational change¹⁶. Another arginine residue (Arg-397 of BcDltA, Arg-396 of BsDltA) also seems to facilitate the conformational change. It forms a salt bridge with the hinge aspartate residue in the thiolation conformation. In the adenylation conformation, this arginine side-chain adopts a more extended rotamer and forms a salt bridge with the β -phosphate group of ATP (Figure 5A). ATP binding would result in re-orientation of both arginine residues and disruption of two salt bridges, thus mobilizing the thiolation conformation.

Structural basis for CoA-enhanced affinity for ATP

The majority of ATP-binding elements lie in the N-terminal domain which remains similar in both adenylation and thiolation conformations. The most significant structural feature in the C-terminal domain for binding ATP is the catalytic Lys-492 of BcDltA (Lys-491 of BsDltA) in the adenylation conformation. In the thiolation conformation, the catalytic residue is replaced by Lys-403 (Lys-402 of BsDltA) (Figure 5). In addition, the thiolation conformation is more ordered than the substrate-free conformation. The favourable DltA/ATP interactions may no longer be used to compensate the energetic cost of stabilizing the disordered hinge region. Therefore the affinity for ATP by the CoA-bound BcDltA in its thiolation conformation should be higher than substrate-free BcDltA.

Structural basis for CoA-enhanced enantiomer selectivity for D-alanine

For both the wild-type and C269A mutant BcDltA, the presence of saturating CoA increases the apparent affinity for D-alanine while decreasing affinity for L-alanine. The majority of alanine-interacting residues lie in the N-terminal domain. The amino group of D-alanine is stabilized by Asp-197 of BcDltA (Asp-196 of BsDltA). Cys-269 of BcDltA (Cys-268 of BsDltA) lies close to α of D-alanine and serves as one major determinant of enantiomer selectivity. The carboxylate group of D-alanine is stabilized by Lys-492 in the

adenylation conformation or by Lys-403 in the thiolation conformation. At the end of the pantetheine channel, Phe-196 of BcDltA (Phe-195 of BsDltA) adopts different rotamers in the two conformations. In the adenylation conformation of BcDltA/D-alanine adenylate, the shortest distance from Phe-196 side-chain to methyl group of D-alanine is 4.15 Å. In the modelled quadruple complex in the thiolation conformation, the Phe-196 side-chain does not contact D-alanine but the thiol group of CoA lies in closer proximity of D-alanine side-chain (3.34 Å), as expected from the pending thiolation reaction between the thiol group of CoA and the carbonyl group of D-alanine-adenylate. On the other hand, the tighter alanine-binding pocket may exert stronger VDW repulsion toward L-alanine, thus lowering further the affinity for the wrong enantiomer. Interestingly, Cys-269 also has a thiol group. The removal of this group in the C269A mutant of BcDltA reduces the affinity for D-alanine and increases the affinity for L-alanine by approximately one order of magnitude, which is almost exactly the opposite to the effect of introducing the thiol group of CoA in the alanine-binding pocket. Since sulphur atoms are larger and more inducible than the second-period elements carbon and oxygen, it is not surprising that the VDW interaction involving a thiol group makes a significant impact on the stability of enzyme/substrate complex.

Hypothesized enzymatic cycle of DltA

Intracellular concentration of D-alanine⁴³ generally exceeds the K_M value in the presence of saturating ATP and CoA. Typical intracellular concentration of ATP lies in the millimolar range⁴⁵, exceeding the K_M value for ATP as well. The concentration of the possibly stress-induced DltC⁴⁴ may also reach saturating level in bacteria when such stress is present. As implied by the CoA-triggered dramatic change in K_M values for ATP and D-alanine, the two adenylation substrates are likely incorporated by the enzyme with pre-bound CoA rather than with merely the other adenylation substrate. In addition, three-dimensional modelling suggests the CoA-bound state of the enzyme can only exist in its thiolation conformation. We therefore hypothesize a two-conformation model for the enzymatic cycle catalyzed by DltA (Figure 6) in the presence of saturating concentration of the three substrates. Our model differs from the three-conformation model proposed for DltA and adenylation domains in non-ribosomal peptide synthetase¹⁶ which includes a third substrate-free conformation. This model also differs from the two-conformation model proposed for and for 4-chlorobenzoate-CoA ligase^{26,46} which includes substrate-free state of the enzyme. The two previously proposed models are consistent with a typical Ping-Pong mechanism. Neither a substrate-free conformation nor a substrate-free state of the enzyme is required in our model for the enzymatic cycle of DltA once the protein enters the reaction cycle. The adenylation reaction starts from a ternary complex with ATP and D-alanine and proceeds with the release of pyrophosphate. A domain rotation around the aspartate hinge residue follows transforming into the thiolation conformation which binds CoA, or DltC in bacteria, and catalyzes the thiolation reaction with D-alanine by displacing AMP. In our model, the resulting complex with the two thiolation products proceeds with AMP release, D-alanine-CoA/CoA

Figure 6. Hypothetical enzymatic cycle of DltA. Non-covalent DltA complexes are shown in text. Forward reaction, substrate binding and release steps are shown by arrows. Steps fulfilled by the adenylation conformation are shown in the green box, and those by the thiolation conformation in brown. The thioester between D-Ala and CoA (D-Ala-CoA) is the final product.

exchange, D-alanine binding, and ATP binding. In the quadruple complex, the ATP-bound magnesium ion would replace Arg-408 of BcDltA in forming a bridge with Glu-298, as observed in the crystal structure BcDltA/ATP complex²⁴. The disruption of the Arg-408 to Glu-298 salt bridge would destabilize the thiolation conformation and facilitate a reverse domain rotation and release of CoA which is not compatible with the adenylation conformation. The hypothesized D-alanine-CoA/CoA exchange step is the central piece of this enzymatic cycle, which reflects the finding that CoA affects DltA's apparent affinities with D-alanine and ATP. If DltA were to become substrate-free for a long enough period of time, it would form a ternary complex with ATP and D-alanine, which contradicts the observed effect by the thiolation substrate CoA. It is worth noting that the sequence of D-alanine and ATP binding is uncertain. Similarly, AMP release has to occur before ATP binding but not necessarily before exchange with CoA or D-alanine binding.

The hypothesized enzymatic cycle involves a second CoA binding step, and a CoA release step in addition to the Ping-Pong mechanism previously proposed for DltA and its homologs. Both additional steps seem unnecessary for the enzymatic reaction itself, but are required to explain our enzymatic data and are consistent with the three-dimensional models of BcDltA and BsDltA. A Ping-Pong mechanism would require that the binding of CoA and the binding of either adenylation substrate be uncompetitive, and therefore the apparent K_M and k_{cat} values for either adenylation substrate both would become larger at higher CoA concentration. While the k_{cat} values for both ATP and D-alanine did become larger at saturating

CoA concentration, the K_M values actually became smaller, therefore contradicting the typical Ping-Pong mechanism. Another property of aminoacyl-CoA synthetases including DltA is the inhibitory effect of CoA at high concentration⁴², which is difficult to explain by a typical Ping-Pong mechanism unless the enzymatic cycle includes an additional CoA-dissociation step as in our model. The closest homologs of DltA are amino acid-activation domains found in non-ribosomal peptide synthetases⁴². Similar to DltA, these homologs also pass the adenylated intermediate to the 4'-phosphopantetheine group attached to a serine residue on a peptide carrier domain. It is possible that such amino acid activation domains may also act as DltA.

The extra binding step for 4'-phosphopantetheine D-alanyl carrier could serve as the sensor for the availability of the carrier. The maximum rate catalyzed by BcDltA is approximately seven times faster in the presence of CoA than in its absence, with respective k_{cat} values 10.9 min⁻¹ and 1.5 min⁻¹. Moreover, the intracellular concentration of D-alanine is typically found in the 100 micromolar range⁴³, which lies above the K_M value for D-alanine in the presence of CoA (~30 μ M) but below the K_M value in the absence of CoA (1100 μ M) (Table 2). The reaction rate should become slower by approximately 100 fold when the thiol carrier is absent. It is worth noting that the adenylated D-alanine intermediate generated by the adenylation reaction is not covalently attached to the enzyme and could be released and wasted when the thiolation substrate is absent. The significant slowing down of the adenylation reaction in the absence of the 4'-phosphopantetheine carrier therefore provides a biological advantage.

Data availability

figshare: Data of pyrophosphate release, ATP-induced fluorescence and thiol depletion for DltA in *Bacillus cereus*, <http://dx.doi.org/10.6084/m9.figshare.1018489>⁴⁷

Author contributions

YL conceived the study, designed the experiments, determined the crystal structure, and wrote the manuscript. LD carried out cloning, expression, crystallization and biochemical assays, and approved the manuscript for publication.

Competing interests

No competing interests were disclosed.

Supplementary file

[PDB validation report](#)

Grant information

This work is supported by a Saskatchewan Health Research Foundation Phase 3 Team Grant to the Molecular Design Research Group at University of Saskatchewan, and by a Natural Sciences and Engineering Research Council Discovery Grant 261981-2010 to YL.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Drs. Gabriele Schatte and Wilson Quail for assistance with the X-ray facility at the Saskatchewan Structural Sciences Centre.

References

- Armstrong JJ, Baddiley J, Buchanan JG, *et al.*: **Composition of teichoic acids from a number of bacterial walls.** *Nature*. 1959; **184**: 247–248.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Hyrylainen HL, Vitikainen M, Thwaite J, *et al.*: **D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*.** *J Biol Chem*. 2000; **275**(35): 26696–26703.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Fischer W: **Physiology of lipoteichoic acids in bacteria.** *Adv Microb Physiol*. 1988; **29**: 233–302.
[PubMed Abstract](#)
- Perego M, Glaser P, Minutello A, *et al.*: **Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation.** *J Biol Chem*. 1995; **270**(26): 15598–15606.
[PubMed Abstract](#)
- Wecke J, Perego M, Fischer W: **D-alanine deprivation of *Bacillus subtilis* teichoic acids is without effect on cell growth and morphology but affects the autolytic activity.** *Microb Drug Resist*. 1996; **2**(1): 123–129.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Peschel A, Otto M, Jack RW, *et al.*: **Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides.** *J Biol Chem*. 1999; **274**(13): 8405–8410.
[PubMed Abstract](#)
- Kristian SA, Lauth X, Nizet V, *et al.*: **Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model.** *J Infect Dis*. 2003; **188**(3): 414–423.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gross M, Cramton SE, Gotz F, *et al.*: **Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces.** *Infect Immun*. 2001; **69**(5): 3423–3426.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Gotz F: ***Staphylococcus* and biofilms.** *Mol Microbiol*. 2002; **43**(6): 1367–1378.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Neuhaus FC, Baddiley J: **A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria.** *Microbiol Mol Biol Rev*. 2003; **67**(4): 686–723.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- May JJ, Finking R, Wiegshoff F, *et al.*: **Inhibition of the D-alanine:D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall.** *FEBS J*. 2005; **272**(12): 2993–3003.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Stachelhaus T, Mootz HD, Marahiel MA: **The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases.** *Chem Biol*. 1999; **6**(8): 493–505.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Conti E, Franks NP, Brick P: **Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes.** *Structure*. 1996; **4**(3): 287–298.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gulick AM, Lu X, Dunaway-Mariano D: **Crystal structure of 4-chlorobenzoate:CoA ligase/synthetase in the unliganded and aryl substrate-bound states.** *Biochemistry*. 2004; **43**(27): 8670–8679.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Reger AS, Wu R, Dunaway-Mariano D, *et al.*: **Structural characterization of a 140 degrees domain movement in the two-step reaction catalyzed by 4-chlorobenzoate:CoA ligase.** *Biochemistry*. 2008; **47**(31): 8016–8025.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Yonus H, Neumann P, Zimmermann S, *et al.*: **Crystal structure of DltA: implications for the reaction mechanism of non-ribosomal peptide synthetase (NRPS) adenylation domains.** *J Biol Chem*. 2008; **283**(47): 32484–91.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Du L, He Y, Luo Y: **Crystal structure and enantiomer selection by D-alanyl carrier protein ligase DltA from *Bacillus cereus*.** *Biochemistry*. 2008; **47**(44): 11473–11480.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Yonus H, Neumann P, Zimmermann S, *et al.*: **Crystal structure of DltA. Implications for the reaction mechanism of non-ribosomal peptide synthetase adenylation domains.** *J Biol Chem*. 2008; **283**(47): 32484–32491.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gulick AM, Starai VJ, Horswill AR, *et al.*: **The 1.75 Å crystal structure of acetyl-CoA synthetase bound to adenosine-5'-propylphosphate and coenzyme A.** *Biochemistry*. 2003; **42**(10): 2866–2873.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Conti E, Stachelhaus T, Marahiel MA, *et al.*: **Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S.** *EMBO J*. 1997; **16**(14): 4174–4183.
[PubMed Abstract](#) | [Free Full Text](#)
- May JJ, Kessler N, Marahiel MA, *et al.*: **Crystal structure of DhhE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases.** *Proc Natl Acad Sci U S A*. 2002; **99**(19): 12120–12125.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Jogl G, Tong L: **Crystal structure of yeast acetyl-coenzyme A synthetase in complex with AMP.** *Biochemistry*. 2004; **43**(6): 1425–1431.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Reger AS, Carney JM, Gulick AM: **Biochemical and crystallographic analysis of substrate binding and conformational changes in acetyl-CoA synthetase.** *Biochemistry*. 2007; **46**(22): 6536–6546.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Osman KT, Du L, He Y, *et al.*: **Crystal structure of *Bacillus cereus* D-alanyl carrier protein ligase (DltA) in complex with ATP.** *J Mol Biol*. 2009; **388**(2): 345–355.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Neuhaus FC, Linzer R, Reusch VM Jr: **Biosynthesis of membrane teichoic acid: role of the D-alanine-activating enzyme and D-alanine: membrane acceptor**

- ligase.** *Ann N Y Acad Sci.* 1974; **235**(0): 502–518.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Wu R, Cao J, Lu X, *et al.*: **Mechanism of 4-chlorobenzoate:coenzyme A ligase catalysis.** *Biochemistry.* 2008; **47**(31): 8026–8039.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 27. Wu Y, He Y, Moya IA, *et al.*: **Crystal structure of archaeal recombinase RadA: a snapshot of its extended conformation.** *Mol Cell.* 2004; **15**(3): 423–435.
[PubMed Abstract](#) | [Publisher Full Text](#)
 28. Brunger AT, Adams PD, Clore GM, *et al.*: **Crystallography & NMR system: A new software suite for macromolecular structure determination.** *Acta Crystallogr D Biol Crystallogr.* 1998; **54**(Pt 5): 905–921.
[PubMed Abstract](#) | [Publisher Full Text](#)
 29. Perrakis A, Morris R, Lamzin VS: **Automated protein model building combined with iterative structure refinement.** *Nat Struct Biol.* 1999; **6**(5): 458–463.
[PubMed Abstract](#) | [Publisher Full Text](#)
 30. McRee DE: **XtalView/Xfit—A versatile program for manipulating atomic coordinates and electron density.** *J Struct Biol.* 1999; **125**(2–3): 156–165.
[PubMed Abstract](#) | [Publisher Full Text](#)
 31. Kraulis P: **MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures.** *J Appl Cryst.* 1991; **24**: 946–950.
[Publisher Full Text](#)
 32. Bacon DJ, Anderson WF: **A Fast Algorithm for Rendering Space-Filling Molecule Pictures.** *J Mol Graph.* 1988; **6**(4): 219–220.
[Publisher Full Text](#)
 33. Berman HM, Westbrook J, Feng Z, *et al.*: **The Protein Data Bank.** *Nucleic Acids Res.* 2000; **28**(1): 235–242.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 34. Berman H, Henrick K, Nakamura H: **Announcing the worldwide Protein Data Bank.** *Nat Struct Biol.* 2003; **10**(12): 980.
[PubMed Abstract](#) | [Publisher Full Text](#)
 35. Bernstein FC, Koetzle TF, Williams GJ, *et al.*: **The Protein Data Bank. A computer-based archival file for macromolecular structures.** *Eur J Biochem.* 1977; **80**(2): 319–324.
[PubMed Abstract](#) | [Publisher Full Text](#)
 36. Itaya K, Ui M: **A new micromethod for the colorimetric determination of inorganic phosphate.** *Clin Chim Acta.* 1966; **14**(3): 361–366.
[PubMed Abstract](#) | [Publisher Full Text](#)
 37. Chang KH, Xiang H, Dunaway-Mariano D: **Acyl-adenylate motif of the acyl-adenylate/thioester-forming enzyme superfamily: a site-directed mutagenesis study with the Pseudomonas sp. strain CBS3 4-chlorobenzoate:coenzyme A ligase.** *Biochemistry.* 1997; **36**(50): 15650–15659.
[PubMed Abstract](#) | [Publisher Full Text](#)
 38. Stuibler H, Buttner D, Ehling J, *et al.*: **Mutational analysis of 4-coumarate:CoA ligase identifies functionally important amino acids and verifies its close relationship to other adenylate-forming enzymes.** *FEBS Lett.* 2000; **467**(1): 117–122.
[PubMed Abstract](#) | [Publisher Full Text](#)
 39. Horswill AR, Escalante-Semerena JC: **Characterization of the propionyl-CoA synthetase (PrpE) enzyme of Salmonella enterica: residue Lys592 is required for propionyl-AMP synthesis.** *Biochemistry.* 2002; **41**(7): 2379–2387.
[PubMed Abstract](#) | [Publisher Full Text](#)
 40. Walker JE, Saraste M, Runswick MJ, *et al.*: **Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold.** *EMBO J.* 1982; **1**(8): 945–951.
[PubMed Abstract](#) | [Free Full Text](#)
 41. Kochan G, Pilka ES, von Delft F, *et al.*: **Structural snapshots for the conformation-dependent catalysis by human medium-chain acyl-coenzyme A synthetase ACSM2A.** *J Mol Biol.* 2009; **388**(5): 997–1008.
[PubMed Abstract](#) | [Publisher Full Text](#)
 42. Linne U, Schafer A, Stubbs MT, *et al.*: **Aminoacyl-coenzyme A synthesis catalyzed by adenylation domains.** *FEBS Lett.* 2007; **581**(5): 905–910.
[PubMed Abstract](#) | [Publisher Full Text](#)
 43. Manning JM, Merrifield NE, Jones WM, *et al.*: **Inhibition of bacterial growth by beta-chloro-D-alanine.** *Proc Natl Acad Sci U S A.* 1974; **71**(2): 417–421.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 44. McCormick NE, Halperin SA, Lee SF: **Regulation of D-alanylation of lipoteichoic acid in Streptococcus gordonii.** *Microbiology.* 2011; **157**(Pt 8): 2248–2256.
[PubMed Abstract](#) | [Publisher Full Text](#)
 45. Beis I, Newsholme EA: **The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates.** *Biochem J.* 1975; **152**(1): 23–32.
[PubMed Abstract](#) | [Free Full Text](#)
 46. Wu R, Reger AS, Lu X, *et al.*: **The mechanism of domain alternation in the acyl-adenylate forming ligase superfamily member 4-chlorobenzoate: coenzyme A ligase.** *Biochemistry.* 2009; **48**(19): 4115–4125.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 47. Liqin Du, Yu Luo: **Data of pyrophosphate release, ATP-induced fluorescence and thiol depletion for DltA in Bacillus cereus.** *Figshare.* 2014.
[Data Source](#)

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 17 June 2014

<https://doi.org/10.5256/f1000research.4389.r5026>

© 2014 Bell C. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Charles Bell

Department of Chemistry and Biochemistry, Ohio State University, Columbus, OH, USA

Ita catalyzes two reactions: (1) an adenylation reaction in which ATP is joined to D-Ala to form D-Ala-AMP (PPi is released), and (2) a thiolation reaction in which D-Ala is transferred from D-Ala-AMP to a phosphopantetheine group of D-alanyl carrier protein (DltC). The reaction can be mimicked in vitro by substituting CoA for DltC (does this also happen in vivo?). Previous crystal structures have indicated that the two reactions are promoted by two distinct conformational states of the enzyme, which differ by a large rearrangement of a C-terminal domain that closes over the active site. In this work, Du & Luo provide kinetic data to suggest that binding of the two substrates for the adenylation reaction (ATP and D-Ala) is promoted by the presence of the substrate (CoA) for the second reaction. The selectivity for binding of D-Ala as opposed to L-Ala is also enhanced significantly by the presence of CoA. A new scheme for the reaction cycle is proposed in which instead of forming an empty enzyme when the final product D-Ala-CoA is released, free CoA is rapidly "exchanged" in for D-Ala-CoA to keep the enzyme in its thiolation conformation. Then new D-Ala and ATP substrates bind (with the enzyme still in its CoA-bound thiolation conformation), and CoA is released to allow the enzyme to adopt the adenylation conformation, which is required for ATP and D-Ala to react. Binding of CoA to the newly formed D-Ala-AMP complex then puts the enzyme back in the thiolation conformation. The new reaction scheme is considerably more complicated than one would expect, but seems to account for the observed kinetic data. The authors propose that the biological purpose for this type of mechanism is to limit binding (and reaction) of ATP and D-Ala to conditions in which the 2nd substrate (presumably DltC in vivo) is available, so as to avoid non-productive formation and release D-Ala-AMP.

A crystal structure of the apo form of DltA is also determined, which shows that in the absence of substrates the enzyme adopts the adenylation conformation, and that several loops on the enzyme are considerably more disordered than in previous structures of various substrate or product-bound states. The new structure does not add a lot, but is meant to support the concept that ATP and D-Ala do not bind tightly to the enzyme unless CoA is around to reduce the overall flexibility.

The manuscript is reasonably well written, scientifically sound, and should be of fairly broad interest, particularly for those studying other types of two-reaction enzymes, such as in non-ribosomal peptide synthesis, and possibly even enzymes like amino-acyl tRNA synthetases. I found the manuscript to be rather uninteresting, until I got to the end of the discussion, where the newly proposed reaction scheme of Figure 6 is presented, and a biological purpose of the observed phenomena is proposed. If there is some way to bring these aspects into the Abstract or the Introduction, a potential reader might be drawn in a little bit earlier than I was.

The proposed scheme is quite unusual in that CoA supposedly promotes binding of ATP and D-Ala, but subsequent release of CoA is required for the two to react. Presumably this means that the rate of the catalytic step for the adenylation reaction must be considerably faster than the rates of ATP and D-Ala dissociation, or else the enzyme could return to the apo state, and presumably the adenylation conformation, when CoA is released. Are these parameters known? Also, is the physiological concentration of DltC acceptor protein, which presumably takes over the role of CoA in vivo, high enough to be consistent with the proposed reaction cycle? It would need to rebind to the enzyme in the exchange step before the enzyme reverts to the adenylation conformation. Presumably the rate of DltC binding is faster than the rate of reversion back to the adenylation conformation? The authors should discuss/clarify these points.

Minor points:

1. The data in this paper demonstrate the effect on the enzyme by CoA, a mimic of the in vivo substrate DltC, but not for DltC itself. Is there some reason why this is difficult to demonstrate with DltC experimentally?
2. Was it previously known from other structures that the apo form of the enzyme is in the adenylation conformation, or is this a new insight? In general the authors could do a little bit of a better job describing the previous structures in the Introduction. Why were the previous structures of the *B. subtilis* enzyme found to be in the thiolation conformation despite the absence of CoA (or DltC)? Is there no structure available with CoA?
3. References 16 and 18 are the same.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response () 23 Jun 2014

Yu Luo, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

As far as we know, the rate constants of substrate binding, reaction and product dissociation steps have not been measured for DltA or its closely related homologs that transfer the activated amino acyl or peptidyl group to a carrier protein. We do not have expertise in measuring such rate constants.

It is generally accepted that DltC is the D-alanine carrier *in vivo*. CoA, however, is a more feasible analog of DltC for enzymatic assays. If DltC were used, the assay would require either millimolar concentration of DltC or addition of a thioesterase to regenerate DltC, for which we haven't resolved the technical difficulties.

The *B. subtilis* DltA in thiolation conformation has been co-crystallized with DltC, although DltC is disordered or absent from the crystal structure. Homologous acyl and aryl CoA synthetases have been known to crystallize in either adenylation or thiolation conformation. Remotely related Japanese firefly luciferase has been crystallized in an apo-form dissimilar to its adenylation conformation. However, such luciferases do not catalyze the second thiolation reaction and recently, DltA from *S. pyogenes* has been crystallized in the absence of any substrate. The structure (PDB entry 3L8C, to be published) resembles the adenylation conformation of *B. cereus* DltA.

Competing Interests: No competing interests were disclosed.

Reviewer Report 16 June 2014

<https://doi.org/10.5256/f1000research.4389.r5123>

© 2014 Wang T. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Ting Fang Wang

Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

This paper provides an intriguing two-conformation hypothesis for the enzymatic cycle of *B. cereus* D-alanyl carrier protein ligase DltA. Overall, both structural determination and biochemical assays were well done.

A minor point: The two-conformation hypothesis (Figure 6) suggests that the inclusion of ATP to the DltA-CoA-Ala complex functions includes CoA release and a large domain rotation. It is important to show that AMP-CPP, a non-hydrolyzable analog of ATP, can indeed induce CoA release.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response () 23 Jun 2014

Yu Luo, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

It would be interesting to carry out an experiment to study ATP-stimulated release of CoA or DltC. One plan is to use a surface plasmon resonance-based technique, or similar techniques, to study the association and dissociation of DltA with DltC under various conditions.

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research