Crystal structures of FolM alternative dihydrofolate reductase 1 from *Brucella suis* and *Brucella canis*

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Members of the bacterial genus *Brucella* cause brucellosis, a zoonotic disease that affects both livestock and wildlife. *Brucella* are category B infectious agents that can be aerosolized for biological warfare. As part of the structural genomics studies at the Seattle Structural Genomics Center for Infectious Disease (SSGCID), FolM alternative dihydrofolate reductases 1 from *Brucella suis* and *Brucella canis* were produced and their structures are reported. The enzymes share ~95% sequence identity but have less than 33% sequence identity to other homologues with known structure. The structures are prototypical NADPH-dependent short-chain reductases that share their highest tertiary-structural similarity with protozoan pteridine reductases, which are being investigated for rational therapeutic development.

1. Introduction

Brucellosis is the most common bacterial zoonotic disease and is caused by the bacterial genus *Brucella*, which infects humans who consume contaminated animal products, or through contact with infected animals and their secretions (Ducrotoy *et al.*, 2016; Godfroid, Al Dahouk *et al.*, 2013). *Brucella* are classified as category B infectious agents that can be aerosolized (de Figueiredo *et al.*, 2015). Serological evidence suggests that human brucellosis is misdiagnosed as malaria or other febrile diseases in sub-Saharan Africa (Ducrotoy *et al.*, 2017). Brucellosis is highly contagious and affects economically important livestock and wild animals globally (Ducrotoy *et al.*, 2017; Godfroid, Garin-Bastuji *et al.*, 2013; Godfroid *et al.*, 2011; Megersa *et al.*, 2011). While brucellosis has been eradicated in cattle and small ruminants in a few countries, it remains endemic globally within a wide range of animal hosts (Moreno, 2014).

Current control approaches for brucellosis include vaccination, education and basic hygiene; however, these strategies have not effectively reduced the disease burden due to cost and other issues (Ariza *et al.*, 2007). Notably, current vaccines are species-specific and are devastating to pregnant livestock, and cultural practices among rural dwellers and nomadic groups that rear animals are often incompatible with disease control (Ducrotoy *et al.*, 2017; Godfroid, Al Dahouk *et al.*, 2013). There is a continued need to develop new cost-effective approaches to treat infected animals, including the rational
design or repurposing of small molecules that target enzymes that are vital for bacterial survival. The Seattle Structural Genomics Center for Infectious Disease (SSGCID) has determined the crystal structures of many target enzymes, including FoM alternative dihydrofolate reductase 1 from two Brucella species, B. suis and B. canis. Dihydrofolate reductase reduces dihydrofolic acid to tetrahydrofolic acid using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor. While this reaction is catalyzed by the enzyme dihydrofolate reductase (DHFR) in mammals and other organisms, some bacteria have an alternative pathway for reduced folate biosynthesis using FoM alternative dihydrofolate reductase 1 (Levin et al., 2004). Here, we present the crystal structures of FoM alternative dihydrofolate reductase 1 from two Brucella species, B. suis (BsFoM) and B. canis (BcFoM).

BsFoM and BcFoM are 95% identical in sequence. BLAST alignment of the protein sequences against the Protein Data Bank (PDB) reveals the most similar proteins to be T0495 from Thermus thermophilus HB8 (Pampa et al., 2014) with ~32% sequence identity and ~85% coverage; Leishmania major pteridine reductase (Schüttekopf et al., 2005) with ~30% sequence identity and ~90% coverage; Mycobacterium smegmatis short-chain reductase (Blaise et al., 2017) with ~33% sequence identity and ~85% coverage; and Trypanosoma cruzi pteridine reductase 2 (Schormann et al., 2005) with ~30% sequence identity and ~88% coverage. The reported crystal structures of BsFoM and BcFoM are the first steps towards identifying new therapeutics for brucellosis.

2. Materials and methods
2.1. Macromolecule production

Cloning, expression and purification were conducted as part of the Seattle Structural Genomics Center for Infectious Disease (SSGCID) following standard protocols described previously (Myler et al., 2009; Stacy et al., 2011; Bryan et al., 2011; Choi et al., 2011; Serzhinsky et al., 2015). The full-length FoM genes from B. suis (UniProt A0A0H3G2T6) and B. canis (UniProt A9MA73) were PCR-amplified from genomic DNA using the primers shown in Tables 1 and 2, respectively. The resultant amplicons were cloned into the ligation-independent cloning (PLIC; Aslanidis & de Jong, 1990) expression vector pBG1861 encoding a noncleavable 6His fusion tag (MAHHHHHHHMLNDPEARMVANCPVLVTGGA).

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2 mM DTT, 0.025% azide). The peak fractions were collected and analyzed by SDS–PAGE. The SEC peak fractions eluted as a single large peak with an oligomeric mass of ~77 kDa, suggesting an oligomer, most likely dimeric, trimeric or tetrameric enzyme. The peak fractions were pooled and concentrated to 28.5 mg ml$^{-1}$ (His-BsFolM) or 32.3 mg ml$^{-1}$ (His-BcFolM) as assessed by the OD$_{280}$ using an Amicon concentration system (Millipore). Aliquots of 200 μl were flash-frozen in liquid nitrogen and stored at −80°C until use for crystallization.

2.2. Crystallization

Purified His-BsFolM and His-BcFolM were screened for crystallization in 96-well sitting-drop plates against the JCSG++ HTS (Jena Bioscience) and MCSG1 (Molecular Dimensions) crystallization screens. Equal volumes of protein solution (0.4 μl) and precipitant solution were set up at 290 K against 80 μl reservoir in sitting-drop vapor-diffusion format. Before crystallization, NADPH was added to the protein solution to a final concentration of 4 mM (BsFolM) or 6 mM (BcFolM). The precipitant solution was MCSG-1 condition A1 (Tables 3 and 4). The crystals were harvested and cryoprotected with crystallization solution supplemented with 20% ethylene glycol before flash-cooling in liquid nitrogen.

2.3. Data collection and processing

Data were collected at 100 K at the Advanced Photon Source, Argonne National Laboratory (Table 5). The data were reduced with XSCALE (Kabsch, 2010). Raw X-ray diffraction images are available at the Integrated Resource for research communications.
2.4. Structure solution and refinement

Both structures were solved by molecular replacement. BcFolM was solved with BALBES (Long et al., 2008) with PDB entry 2uvd, a 3-oxoacyl-(acyl carrier protein) reductase (Ba3989) from Bacillus anthracis (Zaccai et al., 2008), as the search model. BcFolM was solved with MoRDa (Vagin & Lebedev, 2015) using BcFolM (PDB entry 5bt9) as the search model. Both structures were refined using iterative cycles of refinement in Phenix (Liebschner et al., 2019) followed by manual structure-rebuilding cycles in Coot (Emsley & Cowtan, 2004; Emsley et al., 2010). The quality of both structures was checked using MolProbity (Chen et al., 2010). All research communications

Figure 2

Structural and primary-sequence alignment of FolM alternative dihydrofolate reductase 1 from B. suis (PDB entry 5tdg) and B. canis (PDB entry 5bt9) with the molecular-replacement search model 3-oxoacyl-(acyl carrier protein) reductase from Bacillus anthracis (PDB entry 2uvd) and protozoan structures (Trypanosoma brucei pteridine reductase with cyromazine, PDB entry 2x9n; T. brucei pteridine reductase ternary complex with cofactor and inhibitor, PDB entry 4cm8; T. cruzi pteridine reductase, PDB entry 1mxf). The secondary-structure elements are shown as follows: α-helices are shown as large coils, 310-helices are shown as small coils labeled , β-strands are shown as arrows labeled and β-turns are labeled TT. Identical residues are shown on a red background, with conserved residues in red and conserved regions in blue boxes. Regions of greatest variability within the core of the protein are identified with brown lines and labeled SBC due to their proximity to the substrate-binding cavity.

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data-reduction and refinement statistics are shown in Table 6. The BsFolM structure was refined to a resolution of 1.70 Å, while that of BcFolM was refined to 1.50 Å resolution. Figures depicting the structure were analyzed and prepared using PyMOL (version 1.5; Schrödinger). Multiple sequence alignments were performed using Clustal Omega (Li, 2003; Sievers et al., 2011). Coordinates and structure factors have been deposited in the Protein Data Bank (https://www.rcsb.org/) as entries 5tgd and 5bt9 for BsFolM and BcFolM, respectively.

3. Results and discussion

The structures of FolM alternative dihydrofolate reductase 1 from B. suis (BsFolM) and B. canis (BcFolM) were determined in the monoclinic space group P2₁ with four monomers in the asymmetric unit (Fig. 1). PDBsum analysis (http://www.ebi.ac.uk/pdbsum/) indicates that each monomer interacts with three other monomers, with two large interactions and one smaller interaction. The buried surface areas of the interactions are ~1400, ~1300 and ~770 Å² per monomer. These surface areas involve 31, 25 and 14 interface amino acids per monomer, respectively. The interface interactions are mostly hydrogen bonds and other nonbonded contacts. The tetramers are similar and superpose with an r.m.s.d. of ~0.5 Å (Fig. 1c). The tetramer is the prototypical short-chain dehydrogenase/reductase (SDR) tetramer, suggesting that the single SEC peak may indeed correspond to a tetramer.

Each monomer has the extended double-Rossmann fold of NADPH-dependent SDRs with a central seven-stranded parallel β-sheet sandwiched between two pairs of three α-helices. Both the BsFolM and BcFolM structures were co-crystallized with a cofactor (NADPH). The monomers are virtually identical, with an r.m.s.d. of ~0.17 Å on superposing all main-chain atoms of both structures (Fig. 1).

The most similar structures to BsFolM and BcFolM were identified by PDBeFold (http://www.ebi.ac.uk/msd-srv/ssm) research communications Acta Cryst. (2022). F78, 31–38 Imani Porter et al. FolM alternative dihydrofolate reductase 1
analysis using the default threshold cutoffs of 70% for the percentage of the secondary structure of the target chain identified in the query protein and of the secondary structure of the query chain (Krissinel & Henrick, 2004). The most similar structures are protozoan pteridine reductases (Khalaf et al., 2014; Tulloch et al., 2010; Schormann et al., 2005). These structures share ~29% sequence identity with BsFolM and BcFolM, and their main-chain Cα atoms align with an r.m.s.d. of ~1.5 Å. These protozoan pteridine reductases are more similar to BsFolM and BcFolM than to the structures from Bacillus anthracis (Zaccai et al., 2008), Streptomyces (Wang et al., 2014), Serratia marcescens (Liu et al., 2018), Thermus thermophilus (Asada et al., 2009) or other bacteria.

The BsFolM and BcFolM structures are in the closed conformation with ordered substrate-binding loops, as observed in protozoan pteridine reductases (Khalaf et al., 2014; Tulloch et al., 2010; Schormann et al., 2005; Schüttelkopf et al., 2005). Despite being identified as the closest structures by PDBeFold, the Trypanosoma proteins share a lower sequence identity to BsFolM and BcFolM than the MR search model from B. anthracis (Zaccai et al., 2008), which shares ~30% sequence identity with both proteins (Fig. 2). Both structures have structural differences from the molecular-replacement search model, the 3-oxoacyl-(acyl carrier protein) reductase (Ba3989) from Bacillus anthracis, and have an r.m.s.d. of ~2.12 Å on superposing all main-chain atoms (Fig. 3).

While the cofactor-binding cavities of BsFolM, BcFolM and the Trypanosoma proteins are well conserved, there is a loop insertion (labeled in green; Fig. 2). This loop (labeled the cofactor loop in Fig. 3) points away from the cofactor (NADPH) and aligns well in both BsFolM and BcFolM. Interestingly, this loop is conserved in the protozoan enzymes.

Figure 4
LIGPLOT diagrams reveal well conserved NADPH-binding cavities in FolM alternative dihydrofolate reductase 1 from B. suis (PDB entry 5td) and B. canis (PDB entry 5bh), Trypanosoma brucei pteridine reductase with cyromazine (PDB entry 2xn) T. brucei pteridine reductase in a ternary complex with cofactor and inhibitor (PDB entry 4cm) and T. cruzi pteridine reductase (PDB entry 1mx). Identical amino-acid residues are circled.
and forms a 6.5 Å larger cavity than that observed in the Brucella enzymes (both BsFolM and BcFolM; Fig. 3). Apart from this loop region, the cofactor-binding cavity is very similar in these enzymes. Furthermore, the residues involved in NADPH binding are well conserved (Fig. 4).

As expected, the substrate-binding cavity of each protein shows the greatest structural difference (Figs. 2 and 3). This structural variability is believed to allow substrate specificity among SDRs. While the substrates of BsFolM and BcFolM are unknown, their substrate-binding cavities are large enough to accommodate the inhibitors identified by rational therapeutics discovery for human African trypanosomiasis and Chagas disease. There are >150 structures of complexes of protozoan pteridine reductases with unique inhibitors deposited in the Protein Data Bank (Khalaf et al., 2014; Tulloch et al., 2010; Schormann et al., 2005) that can serve as starting points for the discovery of therapeutics for brucellosis.

4. Conclusions
The high-resolution structures of FolM alternative dihydrofolate reductase 1 from B. suis and B. canis have prototypical NADPH-dependent short-chain reductase topology and structural similarity to the well characterized protozoan pteridine reductases. Despite their low sequence identity, their structural similarity to the protozoan pteridine reductases may accelerate drug-repurposing efforts.

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