Structural basis of inhibition of *Mycoplasma tuberculosis* DprE1 by benzothiazine inhibitors


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Resistance against currently used antibacterial therapeutics increasingly undermines efforts to contain the worldwide tuberculosis (TB) epidemic. Recently, benzothiazine (BTZ) inhibitors have shown nanomolar potency against both drug-susceptible and multidrug-resistant strains of the tubercle bacillus. However, their proposed mode of action is lacking structural evidence. We report here the crystal structure of the BTZ target, FAD-containing oxidoreductase *Mycobacterium tuberculosis* DprE1, which is essential for viability. Different crystal forms of ligand-free DprE1 reveal considerable levels of structural flexibility of two surface loops that seem to govern accessibility of the active site. Structures of complexes with the BTZ-derived nitroso derivative CT325 reveal the mode of inhibitor binding, which includes a covalent link to conserved Cys387, and reveal a trifluoromethyl group as a second key determinant of interaction with the enzyme. Surprisingly, we find that a noncovalent complex was formed between DprE1 and CT319, which is structurally identical to CT325 except for an inert nitro group replacing the reactive nitroso group. This demonstrates that binding of BTZ-class inhibitors to DprE1 is not strictly dependent on formation of the covalent link to Cys387. On the basis of the structural and activity data, we propose that the complex of DprE1 bound to CT325 is a representative of the BTZ-target complex. These results mark a significant step forward in the characterization of a key TB drug target.

BTZ and related compounds inhibit the conversion of decaprenylphosphoryl-β-D-arabinose (DPR) to decaprenylphosphoryl-β-D-arabinofuranose (DPA; Scheme 1B), a precursor of mycobacterial cell wall arabinian. This two-step epimerization reaction is catalyzed by the joint or successive action of the FAD-containing oxidoreductase DprE1 (Rv3790) and the NADH-dependent reductase DprE2 (Rv3791) (Scheme 1B) (8). DPA is the sole known donor substrate for a series of membrane-embedded arabinosyltransferases, including the ethambutol targets EmbC, EmbA, and EmbB (9). Essentiality of DPA supply and lack of alternative synthetic pathways position DprE1, which is highly conserved in mycobacteria (Fig. S1A), and DprE2 at a critical intersection of cell wall biosynthesis, a notion confirmed by transposon mutagenesis (10, 11). This situation has led some to speak of DprE1 as a magic drug target (1).

BTZ and DNB class inhibitors both contain a central benzene ring carrying a nitro group at position 3 (Scheme 1A). Inhibition of DprE1 by BTZ/DNB inhibitors has been shown to require conversion of the nitro to a nitroso group, proposed to form a semimercaptal linkage with a conserved cysteine in the active site of DprE1 (Cys387 in *M. tuberculosis*) (12, 13). There has been speculation that BTZ and DNB inhibitors require activation by a separate nitroreductase (14), but more recently it has been proposed that DprE1 itself is able to activate BTZ in a substrate-dependent fashion, prompting their characterization as suicide inhibitors (13).

Efforts to structurally characterize *M. tuberculosis* DprE1 and its interaction with inhibitors were hampered by the failure to obtain sufficient amounts of soluble recombinant protein. We have been able to overcome this roadblock, facilitating crystallization and structure determination of this target. Herein, we report the crystal structure of *M. tuberculosis* DprE1, both in the ligand-free form and bound to the BTZ-derived inhibitors CT325 and CT319 (12).

**Results**

**Coexpression with Mycobacterial Chaperonin Yields Soluble Recombinant DprE1.** Exploring coexpression with chaperones we found that small amounts of soluble DprE1 could be obtained using the *Escherichia coli* Tuner strain (Fig. S2).


The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4FDN, 4FDO, 4DFP, 4FE1, and 4F66).

1To whom correspondence may be addressed. E-mail: g.besra@bham.ac.uk, l.alderwick@bham.ac.uk, or k.futterer@bham.ac.uk.

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The amount of DprE1 was consistently less than that of the chaperonin *E. coli* GroEL, and attempts to separate the two products by chromatography were not successful. Coexpressing DprE1 with the mycobacterial GroEL homolog Cpn60.1 and the cochaperonin *E. coli* GroES (Fig. S2B) gave similarly disappointing results. However, coexpression with *M. tuberculosis* Cpn60.2 and *E. coli* GroES led to a significant increase of soluble DprE1 that could be readily separated from the chaperone component by Ni-NTA and ion exchange chromatography, yielding ∼5 mg of pure DprE1 from a 2-L culture (Fig. S2C and D). Although no FAD was added to cell lysates or purification buffers, concentrated and crystallized DprE1 showed the characteristic intense yellow color of a FAD-containing protein in the oxidized state.

Crystalllographic Structure Determination and Overall Structure of DprE1. Crystallization screens of purified *M. tuberculosis* DprE1 resulted in hits for several reservoir conditions, with crystals in space group symmetries *P*2₁ or *P*6₃ (Tables S1 and S2). Phases for the structure of ligand-free DprE1 were determined by single-wavelength anomalous diffraction of an ytterbium-derivative crystal of monoclinic DprE1 (two molecules per asymmetric unit; Fig. S3A and B and Table S1). Crystals of DprE1 in complex with 3-(nitroso)-N-(1-phenylethyl)-5-(trifluoromethyl) benzamide [CT325 (12)] were obtained both in the hexagonal and monoclinic crystal forms, providing crystallographically independent views of the mode of inhibitor binding. In addition we obtained crystals in complex with 3-(nitro)-N-(1-phenylethyl)-5-(trifluoromethyl) benzamide [CT319 (12)], which carries an NO₂ instead of the NO ring substituent of CT325.

The model of the hexagonal crystal form in complex with CT319 comprises residues 7–461, with one disordered region (residues 268 and 298), whereas the ligand-free forms and the CT325 complex include two disordered regions in the chain (Fig. S3A and B and Fig. S3C). The overall structure of DprE1 resembles closely that of FAD-containing alditol oxidase from *Streptomyces coelicolor* (15) and several other structural homologs of DprE1, the conformation of FAD and its immediate protein environment are highly conserved. However, in DprE1 there is no covalent link between FAD and the protein, as is typical for VAO enzymes (17). The substrate-binding domain consists of an extended, antiparallel β-sheet (strands β1–β4 and β5–β9, respectively), and several helices (α1–α4 and α11–α13). The cofactor is deeply buried in the FAD-binding domain, with the isoalloxazine positioned at the interface to the substrate-binding domain. In comparison with the structure of alditol oxidase (15) and several other structural homologs of DprE1, the conformation of FAD and its immediate protein environment are highly conserved. However, in DprE1 there is no covalent link between FAD and the protein.

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The monoclinic and hexagonal crystal forms both display an apparent dimer of DprE1 (Fig. S4A). Independent of symmetry, dimerization is mediated by a twofold rotation axis that aligns strands β5 (residues 80–84) of two copies of DprE1, related by either space group symmetry (hexagonal lattice) or noncrystallographic symmetry (monoclinic lattice), respectively. The dimerization interface (buried solvent-accessible surface ∼340 Å²) is stabilized by four main chain–main chain hydrogen bonds and hydrophobic interactions between side chains of residues Ile80, Ile83, and Phe108 (Fig. S4B). However, in sedimentation velocity experiments we find a single monomer peak, suggesting that DprE1 does not dimerize appreciably in solution (Fig. S4C).
Structural Flexibility of DprE1. Electron density of both crystal forms obtained for ligand-free DprE1 reveal disorder of two surface loops in the substrate-binding domain. As a consequence of the disorder, the active site is wide open in the ligand-free form of the enzyme (Fig. 2A). At a maximum, residues 269–297 and 316–330 are lacking electron density, but the degree of disorder or extent of the apparent gaps varies between the monoclinic and hexagonal crystal forms. In the hexagonal form of ligand-free DprE1, the first region is disordered between residues 269 and 297, and the second region lacks density for residues 316–322 (Fig. 1B). In the monoclinic crystal form, the density gap in the second region is considerably larger, affecting residues 316–330 (Fig. 1C). In contrast, the density gap in the first region is smaller than in hexagonal crystals but varies between the two crystallographically distinct molecules of monoclinic DprE1: molecule A is lacking density for residues 269–283, whereas in molecule B this gap extends from 269 to 293 (Fig. 1C). As a result, helix α6 (residues 292–298) only forms fully in molecule A of monoclinic DprE1, whereas it appears as a two-turn fragment in molecule B (Fig. 1C) and is altogether absent from the structural model of hexagonal DprE1 (Fig. 1B).

However, there are two conditions under which we observe ordering of region one or region two, but not both simultaneously. In the density for crystal Apo-mcl-dmso, the chain can be completely traced between residues 268 and 284 for molecule A (trace in cyan in Fig. 1C) but not for molecule B. The peptide chain of this region is vauling over the β-sheet of the substrate-binding domain. Contacts with a symmetry-related copy of molecule A in the center of this loop help stabilize this particular conformation. Complete ordering of the second region (residues 316–330) is observed in hexagonal crystal of the DprE1:CT319 complex, where residues 316–322 become ordered when CT319 is bound (trace in cyan in Fig. 1B, red surface in Fig. 2B, and Fig. S5 A and B). As result, residues 317–327 form a three-turn helix (Fig. 1A, trace in cyan), although it does not maintain strict α-helical geometry. Unit cell parameters and crystal symmetry are unchanged between the ligand-free and the CT325- and CT319-bound forms of hexagonal DprE1, despite different reservoir conditions (Table S2). We, therefore, attribute ordering of residues 316–322 to binding of CT319, the position of which in the active site differs subtly but distinctly from that of CT325 (Fig. S5C).

Structures of the Complexes with BTZ Derivatives CT325 and CT319. Although fluorescence-based ligand-binding experiments (Fig. S6A) demonstrated binding of the inhibitor BTZ043 (S) to DprE1, crystals grown in the presence of BTZ043 did not reveal a bound ligand. Treffzar et al. (13) had suggested that formation of the covalent complex between BTZ043 and DprE1 requires prior conversion of the nitro group to the nitroso form. To circumvent the need for inhibitor activation, we synthesized the nitroso-benzothiazinone CT325 (12) for cocrystallization experiments.

We obtained crystals of the DprE1:CT325 complex in both the hexagonal and monoclinic crystal forms (CT325-hex3 and CT325-mcl; Table S1) and recorded diffraction data to resolutions of 2.4 and 2.6 Å (CuKα), respectively. A σA-weighted Fo-Fc difference density map, calculated with model phases before including the ligand in the model, displays prominent density between flavin and Cys387, the residue postulated to be critical for BTZ-mediated inhibition of DprE1 activity and BTZ-dependent cessation of mycobacterial growth (5) (Fig. 3 A and B). We observe bridging density between this cysteine and the density enveloping the inhibitor model, evidence for the formation of a covalent bond between CT325 and Cys387 in both crystal forms. However, the conformation of the
phenylethyl group remained undefined in the CT325 complexes. While attempting to obtain diffraction data of the DprE1:CT325 complex, we noticed that for some crystals the bridging density between CT325 and Cys387 was notably absent, but the whole of the inhibitor molecule was defined by electron density, including the terminal phenylethyl group. We suspected that the reactive nitroso group was unstable and may have reverted to the more stable NO₂ state before binding to the protein, preventing formation of the covalent link to Cys387. To test this assumption we generated crystals of CT319-bound DprE1, which led to a noncovalent complex and ordering of the loop region encompassing residues 316–322 over the active site (Fig. S5A). As a result of the ordered state of residues 316–330, the active site is shielded from bulk solvent (Fig. 2B), whereas in the crystals of the DprE1:CT325 complex, the 316–330 loop region remains in the disordered state.

The consistent density features obtained from crystals in different crystal forms allow one to unequivocally position CT325 in the active site. The inhibitor adopts an extended conformation, running parallel to the isoalloxazine. The trifluoromethyl moiety packs against the backbone of residues 132–134, forming van der Waals interactions with Gly133 and Lys134 (contact distances between 3.3 Å and 3.9 Å; Fig. 3C and Fig. S7A), with additional van der Waals contacts to the side chains of His132, Ser228, and Lys367. The central benzamide group primarily interacts with the phenyl ring and contact distances between 3.0 Å and 3.5 Å, with Gly117 (3.2 Å), and with the side chain of Val365 (shortest contact 3.7 Å). In addition the NO group forms a strong H-bond (2.4 Å) with the amide of Asn385. Compared with the ligand-free and CT319-bound state, the side chain of Cys387 is rotated by 180° about the Cα-Cβ bond, facilitating the covalent bond to the nitroso group, consistent with a previous prediction (12). The terminal phenylethyl group is disordered in the CT325-bound complex but well ordered for CT319, in which case the benzene ring occupies a hydrophobic cavity formed by Leu363 and Val365 in strand β16, Trp230 in strand β11, and by Leu317 and Phe320 in the “316–322” loop (Fig. S7B). In the absence of loop ordering, the cavity applies fewer constraints on the orientation of the phenyl ring, and it is plausible that the terminal benzene ring can assume variable orientations.

CT325 Inhibits Epimerization of DPR to DPA. Although CT319-dependent inhibition of DprE1 had been previously demonstrated for the Mycobacterium smegmatis ortholog (12), activity data were not available to us for CT325. Incubating ¹⁴C-labeled decaprenylphosphoryl-β-D-ribose (¹⁴C-DPR) with purified M. tuberculosis DprE1 and DprE2 and analyzing reaction products by TLC, we observe almost complete conversion of DPR to DPA (Fig. 4A). When incubated with DprE1 alone, the DPR band is reduced in intensity, and a distinct band appears below the DPR (Fig. 4B). A similar result was observed when ¹⁴C-DPR was incubated with DprE1 alone, and conversion to the keto-intermediate DPX was monitored. (C) CT325-inhibited inhibition of growth in batch cultures of M. bovis bacillus Calmette–Guérin and M. smegmatis, monitored for 10 d and 40 h, respectively, by measuring absorbance at 600 nm. SDs are derived from two independent experiments.

Discussion

The recent controversy surrounding the report of a “totally drug resistant” strain of M. tuberculosis in India (19) illustrates the high stakes involved in the effort to control the TB pandemic, in particular because this burden is carried primarily by resource-limited health care systems in the developing world. Thus, the discovery of benzo(thiazinones and dinitrobenzamides as novel classes of potent inhibitors, effective against both drug-susceptible and MDR/XDR forms of TB, and the identification of their target—DprE1—are significant steps forward.

Epimerization of DPR is the final step of DPA synthesis, which involves the conversion of glucose to ribose-5-phosphate via the pentose phosphate pathway, followed by synthesis of phosphoribose-diphosphate (pRpp), nucleophilic replacement of pyrophosphate by decaprenyl-phosphate, and removal of the 5′-phosphate to yield DPR (20). Although not firmly established, it is likely that after attachment of the decaprenyl group, the DPA precursor compounds are anchored into the cell membrane. This raises the question of how DprE1 gains access to substrate. DprE1 may need to be able to partially or wholly sequester the decaprenyl moiety of the substrate. The wide-open active site of ligand-free DprE1 and the two flexible loops contrast with the narrow, funnel-like access channel to the active site in the known structures of ligand-free VAO enzymes, for instance in alditol oxidase (Fig. S7C). Therefore, it seems plausible to invoke a link between flexibility of these loops and DPR binding.
We propose that the structure of CT325-bound DprE1 is a representative of the activated BTZ-target complex. The structure and activity data demonstrate that CT325 binds to DprE1 and that this interaction interferes with the conversion of DPR to DPX in a dose-dependent fashion (Fig. 4B). We have not shown explicitly that inhibition of DprE1 by CT325 compromises cell wall arabinan, but the suppression of DPR to DPA conversion (Fig. 4D) and CT325’s whole cell activity against *M. smegmatis* and *M. bovis* bacillus Calmette–Güerin (Fig. 4C) provides indirect evidence for such an effect.

Covalent binding of BTZ043 to DprE1 has been shown to be dependent on Cys387: substitutions by Gly or Ser at this site interfere with inhibition of DprE1 and that this interaction interferes with the conversion of DPR to DPX in a dose-dependent fashion (Fig. 4B). We have not shown explicitly that inhibition of DprE1 by CT325 compromises cell wall arabinan, but the suppression of DPR to DPA conversion (Fig. 4D) and CT325’s whole cell activity against *M. smegmatis* and *M. bovis* bacillus Calmette–Güerin (Fig. 4C) provides indirect evidence for such an effect.

### Methods

**Overexpression and Purification of DprE1.** DprE1 (Rv3790) was coexpressed with chaperones from *E. coli* (GroES) and *M. tuberculosis* (CPN60.2) in *E. coli* BL21 (DE3). Liquid cultures of *E. coli*, harboring pET28a-Rv3790 and pTrc60.2-GroES, were grown at 37 °C in LB broth (Difco) supplemented with kanamycin (50 μg/mL) and ampicillin (100 μg/mL). At OD₆₀₀nm 0.4–0.6 the temperature was reduced to 16 °C, and protein expression of Rv3790 and chaperones was induced with 0.5 mM isopropylthiogalactoside, followed by incubation overnight (16 °C). Cells were harvested, washed with 0.85% saline, and stored at −20 °C. Frozen cell pellets were thawed on ice and resuspended in 30 ml of 50 mM NaH₂PO₄ (pH 8), 300 mM NaCl, and 10 mM imidazole (buffer A), supplemented with EDTA-free protease inhibitor mixture (Roche). The suspension was sonicated (Sonicator Ultrasonic Liquid Processor XL, Misonix) on ice for a total time of 10 min (20-s pulses, 40-s cooling). The lysate was centrifuged (27,000 × g, 40 min, 4 °C), and the supernatant passed through a 0.45-mm filter. Next, prepacked Ni²⁺-charged HiTrap Chelating HP (GE Healthcare). The column was washed with 50 mL of buffer A plus 20 mM imidazole, and the protein was eluted with a 50–300 mM gradient of imidazole. Fractions containing protein were dialyzed against 20 mM Tris HCl (pH 8.5), 10 mM NaCl, and 10% (vol/vol) glycerol (buffer B). After dialysis, the protein was loaded on a 1-mL QHP ion exchange column (Amersham), washed with 10 mL of buffer B with 50 mM NaCl, and eluted with a gradient of 20–200 mM in buffer B. Fractions containing pure protein were dialyzed overnight against buffer B and concentrated to 35 mg/mL, by ultrafiltration (10-kDa cutoff, Amicon Ultra).

### Crystallization and Structure Determination

Crystals of DprE1 were grown by sitting drop vapor diffusion in 96-well plates (Swissvec), aided by a Mosquito (TTP Labtech) liquid handling robot. Conditions producing diffracting crystals of DprE1 are listed in Table S2. Crystals appeared after 1–3 d and could be mounted from the drop and frozen in liquid nitrogen without additional cryoprotection. The heavy atom derivative was obtained by immersing monoclonic crystals for 12 min in mother liquor supplemented with 50 mM ytterbium acetate, before mounting and flash freezing. Diffraction data were collected using XDS, XSCALE (21) (Table S1). Single-wavelength anomalous diffraction data from the Yb-derivative crystal (Table S1) allowed determination of the heavy atom positions [SHELXL (22)]. Heavy atom sites were found with the batch and phase cycle [SOOT (28), REFMAC5 (26), PHENIX REPAIR (24)]. Structures of the inhibitor-bound enzyme were solved by molecular replacement [PHASER (27)], with model building and refinement according to standard protocols. Differences mapping indicates the presence of CT325 or CT319 were calculated according to protein coordinates alone, before incorporation of the ligand coordinates in the structural model. Figures were prepared using PyMol (www.pymol.org), adopting the Corey-Pauling-Koltun (CPK) coloring scheme: O, red; N, blue; S, sulfur; and C, as indicated in figure legends.

**Activity Assays.** The activity of DprE1, in the presence and absence of DprE2, was assayed using radiolabeled [14C]-DPR substrate, prepared as previously described (30). Reaction mixtures contained 50 μg of each enzyme, 1 mM FAD, 1mM ATP, 1 mM NaCl, and 1 mM NADP, in 50 mM Mops (pH 7.9), 10 mM MgCl₂. To probe inhibition, enzymes were incubated with inhibitors (CT325 2.5 μM final concentration, or BTZ043 at a final concentration of 0.1 mM) for 30 min at 30 °C, followed by addition of 2,000 cpm of [14C]-DPR in 5.5 μL of 1% IGEPA. Reactions were allowed to proceed for a further 90 min and then quenched with 350 μL of CHCl₃:CH₃OH:CH₃OD (2:1, vol/vol) and 55 μL H₂O. The bottom layer of the biphase was dried down, resuspended in 10 μL of CH₃OH:CH₃OD (2:1, vol/vol), and analyzed. Samples were spotted on to a high-performance aluminum-backed TLC plate (Merck) and separated in CH₃OH:CH₃OH:1M CH₃CONH₂:conc. NH₄OH:H₂O (180:140:9:23) (vol/vol) Bands were visualized using a phosphor imaging screen (Fuji) and a phosphor imager (Bio-Rad). CT325 was dissolved in dimethyl sulfoxide to a concentration of 2 mg/mL and added to 2 mL of tryptic soy broth in culture tubes to a final concentration between 0 and 100 μg/mL. After the addition of 10 μL of a cell culture of stationary phase *M. smegmatis* mc²155 or *M. bovis* bacillus Calmette–Güerin, the cultures were incubated at 37 °C for 40 h (mc²155) or 10 d (M. bovis bacillus Calmette–Güerin) with shaking. Growth culture was monitored by measuring absorbance at 600 nm (OD₆₀₀) and the experiment was performed in duplicate.

### Deposition of Coordinates and Structure Factors

Coordinates and structure factors of DprE1 in ligand-free and CT325-bound forms have been deposited in the Protein Data Bank (www.pdb.org).

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