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The crystal structure of dihydrodipicolinate reductase from the human-pathogenic bacterium *Bartonella henselae* strain Houston-1 at 2.3 Å resolution

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In bacteria, the second committed step in the diaminopimelate/lysine anabolic pathways is catalyzed by the enzyme dihydrodipicolinate reductase (DapB). DapB catalyzes the reduction of dihydrodipicolinate to yield tetrahydrodipicolinate. Here, the cloning, expression, purification, crystallization and X-ray diffraction analysis of DapB from the human-pathogenic bacterium *Bartonella henselae*, the causative bacterium of cat-scratch disease, are reported. Protein crystals were grown in conditions consisting of 5% (w/v) PEG 4000, 200 mM sodium acetate, 100 mM sodium citrate tribasic pH 5.5 and were shown to diffract to ~2.3 Å resolution. They belonged to space group $P4_322$, with unit-cell parameters $a = 109.38$, $b = 109.38$, $c = 176.95$ Å. $R_{\text{f.i.m.}}$ was 0.11, R_{work} was 0.177 and R_{free} was 0.208. The three-dimensional structural features of the enzymes show that DapB from *B. henselae* is a tetramer consisting of four identical polypeptides. In addition, the substrate NADP^+ was found to be bound to one monomer, which resulted in a closed conformational change in the N-terminal domain.

1. Introduction

The enzymes involved in the bacterial synthesis of diaminopimelate and lysine are potential targets for the development of antibiotics (Cox *et al.*, 2000). Four pathways have been discovered thus far; the acyl pathways which employs succinylated or acylated intermediates, the dehydrogenase pathway which uses the enzyme *meso*-diaminopimelate dehydrogenase and the L,L-diaminopimelate aminotransferase pathway (Hudson *et al.*, 2006; Dogovski *et al.*, 2009). The synthesis of tetrahydrodipicolinate (THDP) from aspartate (Asp) is common to all four variant pathways (Hudson *et al.*, 2006). The synthesis of *meso*-diaminopimelate (*m*-DAP), the penultimate compound, from THDP defines the uniqueness of the variants. Most bacteria use the acyl pathways, where the intermediates from THDP to L,L-diaminopimelate are either

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acetylated or succinylated by an acylase encoded by the DapD enzyme. This is followed by an aminotransferase reaction catalyzed by DapC, a deacylase reaction catalyzed by DapE and an epimerase reaction catalyzed by DapF (Fig. 1*a*). In another variant, *m*-DAP dehydrogenase (Ddh) catalyzes the synthesis of *m*-DAP directly from THDP, bypassing the acylase, aminotransferase, deacylase and epimerase enzymatic steps that are used in the acyl pathways. The most recent pathway to be discovered is the L,L-diaminopimelate aminotransferase (DapL) pathway. The enzyme DapL utilizes glutamate as the amino donor and THDP as the amino acceptor to synthesize L,L-diaminopimelate (L,L-DAP) in a single step, circumventing the acylase, aminotransferase and deacylase reactions that are present in the acyl pathways (Hudson *et al.*, 2006, 2008; McCoy *et al.*, 2006; Nachar *et al.*, 2012; Dobson *et al.*, 2011). Lastly, the conversion of *m*-DAP to lysine by *meso*-diaminopimelate decarboxylase (LysA) is common to all four variants (Fig. 1*a*).

The enzyme dihydrodipicolinate reductase (DapB; EC 1.3.1.26) catalyzes the second step in the diaminopimelate/lysine pathway. It follows the generation of THDP by the enzyme dihydrodipicolinate synthase (Dobson *et al.*, 2008). The enzyme catalyzes the reduction of dihydrodipicolinate to synthesize THDP utilizing NAD(P)H as an electron source

(Farkas & Gilvarg, 1965; Dommaraju *et al.*, 2010; Fig. 1*b*). Given that most eubacteria use one (or more) of the diaminopimelate pathways for diaminopimelate/lysine biosynthesis and that DapB is present in all four variants, DapB is a potential target for the development and/or discovery of broad-spectrum antibiotics. The genomes of animals do not contain the genes for the synthesis of diaminopimelate/lysine, and therefore lysine is deemed to be one of the nine essential amino acids and must be acquired through dietary means to facilitate protein synthesis. In addition, either lysine (Gram-negative bacteria) or the penultimate intermediate, *m*-DAP (Gram-positive bacteria), serve as a cross-linking amino acid at the third position of the peptide stem in the synthesis of peptidoglycan (PG), since the diamine allows the formation of two peptide bonds to facilitate cross-linking (Hutton *et al.*, 2007; Figs. 1*c* and 1*d*). Since diaminopimelate and lysine are essential for both protein and PG synthesis, inhibition of the enzymes in this pathway has the potential to lead to bacterial cell death owing to cell lysis as a result of the osmotic pressure from the lack of and/or improperly constructed PG (Cox, 1996; Baizman *et al.*, 2000). The lack of protein synthesis would also lead to bactericidal and/or bacteriostatic scenario since lysine cannot be incorporated into the protein synthesis (Triassi *et al.*, 2014). As such, the enzymes associated with the

Figure 1

(a) The acyl pathway for diaminopimelate/lysine biosynthesis as present in *B. henselae*. Abbreviations are as follows: LysC, aspartate kinase; asd, aspartate semialdehyde dehydrogenase; DapA, dihydrodipicolinate synthase; DapB, dihydrodipicolinate reductase; DapD, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate *N*-acyltransferase; DapC, acyl-diaminopimelate aminotransferase; DapE, acyl-diaminopimelate dehydrogenase; DapL, L,L-diaminopimelate aminotransferase; DapF, diaminopimelate epimerase; LysA, *meso*-diaminopimelate decarboxylase; MurE, UDP-*N*-acetylmuramoylalanine-2,6-diaminopimelate ligase. (b) Reaction catalyzed by dihydrodipicolinate reductase (DapB). (c) Monomeric unit of peptidoglycan highlighting the *meso*-diaminopimelate or lysine at the third position of the peptide stem. (d) *meso*-Diaminopimelate (*m*-DAP) serves as the cross-linking amino acid with D-glutamate and D-alanine.

Table 1Data-collection and processing statistics for *BhDapB* (PBD entry 3ijp).

Values in parentheses are for the outer shell.

Diffraction source	ALS beamline 5.0.1
Wavelength (Å)	0.9774
Temperature (K)	100.0
Detector	ADSC Quantum 210 CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	10
Space group	<i>P</i> 4 ₃ 22
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.38, 109.38, 176.95
Mosaicity (°)	0.33
Resolution range (Å)	20.00–2.30 (2.36–2.30)
Total No. of reflections	695143 (47260)
No. of unique reflections	48275 (3502)
Completeness (%)	99.8 (100.0)
Multiplicity	9.6 (8.9)
$\langle I/\sigma(I) \rangle$	19.4 (4.4)
$R_{\text{r.i.m.}}^{\dagger}$	0.11 (0.618)
Overall <i>B</i> factor from Wilson plot (Å ²)	18.06

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

diaminopimelate/lysine pathways are attractive targets for the development of antibiotics given their roles in two essential primary metabolic bacterial pathways (cell-wall and protein synthesis). Our group and others have been actively working towards the design of new inhibitors of this pathway (Boughton, Dobson *et al.*, 2008; Boughton, Griffin *et al.*, 2008; Turner *et al.*, 2005; McKinnie *et al.*, 2014).

The macromolecular structure of DapB from a Gram-negative human-pathogenic bacteria, such as *Bartonella henselae*, the causative agent of cat-scratch disease, provides downstream opportunities to facilitate the development of antibiotics that are specific for DapB. Bacterial infection by *B. henselae* can lead to some adverse and dangerous symptoms, such as skin inflammation, fever, lymphadenopathy, various eye disorders, encephalitis and endocarditis (Mazur-Melewska *et al.*, 2015). Although usually benign and treatable in immunocompetent individuals, a recent case showed that *B. henselae* infection can be fatal owing to perivascular lymphocytic infiltrates and microglial nodules in the brain (Fouch & Coventry, 2007). Here, we present the cloning, expression, purification, crystallization and X-ray diffraction analysis of dihydrodipicolinate reductase (DapB) from the pathogenic bacterium *B. henselae* strain Houston-1 at 2.3 Å resolution.

2. Materials and methods

2.1. Amplification and cloning of the *dapB* open reading frame from *B. henselae*

The cloning, expression and purification of DapB from *B. henselae* were conducted as part of the Seattle Structural Genomics Center for Infectious Disease (SSGCID) following standard protocols described previously (Naqvi *et al.*, 2016; Myler *et al.*, 2009; Stacy *et al.*, 2011; Serbzhinskiy *et al.*, 2015; Bryan *et al.*, 2011; Choi *et al.*, 2011). The full-length open reading frame (ORF) encoding amino acids 1–265 (UniProt

Q6G2G3) was amplified by PCR from genomic *B. henselae* strain Houston-1 DNA (ATCC 49882D-5) using the following primer sequences: *BhdapB*-For, 5'-GGGTCCTGGTTCGA-TGCGCCTTACAGTTGTTGGTGC-3', and *BhdapB*-Rev, 5'-CTTGTTCTGCTGCTGTTTATTATTCGTTTCAGTCCCAAA-ACATCAAG-3'. The *dapB* amplicon was cloned using the ligation-independent cloning into the vector pAVA0421, which carries a cleavable hexahistidine fusion tag followed by the human rhinovirus 3C protease-cleavage sequence (MAHHHHHHMGTLEAQTQGPQS) at the N-terminus followed by the *dapB* ORF (Aslanidis & de Jong, 1990). The bold and underlined glutamine (Q) and glycine (G) residues denote the human rhinovirus 3C protease-cleavage site. The pAVA0421::BhdapB plasmid DNA was transformed into *Escherichia coli* BL21 (DE3) R3 Rosetta cells to facilitate protein expression. The DapB protein was expressed using auto-induction medium (2 l) in a LEX Bioreactor (Epiphyte Three Inc.), as described previously (Studier, 2005; Choi *et al.*, 2011).

2.2. Expression and purification of recombinant DapB from *B. henselae*

The hexahistidine-BhdapB was purified using a four-step protocol. The first step consisted of Ni²⁺-affinity chromatography (IMAC). The second step consisted of the cleavage of the N-terminal hexahistidine tag with 3C protease. The third step consisted of removal of the cleaved hexahistidine tag by passage over a second Ni²⁺-affinity chromatography column, and the fourth and final step consisted of size-exclusion chromatography (SEC). Expression and purification were conducted exactly as described previously for *B. henselae* dihydrodipicolinate synthase (DapA; Naqvi *et al.*, 2016). The final SEC column (Superdex 200, GE Healthcare) was equilibrated with running buffer comprised of 20 mM HEPES pH 7, 0.3 M NaCl, 5% glycerol, 2 mM dithiothreitol. The SEC chromatogram showed one predominant peak, which corresponds to a tetrameric oligomeric state of BhdapB. The retention time of the primary peak was used to estimate the molecular weight compared with known standards. The estimated molecular weight was consistent with a tetrameric assembly in buffer solution. The peak fractions were collected and analyzed for the presence of the protein of interest using SDS-PAGE in a 4–20% gradient gel with Tris-MOPS running buffer for 40 min at 175 V. The peak fractions were pooled and concentrated to 6.05 mg ml^{−1} using an Amicon (Millipore) filtration device. Aliquots of 100 µl were cryocooled in liquid nitrogen and stored at −80°C until use for crystallization.

2.3. Crystallization and data collection

Purified BhdapB was screened for crystallization in 96-well sitting-drop plates against the Wizard I and II (Rigaku Reagents) and ProPlex crystal screens (Molecular Dimensions). Equal volumes of protein solution (0.4 µl) and precipitant solution were set up at 293 K against reservoir (80 µl) in sitting-drop vapor-diffusion format. The final crystallization precipitant was ProPlex condition A12 composed of 5% PEG

4000, 200 mM sodium acetate, 100 mM sodium citrate tribasic pH 5.5. The crystals were cryoprotected in crystallant plus 25% 1,2-ethanediol and were cryocooled by dipping them into liquid nitrogen. Data were collected at 373.15 K on Advanced Light Source (ALS) beamline 5.0.1 using an ADSC Quantum 210 CCD detector with 1° oscillations at a wavelength of 0.9774 Å. Data were reduced with *HKL-2000* (Otwinowski & Minor, 1997). Raw X-ray diffraction images are available at the Integrated Resource for Reproducibility in Macromolecular Crystallography (IRMMc; <http://www.proteindiffraction.org>; Grabowski *et al.*, 2016).

2.4. Structure solution and refinement

The structure was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite of programs using the *E. coli* DapB structure (PDB entry 1arz) as a search model (Scapin *et al.*, 1997). The structure was refined using iterative cycles of *REFMAC5* followed by manual rebuilding of the structure using *Coot* (Murshudov *et al.*, 2011; Emsley *et al.*, 2010). The quality of all of structures was checked using *MolProbity* (Chen *et al.*, 2010). Data-collection and data-

Table 2

Structure solution and refinement of *BhDapB* (PDB entry 3ijp).

Values in parentheses are for the outer shell.

Resolution range (Å)	20.00–2.30 (2.36–2.30)
Completeness (%)	99.8 (100)
σ Cutoff	$F > 0.000\sigma(F)$
No. of reflections, working set	48322 (3311)
No. of reflections, test set	2444 (164)
Final R_{cryst}	0.177 (0.26)
Final R_{free}	0.208 (0.294)
No. of non-H atoms	
Protein	3984
Ligand	56
Water	478
Total	4518
R.m.s. deviations	
Bonds (Å)	0.018
Angles ($^\circ$)	1.589
Average B factors (Å ²)	
Protein	23.19
Ligand	17.92
Water	33.9
Ramachandran plot	
Most favored (%)	95.8
Outliers (%)	0

reduction and refinement statistics are shown in Tables 1 and 2, respectively. The structure was refined to a resolution of 2.3 Å. Electron density was visible for the placement of Na⁺ and Cl[−] ions in chains A and B in addition to NADP⁺, a substrate of the enzyme (Supplementary Fig. S1). Structural features of the enzyme were analyzed and prepared using *PyMOL* (v.1.5; Schrodinger) and *PISA*. *PISA* analysis predicts a stable tetrameric assembly composed of four monomers (an analysis of the interface areas is presented in Fig. 4). The coordinates and structure factors of *BhDapB* have been deposited in the Protein Data Bank (PDB; <http://www.rcsb.org>) under the accession annotation 3ijp.

3. Results and discussion

The *dapB* ortholog from *Bartonella henselae* strain Houston-1 was successfully cloned, expressed and purified to homogeneity using a four-step purification scheme to a final concentration of 6.05 mg ml^{−1}. The purity of the enzyme was assessed by SDS–PAGE analysis (Fig. 2*a*), which showed the predicted molecular weight of ~28 kDa for the monomer. Size-exclusion chromatography (SEC) analysis showed a peak with a molecular weight of ~114 kDa which is consistent with a tetramer (Fig. 2*b*). *BhDapB* was screened for crystallization in 96-well sitting-drop plates, which afforded crystals that diffracted to a resolution of 2.3 Å. The structure was subsequently refined to a resolution of 2.3 Å.

The National Center for Biotechnology Information (NCBI) Conserved Domains Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the Protein Families Database (Pfam) were employed for domain analysis (Finn *et al.*, 2014; Marchler-Bauer *et al.*, 2015). *BhDapB* has two domains, which are annotated as the N-terminal and C-terminal domains (Fig. 3*a*). The N-terminal domain (pfam01113) is involved in binding of the nucleotide

Figure 2

SDS–PAGE and SEC analyses of purified recombinant *BhDapB*. (*a*) Lane 1, protein ladder (molecular masses are labelled in kDa); lanes 2, 3, 4, 5, 6 and 7 contain 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 µg, respectively, of the eluate from the purification. The proteins were resolved on a 10% (w/v) acrylamide gel and stained with Coomassie Blue for visualization. (*b*) SEC analysis of purified recombinant *BhDapB* on a Superdex 200 column showing the tetrameric peak (shaded in yellow) at ~114 kDa. The peaks in red depict the various molecular weights of the protein standards.

NAD(P)H and is comprised of amino acids 1–124. The C-terminal domain (pfam05173) is involved in the binding of the substrate L-dihydrodipicolinate and is comprised of amino acids 128–263 (Fig. 3a). The N- and C-terminal domains are based on the *BhDapB* amino-acid numbering, which is similar to the domain mapping of the DapB ortholog from methicillin-resistant *Staphylococcus aureus* (Dogovski *et al.*, 2012).

Analysis of the *BhDapB* structure suggests that the functional enzyme is a homotetramer, which is consistent with other bacterial DapB enzymes from *E. coli* (PDB entry 1arz; Scapin *et al.*, 1997), *Mycobacterium tuberculosis* (PDB entry 1c3v; Cirilli *et al.*, 2003), *S. aureus* (PDB entry 3qy9; Girish *et al.*, 2011) and *Thermotoga maritima* (PDB entry 1vm6; Dogovski *et al.*, 2012). A multiple protein sequence alignment shows the percentage identities of the orthologs listed to that

of *BhDapB* to be 41.2% (*E. coli*), 22.8% (*M. tuberculosis*), 30.5% (*S. aureus*) and 32.9% (*T. maritima*) (Fig. 3b).

In our structure, NADP⁺ is found bound to one monomer within the asymmetric unit, but not the other. It interacts largely with the N-terminal domain (Fig. 4a and 4b) with both charged and π -stacking interactions (Fig. 4c). An overlay of the C-terminal domain (Fig. 4d) demonstrates that binding of NADP⁺ has very little impact on the C-terminal domain, but shows that the N-terminal domain closes over the C-terminal domain by pivoting $\sim 40^\circ$ around a point close to the N-terminal and C-terminal interface (marked with an X). The closed conformation is consistent with the structure of the DapB ortholog from *M. tuberculosis* that was solved with bound substrates (Janowski *et al.*, 2010). Interestingly, it is not uncommon for DapB structures to show this asymmetry and it

Figure 3

Domain mapping and multiple protein sequence alignment. (a) Domain mapping of *BhDapB* using the Conserved Domains Database (CDD) and the Protein Families (Pfam) database, highlighting the amino acids that constitute the N- and C-terminal domains of the enzyme. (b) Multiple protein sequence alignment of DapB orthologs from *Bartonella henselae* (Bh), *Escherichia coli* (Ec), *Mycobacterium tuberculosis* (Mt), *Staphylococcus aureus* (Sa) and *Thermotoga maritima* (Tm). The dark-shaded areas show complete conservation, while the lighter shaded areas show moderate to low conservation. Protein alignment was performed employing the UniProt hub using the *Clustal Omega* feature (<http://www.uniprot.org/align/>; The UniProt Consortium, 2015).

Figure 4

(a) Monomeric structure of chain A of *BhdapB*, illustrating the N-terminal domain (purple) and the C-terminal domain (orange). NADP⁺ is found bound to the N-terminal domain. The sodium ion (Na⁺) and chloride ion (Cl⁻) are also shown. (b) The tetrameric structure of *BhdapB* showing the central β -barrel that forms the tetrameric interface. NADP⁺ is found in two of the four active sites. The surface areas buried by the interfaces of the tetramer, as reported by PISA analysis, are as follows: the average buried surface area between monomers A and B (and C and D) is 1208 Å² and the average buried surface area between monomers A and D (and B and C) is 1518 Å². (c) Scheme showing the interactions that hold NADP⁺ in the active site. (d) An overlay of the two monomers in the asymmetric unit, one of which has NADP⁺ bound in the active site. The overlay was performed on the C-terminal domain only to illustrate the movement of the N-terminal domain upon NADP⁺ binding, which swings ~40° around a pivot point close to the interface of the N- and C-terminal domains.

is likely to be a result of crystal packing. Note that the four monomeric units form a tetramer interface facilitated by a β -barrel of 16 strands, with each monomer contributing four β -strands located in the C-terminal domain (Fig. 4b). This is a consistent feature in all of the DapB ortholog structures that have been solved to date.

In conclusion, we present the cloning, purification, crystallization and X-ray diffraction and structural analyses of DapB from the pathogenic bacterium *B. henselae* strain Houston-1. The data provide a structural characterization of DapB from a pathogenic bacterium such as *B. henselae*. In addition, the data have the potential to facilitate the discovery or the design of

compounds that are deemed bactericidal as a broad-spectrum antibiotic given the fact that DapB is present in all eubacteria that employ the diaminopimelate/lysine pathways for the synthesis of peptidoglycan and proteins.

4. Related literature

The following reference is cited in the Supporting Information: Zheng *et al.* (2014).

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