Crystallographic insights into the structure–activity relationships of diazaborine enoyl-ACP reductase inhibitors

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Enoyl-ACP reductase, the last enzyme of the fatty-acid biosynthetic pathway, is the molecular target for several successful antibiotics such as the tuberculosis therapeutic isoniazid. It is currently under investigation as a narrow-spectrum antibiotic target for the treatment of several types of bacterial infections. The diazaborine family is a group of boron heterocycle-based synthetic antibacterial inhibitors known to target enoyl-ACP reductase. Development of this class of molecules has thus far focused solely on the sulfonyl-containing versions. Here, the requirement for the sulfonyl group in the diazaborine scaffold was investigated by examining several recently characterized enoyl-ACP reductase inhibitors that lack the sulfonyl group and exhibit additional variability in substitutions, size and flexibility. Biochemical studies are reported showing the inhibition of *Escherichia coli* enoyl-ACP reductase by four diazaborines, and the crystal structures of two of the inhibitors bound to *E. coli* enoyl-ACP reductase solved to 2.07 and 2.11 Å resolution are reported. The results show that the sulfonyl group can be replaced with an amide or thioamide without disruption of the mode of inhibition of the molecule.

1. Introduction

Fatty acids are critical components of several types of lipids. Synthesis of these fatty acids from acetyl-CoA and malonyl-CoA is an essential cellular process in all organisms. New fatty-acid chains are built by sequential condensation of acetyl units by fatty-acid synthase through a repeating four-step reaction sequence that is highly conserved among different organisms. Two main cellular strategies are used to carry out this reaction sequence: the type I fatty-acid synthesis (FAS) pathway used by mammalian and fungal cells, and the type II FAS pathway found in bacteria and plants. While the FAS II pathway consists of multiple enzymes that each catalyse a single reaction, the FAS I pathway consists of one or two multifunctional proteins that catalyse all of the reactions in the pathway.

Research has demonstrated that disruption of fatty-acid biosynthesis impacts bacterial lipid metabolism, most notably in the synthesis of lipopolysaccharide (Högenauer & Woisetschlager, 1981; Baldock, de Boer *et al.*, 1998), a major component of the Gram-negative bacterial membrane. The major differences between the bacterial FAS II and mammalian FAS I biosynthetic machineries make this process a good therapeutic target, as inhibitors can be designed against the

FAS II machinery that do not affect the FAS I pathway (Wright & Reynolds, 2007; Lu *et al.*, 2011). Fatty-acid biosynthesis is somewhat underutilized as a drug target, as not all pathogens contain all of the FAS II homologs (Payne *et al.*, 2006) and some Gram-positive bacteria can surmount inhibition of the pathway by scavenging fatty acids from the host organism (Parsons & Rock, 2011). However, the pathway is still a promising target for the development of narrow-spectrum antibiotics, as diversity among the FAS II enzymes of different organisms could be exploited to target bacteria specifically (Massengo-Tiassé & Cronan, 2009; Parsons & Rock, 2011).

The last step in the FAS II elongation cycle is catalyzed by the enzyme enoyl-acyl carrier protein (ACP) reductase (ENR). ENRs use NADPH or NADH to reduce the double bond of an enoyl-ACP FAS II intermediate to yield the corresponding saturated acyl-ACP. In this reaction, hydride is transferred from the dihydronicotinamide ring to carbon 3 of the acyl chain to form an enolate. This intermediate is protonated by Tyr156 at carbon 2 to yield the product (Rafferty *et al.*, 1995). Inhibition of the bacterial enoyl-ACP reductases can effectively block fatty-acid biosynthesis (Kater *et al.*, 1994). This is the mode of action of several antibacterial inhibitors or antibiotics currently in use (triclosan, a broad-spectrum antibacterial compound incorporated into many consumer products, and isoniazid, a front-line tuberculosis treatment) or under development (Tsuji *et al.*, 2013; Wright & Reynolds, 2007; Lu *et al.*, 2011; Afanador *et al.*, 2013; Schiebel *et al.*, 2014). Several atomic-level structural models of this enzyme from *Escherichia coli* (Baldock *et al.*, 1996),

Mycobacterium tuberculosis (Li *et al.*, 2014) and other organisms [*Helicobacter pylori* (Lee *et al.*, 2007), *Bacillus subtilis* (Kim *et al.*, 2011) and others] are available to aid in this development.

The diazaborines are a family of boron-containing molecules that can specifically inhibit ENR (Lu *et al.*, 2011; Lu & Tonge, 2008) in an NAD⁺-dependent manner (Kater *et al.*, 1994). These molecules have been under examination as ENR inhibitors for over two decades, and are known to function by forming a B–O covalent adduct with the NAD⁺ cofactor through its ribose 2'-hydroxyl (Baldock *et al.*, 1996; Baldock, de Boer *et al.*, 1998; Lu & Tonge, 2008). Structurally, the diazaborine class of ENR inhibitors contains a six-membered diazaborine ring fused to a five- or six-membered ring and an alkyl or aryl side chain attached *via* a sulfonyl linker to N2 of the diazaborine ring (Fig. 1). Structure–activity studies on these molecules have focused on substitutions and variations in ring Y and the side chain R, along with substitutions *para* to the B atom in the diazaborine ring (Grassberger *et al.*, 1984; Baldock, de Boer *et al.*, 1998; Levy *et al.*, 2001). The nature of the fused ring has a significant impact on biological activity (Table 1). As a general rule, thieno-diazaborines have significantly stronger antibacterial activity than benzo-, furo- and pyrrolo-diazaborines (Grassberger *et al.*, 1984; Baldock, de Boer *et al.*, 1998). Additionally, decreasing the length of the organosulfonyl side chain generally results in decreasing activity (Table 1), with a propylsulfonyl side chain having maximal activity. Crystallographic studies have shown that the sulfonylated diazaborines 6-methyl-2(propane-1-sulfonyl)-2H-thieno[3,2-*d*][1,2,3]diazaborinin-1-ol (referred to here

Figure 1

Structures of the diazaborine ENR inhibitors used in this study and in previous crystallographic studies. Top left, the simplified diazaborine scaffold (Y represents a fused five- or six-membered aromatic or heteroaromatic ring; R represents an alkyl- or arylsulfonyl side chain; Baldock, de Boer *et al.*, 1998).

Table 1

Diazaborine antibacterial activity.

Minimum inhibitory concentrations (MICs) against *E. coli*, *Klebsiella pneumoniae* and *M. smegmatis* previously reported in the literature are tabulated here unless not determined in the studies (ND).

Molecule	MIC values ($\mu\text{g ml}^{-1}$)			Reference
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>M. smegmatis</i>	
2-Propylsulfonyl-thienodiazaborine	1.25	0.39	ND	Grassberger <i>et al.</i> (1984)
2-Tosyl-benzodiazaborine	25	3.12	ND	Grassberger <i>et al.</i> (1984)
2-Methylsulfonyl-benzodiazaborine	>50	ND	ND	Grassberger <i>et al.</i> (1984)
2-Methylsulfonyl-6-methylbenzodiazaborine	25	6.25	ND	Grassberger <i>et al.</i> (1984)
14b	16	ND	>32	Kanichar <i>et al.</i> (2014)
18c	32	ND	4	Kanichar <i>et al.</i> (2014)
35b	16	ND	>32	Kanichar <i>et al.</i> (2014)
39	32	ND	>32	Kanichar <i>et al.</i> (2014)
Triclosan	0.250	ND	0.5	Kanichar <i>et al.</i> (2014)

as 2-propylsulfonyl-thienodiazaborine) and 2-(toluene-4-sulfonyl)-2*H*-benzo[*d*][1,2,3]diazaborinin-1-ol (referred to here as 2-tosyl-benzodiazaborine) shown in Fig. 1 bind in the same general area as the substrate is expected to bind during catalysis (Baldock *et al.*, 1996; Baldock, de Boer *et al.*, 1998), competitively inhibiting the enzyme through noncovalent interactions.

Building upon these studies, recent work examined the effect of replacing the sulfonyl moiety of the diazaborine scaffold with an acyl group on the antibacterial activity of the molecule (Kanichar *et al.*, 2014). Several of those molecules retained activity against *E. coli* or *M. smegmatis* (Table 1). These molecules were diazaborines 14b, 18c, 39 (Kanichar *et al.*, 2014) and 35b, which was originally synthesized by others searching for antifungal boron heterocycles (Hicks *et al.*, 2008). These four molecules are all benzodiazaborines (Fig. 1), and in addition to the absence of the sulfonyl group they vary from the previously studied diazaborines in the substitution patterns of the diazaborine ring and the molecular size and conformational flexibility of the molecule. As expected for a benzodiazaborine, their antibacterial activity is much weaker than that of a thienodiazaborine, but surprisingly 14b and 35b demonstrated better activity than the corresponding sulfonylated molecule, 2-methylsulfonyl-benzodiazaborine (Table 1; Grassberger *et al.*, 1984; Kanichar *et al.*, 2014).

To confirm that these molecules specifically inhibit ENR and to provide insight into how they retain antibacterial activity even without a sulfonyl linker, we studied the interaction between these four diazaborines and ENR from *E. coli* (ecFabI). Biochemical activity assays confirmed that molecules 14b, 18c, 35b and 39 all inhibit ecFabI, and X-ray crystallographic studies yielded models of ecFabI in the apo form (apo FabI) and bound to the 14b–NAD (14b–FabI) and 35b–NAD (35b–FabI) inhibitor complexes. These models demonstrate that the diazaborine sulfonyl group can be replaced by a carbonyl or thiocarbonyl group without disruption of the mode of inhibition. They also support the proposal that the longer alkyl side chains of the diazaborine scaffold are required for ordering of an active-site loop (Baldock, de Boer *et al.*, 1998) and suggest that the formation of an additional hydrogen bond between the inhibitor and protein helps to increase the inhibitory strength of molecules 14b and 35b.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the ecFabI protein was cloned *via* the ligation-independent cloning (LIC) strategy into the expression vector pET-30 Ek/LIC using a LIC cloning kit according to the manufacturer's instructions (Novagen Technical Bulletin No. 163). Briefly, PCR primers were designed such that each primer contained an additional tail at the 5' end consisting of a unique stretch of bases for LIC procedures (Novagen Technical Bulletin No. 163). The *fabI* gene was amplified *via* PCR using genomic DNA from *E. coli* strain MG1655 as the template. The amplified DNA fragment was treated with T4 DNA polymerase in the presence of dATP and annealed to linearized pET-30 Ek/LIC vector (Novagen Technical Bulletin No. 163). The annealed products were transformed into *E. coli* NovaBlue competent cells (Novagen Technical Bulletin No. 163) and confirmed. The recombinant plasmid (pET30-ecFabI) was isolated and retransformed into the expression host *E. coli* BL21 (DE3) (Supplementary Table S1). Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 37°C. Cells were lysed by treatment with lysozyme and sonication in 50 mM Tris, 150 mM NaCl pH 7.5 with 10 $\mu\text{g ml}^{-1}$ DNase, 10 $\mu\text{g ml}^{-1}$ RNase and 1 mM PMSF. The crude extract was fractionated on a GE Healthcare HisTrap HP column with imidazole elution. After purification by nickel affinity, the protein sample was dialyzed into 20 mM Tris, 5% glycerol, 1 mM DTT pH 7.5 and concentrated to 10 mg ml⁻¹. Typical yields were approximately 15 mg purified ecFabI per litre of culture.

2.2. Crystallization

Initial ecFabI crystallization conditions were identified by sparse-matrix screening using Crystal Screen and Index from Hampton Research. Preliminary crystals were grown by the hanging-drop vapor-diffusion method from 2 μl drops consisting of 1 μl protein solution (10 mg ml⁻¹ in ecFabI storage buffer: 20 mM Tris, 5% glycerol, 1 mM DTT pH 7.5) and 1 μl crystallization solution (100 mM citrate pH 7.0, 100–250 mM ammonium sulfate, 22–27% (w/v) PEG 3350 or PEG 2000) equilibrated against 750 μl crystallization solution in standard 24-well Linbro plates. Standard grid optimization of

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

Model	Apo FabI (PDB entry 5cfz)	14b-FabI (PDB entry 5cg1)	35b-FabI (PDB entry 5cg2)
Diffraction source	Beamline 12-2, SSRL	Beamline 12-2, SSRL	Beamline 12-2, SSRL
Wavelength (Å)	0.95370	0.97950	0.97950
Temperature (K)	100	100	100
Detector	Pilatus 6M CCD, Dectris	Pilatus 6M CCD, Dectris	Pilatus 6M CCD, Dectris
Crystal-to-detector distance (mm)	425	440	450
Rotation range per image (°)	0.15	0.15	0.15
Total rotation range (°)	60	90	90
Exposure time per image (s)	0.2	0.2	0.2
Space group	<i>P</i> ₆ ,22	<i>P</i> ₆ ,22	<i>P</i> ₆ ,22
<i>a</i> , <i>b</i> , <i>c</i> (Å)	80.15, 80.15, 323.94	80.07, 80.07, 325.02	79.91, 79.91, 323.79
α , β , γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Mosaicity (°)	0.16	0.13	0.14
Resolution range (Å)	38.90–1.97 (2.08–1.97)	38.87–2.07 (2.18–2.07)	39.66–2.11 (2.23–2.11)
Total No. of reflections	523117 (56136)	342450 (11829)	624835 (69022)
No. of unique reflections	44807 (6174)	38218 (4875)	36240 (4921)
Completeness (%)	99.4 (96.2)	98.1 (88.6)	99.3 (95.0)
Multiplicity	11.7 (9.1)	9.0 (7.5)	17.2 (14.0)
$\langle I/\sigma(I) \rangle$	26.6 (10.1)	27.4 (13.5)	42.6 (18.1)
$R_{\text{r.i.m.}}$ [†]	0.061 (0.172)	0.056 (0.111)	0.049 (0.119)
$R_{\text{p.i.m.}}$	0.017 (0.053)	0.019 (0.039)	0.012 (0.030)
Overall <i>B</i> factor from Wilson plot (Å ²)	21.2	21.8	26.6

[†] The redundancy-independent merging *R* factor $R_{\text{r.i.m.}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

each crystallization component was used to improve crystal size and quality. Early optimizations often yielded many interlocking, twinned crystals. Decreasing the precipitant concentration improved this phenomenon, likely by decreasing the frequency of crystal nucleation and the rate of vapor diffusion to allow the growth of larger, more complete crystals.

The diazaborine–NAD⁺-bound ecFabI crystals were obtained by cocrystallization of ecFabI pre-incubated with 450 μM NAD⁺ and 1.13 *mM* inhibitor using inhibitor stocks made up in 100% DMSO. Inhibitor and NAD⁺ were added to the protein stock solution and incubated on ice for 30 min. The pre-incubated protein stocks were centrifuged at 4°C for 10 min and transferred to a fresh tube prior to use in crystallization experiments. Inhibitor-bound ecFabI crystallized with the same general crystal morphology in the same conditions as used to crystallize apo FabI. However, the data presented here were collected from crystals obtained in 100 *mM* HEPES pH 7.5, 2.0 *M* ammonium sulfate (35b-FabI) and 100 *mM* Tris pH 8.5, 2.0 *M* ammonium sulfate (14b-FabI); both crystals were harvested directly from Index trays.

2.3. Data collection and processing

All crystals were cryoprotected with crystallization solution supplemented with 10% glycerol and subsequently flash-cooled in liquid nitrogen prior to data collection. X-ray diffraction data were collected remotely on beamline 12-2 of the Stanford Synchrotron Radiation Laboratory at the Stanford Light Accelerator Center, Menlo Park, California, USA using the *Blu-Ice* (Stepanov *et al.*, 2011) software. The data sets were processed using *XDS* (Kabsch, 2010) or *HKL-2000* (Otwinowski & Minor, 1997).

2.4. Structure determination, refinement and structural analysis

The ecFabI data were phased by molecular replacement in *Phaser* (McCoy *et al.*, 2007) from the *CCP4* program suite (Winn *et al.*, 2011) using a previous ecFabI structure as the search model (PDB entry 1qsg; Stewart *et al.*, 1999). *PRODRG* (van Aalten *et al.*, 1996) and the *Grade* web server (*Grade* v.1.2.9; <http://grade.globalphasing.org>) were used to create parameter and topology library files for refinement of the NAD–inhibitor covalent complexes. The ecFabI models were built in *Coot* (Emsley & Cowtan, 2004) and refined in both *CNS* (Brünger *et al.*, 1998) and *REFMAC5* (Murshudov *et al.*, 2011). Automatically generated local NCS restraints were used throughout the refinement in *REFMAC5*. OMIT electron-density maps were calculated from models refined without the NAD–inhibitor covalent complexes to demonstrate the presence of the inhibitors in each crystal. Figures were prepared using *PyMOL* (v.1.3r1; Schrödinger).

2.5. Enzyme-activity assays

Enzymatic activity was determined by following NADH consumption. Assay mixtures consisted of 20 *mM* HEPES pH 7.4, 150 *mM* NaCl, 250 μM crotonoyl-CoA and 4.8 μM ecFabI in a total volume of 120 μl . Each reaction was initiated by adding 161 μM NADH, and the absorbance at 340 nm was monitored on a Cary 60 spectrophotometer for 1 min to obtain initial reaction rates.

This activity assay was modified to study the inhibition of ecFabI by the diazaborine inhibitors 14b, 18c, 35b and 39. Each assay is assembled as described above. Before addition of NADH to initiate the reaction, 52 μM inhibitor (prepared in DMSO and stored in the dark) and 50 μM NAD⁺ were

Table 3
Structure solution and refinement.

Values in parentheses are for the outer shell.

Model	Apo FabI	14b–FabI	35b–FabI
Resolution range (Å)	38.90–1.97 (2.02–1.97)	38.87–2.07 (2.12–2.07)	39.66–2.11 (2.17–2.11)
Completeness (%)	99.4	97.3	98.8
σ Cutoff	$F > 0.000\sigma(F)$	$F > 0.000\sigma(F)$	$F > 0.000\sigma(F)$
No. of reflections, working set	42431 (2548)	35992 (1834)	34171 (1986)
No. of reflections, test set	2255 (124)	1944 (105)	1836 (111)
Final R_{work} (%)	19.1 (22.0)	20.4 (22.5)	20.2 (19.7)
Final R_{free} (%)	22.5 (28.8)	24.7 (27.4)	24.1 (22.8)
No. of non-H atoms			
Protein	3653	3643	3510
Ligand	0	116	116
Solvent	226	363	213
Total	3879	4122	3839
R.m.s. deviations			
Bonds (Å)	0.020	0.013	0.012
Angles (°)	2.011	1.539	1.492
Average B factors (Å ²)			
Protein	21.0	20.2	20.2
Ligand	n/a	30.2	29.8
Water	29.1	31.7	31.6
Ramachandran plot			
Most favored (%)	98.0	97.0	97.0
Allowed (%)	2.0	3.0	3.0

added to the assay mixture and incubated for 10 min in the dark to allow the inhibitor–NAD⁺ complex to form and bind to the enzyme. The control experiments used here included 3% DMSO and 50 μM NAD⁺ with no inhibitor.

3. Results

3.1. Purification and crystallization of ecFabI

The construct generated by ligation-independent cloning into pET-30 Ek/LIC has a thrombin-cleavable N-terminal six-histidine tag (Supplementary Table S1). The ecFabI protein was purified using nickel-affinity chromatographic methods. The protein crystallized readily in 100 mM citrate pH 7.0,

Figure 2
Inhibition of the ecFabI-catalyzed reduction of crotonyl-CoA by the inhibitors 14b, 18c, 35b, 39 and triclosan. The averaged rate of the ecFabI reaction in the presence of each inhibitor is shown along with the control reaction rate, which included DMSO and NAD⁺. Each rate is the average of three independent determinations.

100 mM ammonium sulfate, 22%(w/v) PEG 3350 (Supplementary Table S2). The initial ecFabI crystals were thin needles that grew in 2–4 d, often with significant imperfections, and were optimized by standard grid screening of buffer pH and citrate, ammonium sulfate and PEG concentrations. The imperfections in the crystals (hollow or malformed crystals) suggested that crystal growth was occurring too quickly, and optimizations focused on slowing crystal growth to promote the formation of well ordered crystals. After optimization, the average ecFabI crystal had dimensions of $250 \times 250 \times 500 \mu\text{m}$ (Supplementary Fig. S1) and diffracted to between 2 and 3 Å resolution (Supplementary Fig. S2) despite some remaining imperfections. Surprisingly, the best diffraction obtained for 14b–FabI and 35b–FabI crystals was collected from crystals grown from the Index screen with no optimization. See Table 2 for data-collection and processing statistics. Extensive cocrystallization experimentation with inhibitors 18c and 39, which included optimization of the molar ratio of protein to NAD⁺ to inhibitor, was carried out but did not yield crystals.

3.2. Inhibition of ecFabI by diazaborines 14b, 18c, 35b and 39

Reductase activity was determined by monitoring NADH consumption at 340 nm. The ecFabI samples used were active (with a specific activity of 9.3 U mg^{-1} under our assay conditions, where one unit of ecFabI activity was defined as $1 \mu\text{mol s}^{-1}$), indicating proper folding. DMSO, the solvent used to solubilize the four inhibitors, and NAD⁺, which is known to be required for ENR inhibition by diazaborine molecules, both decreased ecFabI reaction rates on their own. The reaction rates were determined in the presence of each inhibitor and were compared with samples that included both DMSO and NAD⁺ to control for the effect of these molecules on ecFabI kinetics. All four of the inhibitors examined here inhibited ecFabI specifically and to similar extents (Fig. 2), decreasing ecFabI reaction rates by up to 32%. Inhibition by each of these four molecules was NAD⁺-dependent, as observed for the sulfonylated diazaborines.

3.3. Quality of the ecFabI models and overall protein fold

The three ecFabI crystal structures reported here (Table 3) were completed and validated using the *PDB Validation Server* to judge the overall quality of the models. These models include (i) ecFabI with no ligands bound (apo FabI; solved to 1.97 Å resolution with R and R_{free} factors of 19.1 and 22.5%, respectively), (ii) ecFabI in complex with NAD⁺ and inhibitor 14b (14b–FabI; 2.07 Å resolution, with R and R_{free} factors of 20.4 and 24.7%, respectively) and (iii) ecFabI in complex with NAD⁺ and inhibitor 35b (35b–FabI; 2.11 Å resolution, with R and R_{free} factors of 20.1 and 24.1%, respectively). ecFabI is a homotetramer made up of 33 kDa subunits (Fig. 3). In all three models the asymmetric unit contains one dimer of the homotetramer. The individual chains of each model are, as expected, very similar to each other overall, with r.m.s.d.s of 0.124 Å over 211 atoms for apo FabI, 0.129 Å over 215 atoms

Figure 3

Overviews of the ecFabI structure. (a) The ecFabI tetramer is shown in cartoon representation with a transparent surface representation overlaid. Chain A is shown in color, with α -helices in light cyan, β -strands in brown, helix $\alpha 6'$ (see below) in light pink and the flipping loop in hot pink. Each end of the single chain break is marked with a sphere. Chain B is shown in black cartoons, chain C (symmetry equivalent of chain A) in gray and chain D in white. Bound inhibitor–NAD⁺ complexes are shown in stick representation with the C atoms of NAD⁺ of chain A in yellow, inhibitor C atoms in green, N atoms in blue, O atoms in red and S atoms in gold. The inhibitor–NAD⁺ complexes of chains B–D are shown in stick representation in the same color as the rest of the corresponding chain. (b) Chain A of the ecFabI monomer is shown in cartoon representation with bound 14b–NAD⁺, as described in (a). See Supplementary Fig. S3 for a stereoview of the ecFabI monomer with secondary-structural elements labelled.

for 14b–FabI and 0.136 Å over 211 atoms for 35b–FabI. Also as expected, each of the three models is very similar to the others, with an average r.m.s.d. of 0.127 ± 0.019 Å over 441 atoms. All three of the models comprise residues 2–257 of 305 amino acids. The N-terminal histidine tag and linker conferred by pET-30 Ek/LIC were disordered in all three structures. Additionally, one chain break at the active site occurs in each individual chain. This disordered loop is in a slightly different position in each chain (see Supplementary Table S3), but generally includes residues 195–202. This region of the protein is known to become ordered and close off the active site when select inhibitors are bound (see below).

The overall fold observed in our *E. coli* FabI models is virtually identical to those shown by previous studies, with

Figure 4

Inhibitor-binding sites. (a) The 14b–NAD⁺ inhibitor-binding site is shown focusing on 14b binding. The bound inhibitor and NAD⁺ are displayed as in Fig. 3(a) with an $F_o - F_c$ OMIT electron-density map contoured at 2.0σ shown as a mesh colored blue (positive difference density) and red (negative difference density). The OMIT map shown here was calculated after removing the inhibitor and NAD⁺ and carrying out a round of refinement in REFMAC. The ecFabI residues that interact with the inhibitor are shown in stick representation with gray C atoms. (b) The 35b–NAD⁺ inhibitor-binding site displayed as described in (a).

some minor deviations near the active-site pocket. Each chain consists of a single Rossmann domain that has a total surface area of approximately $11\,250\text{ \AA}^2$. Approximately $3500\text{ \AA}^2$ of the surface area of each monomer is buried upon oligomerization. Although there are relatively extensive interactions between each of the chains of the tetramer, each active site is formed entirely by one domain from loops at the top of the Rossmann β -sheet (Fig. 3*b* and Supplementary Fig. S3). These active sites are well separated in the tetramer, with $35\text{ \AA}$ distance between each nicotinamide ring.

3.4. Inhibitor-binding modes

Both the 14b and 35b inhibitors are well ordered (Fig. 4) and are bound in the same site and with the same general conformation as 2-tosyl-benzodiazaborine and 2-propyl-sulfonyl-thienodiazaborine in the previously published diazaborine-inhibited ecFabI models (Baldock *et al.*, 1996; Levy *et al.*, 2001). As in those structures, both inhibitors form a covalent linkage to NAD^+ through its 2'-hydroxyl (Fig. 5). The majority of the contacts between the NAD^+ -inhibitor complexes and the protein are made by NAD^+ ; accordingly, there are relatively few contacts made between the inhibitor itself and ecFabI. The contacts that are made are primarily hydrophobic interactions, with two hydrogen-bonding interactions as well. One surface of the inhibitor diazaborine ring stacks against the NAD^+ nicotinamide ring, while the other is largely solvent-exposed (Fig. 4). Tyr146 and Tyr156 pack against the edge of the ring, while residues including Ala95, Leu100 and Met159 form another hydrophobic cavity nearby.

The longer sulfonyl-linked *R* group of the other diazaborine inhibitors exploits this second hydrophobic surface; here, the O or S atom of the amide of 14b or the thioamide of 35b packs near the methionine S atom, leaving the majority of the hydrophobic surface open to solvent.

Hydrogen bonds to Ile192 and Lys163 through the NAD^+ amide and ribose hydroxyls, respectively, hold the nicotinamide nucleotide properly in the active site. Lys163 hydrogen-bonds the NAD^+ 2'-hydroxyl, which bridges the NAD^+ ribose and the inhibitor B atom. Inhibitors 14b and 35b also form a hydrogen bond between Tyr156 and the boron hydroxyl, as observed in the other diazaborine inhibitors (Fig. 4). They form an additional hydrogen bond to the Gly93 backbone carbonyl (Fig. 4), an interaction that is not possible for the sulfonyl alkyl side-chain-containing inhibitors.

The effect of the substitution of the amide O atom of 14b with an S atom in 35b does not appear to change the binding mode of the inhibitor to a significant extent, although there are clearly some minor structural shifts that occur to accommodate the larger atomic radius of the S atom. A slight shift of the inhibitor- NAD^+ complex in the active site occurs, which is reflected by small differences in hydrogen-bonding distances between the NAD^+ -inhibitor complex and Lys163 ($2.85\text{ \AA}$ to the NAD^+ 3'-hydroxyl in 14b-FabI *versus* $3.00\text{ \AA}$ in 35b-FabI, and $3.08\text{ \AA}$ to the NAD^+ 2'-hydroxyl in 14b-FabI *versus* $3.33\text{ \AA}$ in 35b-FabI). These changes appear to facilitate the accommodation of the larger S atom. Notably, neither these minor shifts nor a larger amount of disorder in the 35b-FabI substrate-binding loop (see below) appear to affect the inhibitor strength significantly.

Figure 5

Stereoview of the inhibitor- NAD^+ covalent linkage. The bound inhibitor and NAD^+ are displayed as in Fig. 3(*a*) with an $F_o - F_c$ OMIT electron-density map contoured at 2.0σ shown as a mesh colored blue (positive difference density) and red (negative difference density). The OMIT map shown here was calculated after removing the inhibitor and NAD^+ and carrying out a round of refinement in *REFMAC*. The ecFabI residues that interact with the inhibitor are shown in stick representation with gray C atoms.

3.5. Mobile active-site loop region

The ecFabI active site is 'closed' by the loop connecting β_6 and α_6' (residues 190–203; see Supplementary Fig. S3 for secondary-structural element numbering). This loop is ordered in many of the inhibitor-bound ENR models, and can assume alternate conformations over residues 192–198 (referred to as the 'flipping loop') depending on the shape of the bound inhibitor (Qiu *et al.*, 1999). For example, the diazaborine-bound models (Baldock *et al.*, 1996; Levy *et al.*, 2001) and the triclosan-bound forms (Stewart *et al.*, 1999; Qiu *et al.*, 1999) have significantly different backbone pathways in this region (Supplementary Fig. S4). Meanwhile, this active-site lid region is disordered in some ENR structures, including the NAD⁺-bound form of the enzyme (Baldock *et al.*, 1996; Baldock, Rafferty *et al.*, 1998). This disorder can extend into helix α_6' as well. The β_6 – α_6' active-site loop is disordered to a different extent in each of our three models (Fig. 6). 35b–FabI has the largest disordered region, with the entire helix α_6' being too mobile to include in the model. Interestingly, the apo FabI model has a significant amount of helix α_6' ordered, slightly more so than the NAD⁺-bound ecFabI structure (Baldock, Rafferty *et al.*, 1998). 14b–FabI has the shortest disordered region. The electron density associated with this model was actually strong enough to allow the construction of a complete loop in each chain with reasonable statistics (R and R_{free} factors of 19.8 and 24.5%, respectively). In this model, the two chains have different 'flipping loop' conformations (Supplementary Figs. S5 and S6). However, the electron

density was ultimately too weak, as judged by the RSRZ outliers reported by the *PDB Validation Server*, to include this loop in the final 14b–FabI model.

4. Discussion

The studies presented here have confirmed that the diazaborine inhibitors 14b, 18c, 35b and 39 are all specific, but weak, ecFabI inhibitors that inhibit the enzyme in an NAD⁺-dependent manner (Fig. 2). The weak inhibition observed here is consistent with the larger MIC values against *E. coli* reported in the literature in comparison to triclosan and the thieno-diazaborines (Table 1). These data support the proposal that these antibacterial inhibitors function by directly inhibiting ecFabI, rather than by off-target effects on bacterial growth such as inhibition of an alternative enzyme. In addition, this work presents three new ecFabI structures that suggest new strategies for the development of additional diazaborine ENR inhibitors. The inhibitors bound in our ecFabI structures (Fig. 1) differ from 'typical' diazaborine ENR inhibitors in that the sulfonyl-linked substituent present in the majority of the diazaborine inhibitors was truncated significantly to a simple amide (inhibitor 14b) or a thioamide (inhibitor 35b), both of which lack the sulfonyl group. Interestingly, the loss of the sulfonyl linker alone does not correspond to a large decrease in antibacterial or inhibitory activity in these molecules, as might have been expected (Table 1 and Fig. 2). We attribute this observation to the fact that 14b and 35b form an additional hydrogen-bonding interaction with the protein in comparison to the previously studied diazaborines (see below).

A key finding of these studies is that the diazaborine sulfonyl group can be replaced by a carbonyl or thiocarbonyl with retention of the mode of inhibition. The three ecFabI structures reported in this paper confirm that inhibitors 14b and 35b are capable of forming the usual covalent linkage to the NAD⁺ cofactor and hydrogen bond to Tyr165, as do 2-tosyl-benzodiazaborine and 2-propylsulfonyl-thienodiazaborine, which have both been characterized in complex with ecFabI previously (Baldock *et al.*, 1996). Therefore, we can assert that these inhibitors act using the same diazaborine mechanism of forming a covalent complex with NAD⁺ and blocking the active site to competitively inhibit the enzyme. Previous studies proposed that the sulfonyl group, which is present in all of the characterized diazaborine ENR inhibitors, with extremely limited exceptions (Davis *et al.*, 1998), was required for stabilization of the negatively charged boron in the NAD⁺ adduct (Levy *et al.*, 2001). In addition, researchers proposed that the geometry of the sulfonyl group allows the attached substituent to extend into a hydrophobic cavity in the active site and exploit the hydrophobic interactions provided by this surface for additional binding energy (Levy *et al.*, 2001). While the second point made on the sulfonyl geometry is indeed true (see below), our results show that the less strongly electron-withdrawing carbonyl or thiocarbonyl is sufficient to stabilize the anionic tetrahedral boron.

Figure 6
The disordered active-site loops of apo FabI, 14b–FabI and 35b–FabI. Each model is shown in cartoon representation colored white, with the exception of the substrate-lid region (residues ~190–212), which is colored blue in apoFabI, hot pink in 14b–FabI and red in 35b–FabI. The 14b–NAD⁺ complex is shown as described in Fig. 3(a). See Supplementary Figs. S4 and S5 for representative fully ordered flipping loops for comparison.

In addition to the sulfonyl group, molecules 14b and 35b also lack the alkyl substituent usually attached *via* the sulfonyl linker on 'typical' diazaborines. This side chain on the sulfonylated diazaborines makes hydrophobic interactions with Gly199 and Ile200 of the mobile active-site loop (Baldock *et al.*, 1996). In our structures without these interactions the loop fails to fully order (Fig. 6). Therefore, our structures confirm that the presence of the alkyl substituent is critical to the ordering of the mobile active-site loop, as proposed previously (Baldock, de Boer *et al.*, 1998). The obvious question raised by this observation is could the amide/thioamide side chain of molecule 14b/35b be extended with an alkyl group to make these critical interactions with Gly199 and Ile200? The hydrophobic surface that the alkyl group would need to pack against is located directly above the plane of the (thio)-carbonyl. An alkyl chain attached on the N atom of the (thio)amide could extend into the hydrophobic pocket, with rotation of the bond connecting N2 of the diazaborine to the C atom of the (thio)carbonyl (Supplementary Fig. S7). Such a rotation could result in loss of the hydrogen-bonding interaction to Gly93 owing to the altered distance from the inhibitor amine to the Gly93 carbonyl, which can vary between 2.7 and 3.1 Å depending on the degree of torsional rotation about the bond and the orientation of the two atoms with respect to each other. Taken together, it appears that the diazaborine sulfonyl moiety is not required for formation of the NAD⁺ adduct or for specific interactions with ecFabI.

Previous studies have shown that the length of the sulfonylated diazaborine alkyl substituent has a major effect on antibacterial activity, with shorter chains generally associated with lower activity, most likely owing to loss of the hydrophobic interactions with Gly199 and Ile200 and the resulting disorder in the mobile active-site loop, as discussed above. Surprisingly, molecules 14b and 35b had higher antibacterial activity than their sulfonylated counterparts (Table 1) and had MIC values that were generally comparable with several benzodiazaborines that do have those longer alkyl chains (Grassberger *et al.*, 1984; Kanichar *et al.*, 2014). Our structures suggest an explanation for this observation. The amines of the 14b and 35b side chains appear to form a hydrogen bond to the Gly93 backbone carbonyl O atom (Fig. 4), which signifies an additional interaction made by these inhibitors. This hydrogen bond may provide the extra binding energy required to make up for these missing hydrophobic interactions with Gly199 and Ile200. None of the sulfonylated diazaborines would be capable of forming this hydrogen bond. This explanation is consistent with the trends observed in the MIC values tabulated in Table 1.

As a final comment, molecules 18c and 39 do inhibit ecFabI (Fig. 2), but we did not obtain crystals of ecFabI with either molecule bound; the few crystals obtained from 18c or 39 cocrystallizations either did not have bound inhibitor–NAD (as judged by examining the active-site electron density) or did not diffract. This may be because apo FabI crystals formed in these drops and crystal lattice contacts prevented ligand binding. Alternatively, these molecules may have been too large to fit into the active site. From our examination of the

structures, we would expect steric clashes between the inhibitor and the protein chain, particularly at Gly93. However, this possibility is inconsistent with the fact that both 18c and 39 do inhibit ecFabI in an NAD⁺-dependent manner. Another explanation with respect to molecule 18c, that it behaves as a prodrug and hydrolyzes to yield isoniazid (Kanichar *et al.*, 2014), is also somewhat inconsistent with our results, since in that case we would expect to obtain crystals with that inhibitor bound. Last, it is possible that the binding of molecules 18c or 39 disrupted the protein structure in such a way as to impede crystallization; for example, by causing an increase in disorder at protein surfaces that interfered with the ability of the protein to pack into an ordered lattice. Further experimentation is required to clarify the inhibitory capabilities of these two molecules.

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