Structure of the *Bacillus anthracis* dTDP-L-rhamnose-biosynthetic enzyme dTDP-4-dehydrorhamnose reductase (RfbD)

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Bacillus anthracis is the causative agent of the deadly disease Anthrax. Its use in bioterrorism and its ability to re-emerge have brought renewed interest in this organism. *B. anthracis* is a Gram-positive bacterium that adds L-rhamnose to its cell-wall polysaccharides using the activated donor dTDP- β -L-rhamnose. The enzymes involved in the biosynthesis of the activated donor are absent in humans, which make them ideal targets for therapeutic development to combat pathogens. Here, the 2.65 Å resolution crystal structure of the fourth enzyme in the dTDP- β -L-rhamnose-biosynthetic pathway from *B. anthracis*, dTDP-4-dehydro- β -L-rhamnose reductase (RfbD), is presented in complex with NADP⁺. This enzyme catalyzes the reduction of dTDP-4-dehydro- β -L-rhamnose to dTDP- β -L-rhamnose. Although the protein was co-crystallized in the presence of Mg²⁺, the protein lacks the conserved residues that coordinate Mg²⁺.

1. Introduction

Anthrax is a disease caused by the Gram-positive bacterium *Bacillus anthracis*. In recent years, concern has been raised regarding its ability to re-emerge or to be used in bioterrorism, especially since its spores can survive for long periods of time (Spencer, 2003). There are three main routes by which humans become exposed to this pathogen, subcutaneous, gastrointestinal and pulmonary (Carlson *et al.*, 2015), the latter of which is most likely to be used as an agent of terror. Inhibiting enzymatic reactions that are important for the integrity of bacterial cell walls is one way to combat bacterial infections (Mistou *et al.*, 2016). Many bacteria decorate their cell walls with different sugars, which are critical for their protection in diverse environments, affect their shape and are important for communication and interaction with other bacteria (Blankenfeldt *et al.*, 2002; Dong *et al.*, 2003; Mistou *et al.*, 2016; Graninger *et al.*, 2002). L-Rhamnose is a cell-wall sugar that plays an important role in many bacteria (Dong *et al.*, 2003; Giraud & Naismith, 2000). The activated donor that is used to transfer L-rhamnose to peptidoglycan is dTDP- β -L-rhamnose, which is synthesized using a series of enzymes that comprise the dTDP-L-rhamnose-biosynthetic pathway. Since L-rhamnose is unique to bacteria and is not present in humans, the enzymes within this pathway are attractive targets for therapeutic drugs to combat bacterial pathogens (Blankenfeldt *et al.*, 2002; Giraud & Naismith, 2000; Mistou *et al.*, 2016).

In this work, we determined the crystal structure of the fourth and final enzyme of the dTDP- β -L-rhamnose-biosynthetic pathway, dTDP-4-dehydro- β -L-rhamnose reductase (RfbD), in complex with NADP⁺. This enzyme catalyzes

Table 1

Macromolecule-production information.

The <i>rfbD</i> gene encodes dTDP-4-dehydro- β -L-rhamnose reductase.	
Source organism	<i>B. anthracis</i> strain Ames
Cloning/expression vector	pMCSG7 (ampicillin resistant)
Expression host	<i>E. coli</i> BL21(DE3) Magic cells (kanamycin resistant)
Complete amino-acid sequence of the construct produced	MHHHHHSSGVDLGTENLYFQSNAMKERV ITGANGQLGKQLQEELNP EYDIYPFDK KLLDITNISQVQVQVEIRPHIIHCAA YTKVDQAEKERDLAYVINAIGARNVAVA SQLVGAKLVYISTDYVFQGDREPEGYDEF HNPAPINIYGASKYAGEQFVKELHNKYF IVRTSWLYGKYGNFVKTMIRLGKERE ISVVADQIGSPTYVADLNMINKLIHTS LYGTYHVSNTGSCSWFEFAKKIFSANM KVNVLVSTEEFGAAAARPKYSIFQHN LRLNGFLQMPSEWEEGLERFFIETKSH

the reduction of dTDP-4-dehydro- β -L-rhamnose to dTDP- β -L-rhamnose (Graninger *et al.*, 2002) and is often referred to as RmlD in other bacteria. All four genes that encode the enzymes of the pathway in *B. anthracis* strain Ames reside on the same operon in the order *rfbACBD* (locus tags BA_1228–BA_1231, respectively). This is in contrast to the described dTDP-4-dehydro-L-rhamnose reductase homolog GacA from *Streptococcus pyogenes*, which resides on a different operon to the other enzymes of the pathway (van der Beek *et al.*, 2015). In Gram-negative bacteria the dTDP- β -L-rhamnose product acts as an allosteric inhibitor of the first enzyme of the pathway, glucose-1-phosphate thymidyltransferase (RmlA; Giraud & Naismith, 2000), but it is currently not known whether this pathway is allosterically regulated in Gram-positive bacteria. Here, we present a structural comparison of RfbD from *B. anthracis* with its homologs from both Gram-positive and Gram-negative bacteria to guide future studies of this enzyme.

2. Materials and methods

2.1. Protein expression and purification

The *B. anthracis* strain Ames *rfbD* gene was cloned into the pMCSG7 vector as described previously (Kwon & Peterson, 2014) and transformed into *Escherichia coli* BL21(DE3) Magic cells for protein expression (Table 1). The cells were grown to an OD_{600 nm} between 0.6 and 0.8 in 1.5 l Terrific Broth at 310 K and then cooled on ice. 0.5 mM IPTG was then added to induce protein expression at 298 K overnight. The cells were harvested as described previously (Kuhn *et al.*, 2013), resuspended in 100 ml lysis buffer [10 mM Tris–HCl pH 8.3, 500 mM NaCl, 10% glycerol, 0.01% IGEPAL CA630, 5 mM β -mercaptoethanol (BME)] and sonicated. After centrifugation, the crude extract was loaded onto a 5 ml Ni-NTA affinity column and the RfbD protein was purified as described previously (Kuhn *et al.*, 2013). The yield of pure protein was 43 mg l^{−1}. The N-terminal polyhistidine tag was removed using TEV protease as described previously (Kuhn *et al.*, 2013; Majorek *et al.*, 2013), except that the cleavage was initiated at 298 K instead of 310 K for 2 h, and the protein was

Table 2

Crystallization.

Method	Vapor diffusion, sitting drop
Plate type	96-well microplate
Temperature (K)	297
Protein concentration (mg ml ^{−1})	7.5
Buffer composition of protein solution	10 mM Tris–HCl pH 8.3, 500 mM NaCl, 5 mM BME, 10% glycerol, 1 mM NADP ⁺ , 5 mM MgCl ₂
Composition of reservoir solution	0.2 M lithium sulfate, 0.1 M Tris pH 8.5, 25%(w/v) PEG 5000 MME (The PEGs II Suite condition G1)
Volume and ratio of drop	1:1 μ l
Volume of reservoir (μ l)	100

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	APS beamline 21-ID-D
Wavelength (Å)	0.97856
Temperature (K)	100
Detector	MAR Mosaic 300 mm CCD
Space group	<i>P</i> ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	79.45, 113.34, 144.95
α , β , γ (°)	90.00, 91.18, 90.00
Resolution range (Å)	30.00–2.65 (2.70–2.65)
No. of unique reflections	75200 (3724)
Completeness (%)	100.0 (100.0)
Multiplicity	3.8 (3.8)
$\langle I/\sigma(I) \rangle$	11.49 (2.35)
$R_{\text{r.i.m.}}$ [†]	0.069 (0.417)
Overall <i>B</i> factor from Wilson plot (Å ²)	67.1

[†] Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

then stored overnight at 277 K to minimize precipitation. The cleaved protein was purified and concentrated as described previously (Majorek *et al.*, 2013).

2.2. Crystallization

Concentrated RfbD protein was diluted to 7.5 mg ml^{−1} in buffer consisting of 10 mM Tris–HCl pH 8.3, 500 mM NaCl, 5 mM BME. The final concentrations of additional components were 5 mM MgCl₂, 1 mM NADP⁺ and 10% glycerol. The protein was then added to reservoir solution in a 1 μ l:1 μ l ratio using the sitting-drop vapor-diffusion method in 96-well microplates and screened against the PEGs II Suite and the AmSO₄ Suite crystallization screens from Qiagen. Crystals grew from 0.2 M lithium sulfate, 0.1 M Tris pH 8.5, 25%(w/v) PEG 5000 MME (condition G1 of The PEGs II Suite; Table 2).

2.3. Data collection and processing

Crystals of RfbD were harvested and transferred to reservoir solution prior to flash-cooling in liquid nitrogen. The crystals were considered to be cryoready owing to the high concentration of PEG that was present in the drop. X-ray data were collected on the Advanced Photon Source 21-ID-D beamline at Argonne National Laboratory and were indexed, scaled and integrated with *HKL-2000* (Otwinowski & Minor, 1997). Statistics for data collection and processing are described in Table 3.

Table 4
Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	29.90–2.65 (2.72–2.65)
Completeness (%)	99.9 (99.45)
No. of reflections, working set	70861 (5134)
No. of reflections, test set	3745 (277)
Final R_{cryst}	0.215 (0.338)
Final R_{free}	0.258 (0.407)
No. of non-H atoms	
Protein	13571
Ligand	373
Solvent	240
Total	14184
R.m.s. deviations	
Bonds (Å)	0.006
Angles (°)	1.359
Average B factors (Å ²)	
Protein	57.0
Ligand	
NAP	56.67
SO ₄	80.009
Water	30.21
Ramachandran plot†	
Favored regions (%)	87.5
Additionally allowed (%)	12.5
Outliers (%)	0.0

† Ramachandran plot statistics are based on *PROCHECK* (Laskowski *et al.*, 1993).

2.4. Structure solution and refinement

The RfbD structure (PDB entry 3sc6) was determined using molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* package (Winn *et al.*, 2011). A single monomer from PDB entry 1vl0 (47% sequence identity to 3sc6; Joint Center for Structural Genomics, unpublished work) was used as the model for molecular replacement. *ARP/wARP* (Morris *et al.*, 2003) was used to rebuild and generate solvent molecules. The model was inspected using *Coot* (Emsley & Cowtan, 2004; Emsley *et al.*, 2010), and NADP⁺ and SO₄^{2−} were added to the model. *REFMAC* v.5.5 was used to refine the structure. During the final stages of refinement, translation–libration–screw (TLS) groups were obtained using the *TLSMD* server (Painter & Merritt, 2006; <http://skuldbmsc.washington.edu/~tlsmd/>) and used. *MolProbity* (Chen *et al.*, 2010; Davis *et al.*, 2007; <http://molprobity.biochem.duke.edu/>) and the PDB validation server (<http://deposit.pdb.org/validate/>) were used to determine the quality of the final structure. All statistics for structure refinement are presented in Table 4.

3. Results and discussion

3.1. Structural characteristics of RfbD

The crystals of RfbD from *B. anthracis* belonged to space group $P2_1$ with six monomers in the asymmetric unit. The crystal structure was determined at 2.65 Å resolution and the coordinates were deposited in the Protein Data Bank (PDB) as entry 3sc6. This protein has two domains: an N-terminal NAD(H) or NADP(H) coenzyme-binding domain and a C-terminal nucleotide-sugar-binding domain. All monomers in the structure have NADP⁺ bound to the N-terminal coenzyme-binding domain and sulfate molecules bound at the solvent-exposed surface of the protein. The RfbD structure

displays a characteristic Rossmann-like fold with a β – α – β motif, in which a central β -sheet is surrounded by a series of α -helices (Fig. 1a). The β – α – β motif is located near the N-terminus and contains the Wierenga sequence GXXGXXG (Blankenfeldt *et al.*, 2002; King *et al.*, 2007; van der Beek *et al.*, 2015), where the two phosphates of the coenzyme NADP⁺ bind. RfbD possesses three important residues for its catalytic activity, Thr103, Tyr127 and Lys131, consistent with previously described RfbD homologs (Blankenfeldt *et al.*, 2002; van der Beek *et al.*, 2015).

3.2. Identification and selection of structural homologs

To identify structural homologs of RfbD, we performed a search using the *DALI* server (Holm & Rosenström, 2010; http://ekhidna.biocenter.helsinki.fi/dali_server/start). We selected six homologs based on whether they were from Gram-positive or Gram-negative bacteria and whether coenzyme was bound in their structures. The proteins chosen for comparison included the group A dTDP-4-dehydro- β -L-rhamnose reductase (RmlD) GacA from *S. pyogenes* (van der Beek *et al.*, 2015; PDB entry 4wpg), two dTDP-4-dehydro- β -L-rhamnose reductases (RmlD) from *Salmonella enterica* (Blankenfeldt *et al.*, 2002; PDB entries 1n2s and 1kc3), a dTDP-4-dehydro- β -L-rhamnose reductase (RfbD) from *Clostridium acetobutylicum* (PDB entry 1vl0), a dTDP-glucose-4,6-dehydratase (RfbB) from *B. anthracis* (PDB entry 4egb) and a WbmG short-chain dehydrogenase/reductase-family protein from *Bordetella bronchiseptica* (King *et al.*, 2007; PDB entry 2pzl) (Fig. 1b). The 4wpg, 1vl0 and 4egb structures are from Gram-positive bacteria and 1n2s, 1kc3 and 2pzl are from Gram-negative bacteria. The two structures of the RmlD protein from *S. enterica* have NADH or NADPH coenzyme bound, and the RfbB protein from *B. anthracis* (the second enzyme of the dTDP-L-rhamnose-biosynthetic pathway) has NAD⁺ bound. The 1kc3 structure from *S. enterica* has 2'-deoxythymidine- β -L-rhamnose bound and 2pzl from *B. bronchiseptica* has UDP bound. The C α atoms of selected structures aligned with RfbD with r.m.s.d.s of 0.99 Å (1vl0), 1.23 Å (4wpg), 1.26 Å (1n2s), 1.44 Å (1kc3), 1.80 Å (2pzl) and 3.20 Å (4egb).

3.3. Coenzyme substrate specificity

RfbD belongs to the short-chain dehydrogenase/reductase (SDR) superfamily, which is predominantly comprised of oxidoreductases, lyases and epimerases (Kavanagh *et al.*, 2008). The NAD(H) and NADP(H) coenzyme specificity of these enzymes is dictated by the identity of the residue that binds the ribose of the nucleotide-sugar and the presence or absence of an additional loop in the Rossmann-fold architecture (Kavanagh *et al.*, 2008). Typically, homologs that bind NADP(H) are also capable of binding NAD(H) as the binding pocket for NADP(H) is somewhat larger. Previous experimental results with some homologs, such as the RmlD enzyme from *S. enterica* (PDB entries 1n2s and 1kc3) and GacA from *S. pyogenes* (PDB entry 4wpg), show that both coenzymes can bind to the same site (Blankenfeldt *et al.*, 2002; van der Beek

Figure 1

Overall structure of RfbD from *B. anthracis* and sequence alignment with structural homologs. (a) Structure of RfbD (PDB entry 3sc6) in complex with NADP⁺ (green sticks) colored from the N-terminus (blue) to the C-terminus (red). Secondary-structural elements are labeled. $\beta 8$ and $\beta 11$ are not visible in this orientation. (b) Sequence alignment of selected RfbD homologs, indicated by PDB code (3sc6, RfbD from *B. anthracis*; 4egb, RfbB from *B. anthracis*; 4wpg, RmlD from *Streptococcus pyogenes*; 2pzl, WbmG from *Bordetella bronchiseptica*; 1kc3, RmlD from *Salmonella enterica*; 1vl0, RfbD from *Clostridium acetobutylicum*). Descriptions of each homolog are presented in §2.3 of the Results and discussion section.

et al., 2015). However, enzymes that are specific for binding NAD(H), such as the *B. anthracis* RfbB protein (PDB entry 4egb), exclude the entrance of NADP(H) through the addition of an α - β loop and the conformation of a conserved aspartic acid residue (Asp33 in RfbB and RfbD; van der Beek *et al.*, 2015; Blankenfeldt *et al.*, 2002; Dong *et al.*, 2003; King *et al.*, 2007).

Since we co-crystallized RfbD with NADP⁺, we compared its structure with the structures of its homologs to determine whether it shares similar coenzyme-binding characteristics and could potentially bind NAD⁺. First, we compared the struc-

tures of proteins from Gram-positive bacteria: RfbD from *B. anthracis* (PDB entry 3sc6; NADP⁺ bound) and GacA from *S. pyogenes* (PDB entry 4wpg; no coenzyme bound). GacA was previously shown to bind both the NAD(H) and NADP(H) coenzymes *in vitro*, although the coenzymes were not present in its structure (van der Beek *et al.*, 2015). The RfbD and GacA structures exhibited similar characteristics and only minor differences were observed, with RfbD having a slightly shorter α 1 helix and longer β 1 and β 2 strands compared with GacA. Next, we compared the structures of proteins from Gram-positive and Gram-negative bacteria: RfbD from

Figure 2

Comparison of RfbD and RfbB structures from *B. anthracis*. (a) RfbD structure in complex with NADP⁺. (b) RfbB structure in complex with NAD⁺. The α - β loop that restricts coenzyme binding is shown in purple. (c) Overlay of RfbD and RfbB and enlarged view of the coenzyme-binding site. The α - β loop from RfbB is shown in purple. NAD⁺ is shown in blue sticks and NADP⁺ is shown in green sticks. The conserved Asp33 in both RfbD and RfbB is also shown. Residues Leu35–Glu59 of RfbD and Glu93–Ser94 of RfbB are hidden for clarity in the enlarged view.

Figure 3 Comparison of residues that coordinate Mg^{2+} in RfbD homologs. (a) RmlD dimer from *S. enterica* (gray; PDB entry 1n2s) with Mg^{2+} as a yellow sphere. Residues that coordinate Mg^{2+} from each monomer are shown. (b) Modeled RfbD dimer constructed by overlaying two RfbD monomers (green) onto the 1n2s dimer. Residues in identical positions as the RmlD structure that coordinate Mg^{2+} are shown. (c) Overlay of the RfbD (green) and RmlD (gray) structures around the Mg^{2+} -binding site.

B. anthracis (PDB entry 3sc6; $NADP^+$ bound) and RmlD from *S. enterica* (PDB entries 1n2s and 1kc3; NADH and NADPH bound, respectively). Similar to GacA, RmlD is also capable of using both coenzymes, and crystal structures of this protein in complex with each coenzyme have been determined previously (Blankenfeldt *et al.*, 2002). RfbD had slightly shorter $\alpha 1$, $\alpha 8$, $\alpha 9$ and $\alpha 12$ helices compared with RmlD.

Finally, we compared two enzymes of the dTDP-L-rhamnose-biosynthetic pathway from *B. anthracis* that have different coenzymes bound in their structures: RfbB (PDB entry 4egb; NAD^+ bound) and RfbD (PDB entry 3sc6; $NADP^+$ bound) (Fig. 2). We found that RfbD lacks the α - β loop that is present in RfbB to limit the size of coenzyme that can bind (Fig. 2). Additionally, Asp33 of RfbB interacts with the 2'-hydroxyl and 3'-hydroxyl groups of the ribose of the coenzyme when NAD^+ is bound, but is rotated away from the coenzyme when $NADP^+$ is bound (Blankenfeldt *et al.*, 2002; van der Beek *et al.*, 2015) (Fig. 2c). The absence of the α - β loop combined with the rotational flexibility of Asp33 indicates that RfbD could theoretically utilize either $NADP(H)$ or $NAD(H)$ to catalyze its reaction. The results of our structural comparison also suggest that RmlD/RfbD homologs from both Gram-positive and Gram-negative bacteria share evolutionarily conserved coenzyme-binding capabilities and specificity.

3.4. RfbD lacks conserved residues to coordinate Mg^{2+} and dimerize

Many Gram-negative RmlD/RfbD bacterial homologs have been shown to be functionally active as dimers and require

Mg^{2+} for activity; however, van der Beek and coworkers showed that GacA from *S. pyogenes* was functional as a monomer and that the addition of Mg^{2+} did not cause the protein to dimerize (van der Beek *et al.*, 2015). Mg^{2+} was also not required for optimal kinetic activity (van der Beek *et al.*, 2015). Their analysis of RmlD/RfbD homologs indicated that although many Gram-negative bacterial homologs act as dimers, nearly all Gram-positive bacterial homologs and a large proportion of Gram-negative bacterial homologs are predicted to act as monomers. This is primarily owing to differences in their residues at the dimerization interface and a lack of the conserved residues that coordinate Mg^{2+} . We found six copies of RfbD from *B. anthracis* in the asymmetric unit of our crystals, arranged as two trimers, and no Mg^{2+} was seen in the structure despite the addition of 5 mM $MgCl_2$ during co-crystallization. To determine whether RfbD from *B. anthracis* contained the conserved residues for coordinating Mg^{2+} , we constructed a model of the dimer of the RfbD structure using the RmlD structure (PDB entry 1n2s) from *S. enterica* that has Mg^{2+} bound (Fig. 3). We found that RfbD indeed lacks the conserved residues that coordinate Mg^{2+} (Glu15, Glu190 and Glu292 of the 1n2s structure). Instead, RfbD has Gln17, Ala189 and Glu280, which are not capable of coordinating Mg^{2+} (Figs. 1b and 3). Therefore, we suspect that the catalytically active RfbD protein from *B. anthracis* does not require Mg^{2+} for catalysis.

Although RfbD from *B. anthracis* does not contain the conserved residues to coordinate Mg^{2+} , van der Beek and coworkers showed that dimerization was more likely for

RmlD homologs from Gram-negative bacteria owing to the conservation of interfacial residues that are not present in Gram-positive RfbD homologs (van der Beek *et al.*, 2015). These residues are not strictly conserved in RfbD. To determine whether RfbD could potentially form a higher ordered oligomeric state, we performed an analysis using the *PISA* (*Protein Interfaces, Surfaces and Assemblies*) server at the European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html; Krissinel & Henrick, 2007) and the RfbD structure. *PISA* calculated the surface area buried between each monomer in the trimer as approximately 600 Å², and predicted that the trimers seen in the crystal would not be stable in solution. RmlD (PDB entry 1n2s) was predicted to form a dimer. Further analysis of our artificially built dimer of RfbD superimposed onto the RmlD dimer showed significant clashes between residues in specific regions of each RfbD monomer. Three main regions of clashes were identified: (i) residues of the remnant of the His tag remaining at the N-terminus of one monomer clash with residues at the end of α 11 of the second monomer; (ii) residues located in the loop between α 11 and α 12 clash with residues in the loop between α 1 and β 2; and (iii) residues in α 1 of one monomer clash with residues in α 12 of the second monomer. Therefore, unless significant structural changes occur, it is likely that RfbD from *B. anthracis* acts as a monomer rather than as a dimer. If structural changes do occur, we cannot rule out the possibility that RfbD may form different interactions in a higher oligomeric state since it appears to have crystallized as two trimers and exhibited a calculated molecular weight of 78.9 kDa from size-exclusion chromatography, which corresponds to a dimer or trimer (see Supporting Information).

4. Conclusions

Based on our structural comparison of RfbD from *B. anthracis* with its homologs, we found that RfbD is likely to utilize either NAD(H) or NADP(H) as a coenzyme, act as a monomer owing to its lack of the conserved interfacial residues needed for dimerization and does not require Mg²⁺ for activity owing to its lack of conserved Mg²⁺-binding residues. As a result, this structure provides further evidence that RfbD homologs in Gram-positive bacteria exhibit key differences in the residues important for oligomerization and metal utilization compared with those from many Gram-negative bacteria. These differences may provide new avenues for targeted therapies to combat bacterial infections.

Note added in proof: the structure 4egb in the Protein Data Bank has now been superseded by 6bi4.

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