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Elongation of the Poly-γ-glutamate Tail of F$_{420}$ Requires Both Domains of the F$_{420}$-γ-Glutamyl Ligase (FbiB) of Mycobacterium tuberculosis*

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Cofactor F$_{420}$ is an electron carrier with a major role in the oxidoreductive reactions of Mycobacterium tuberculosis, the causative agent of tuberculosis. A γ-glutamyl ligase catalyzes the final steps of the F$_{420}$ biosynthesis pathway by successive additions of ω-glutamate residues to F$_{420}$-0, producing a poly-γ-glutamate tail. The enzyme responsible for this reaction in archaea (CofE) comprises a single domain and produces F$_{420}$-2 as the major species. The homologous M. tuberculosis enzyme, FbiB, is a two-domain protein and produces F$_{420}$ with predominantly 5–7 ω-glutamate residues in the poly-γ-glutamate tail. The N-terminal domain of FbiB is homologous to CofE with an annotated γ-glutamyl ligase activity, whereas the C-terminal domain has sequence similarity to an FMN-dependent family of nitroreductase enzymes. Here we describe that full-length FbiB adds multiple ω-glutamate residues to F$_{420}$-0 in vitro to produce F$_{420}$-5 after 24 h; communication between the two domains is critical for full γ-glutamyl ligase activity. We also present crystal structures of the C-terminal domain of FbiB in apo-, F$_{420}$-0-, and FMN-bound states, displaying distinct sites for F$_{420}$-0 and FMN ligands that partially overlap. Finally, we discuss the features of a full-length structural model produced by small angle x-ray scattering and its implications for the role of N- and C-terminal domains in catalysis.

The cofactor F$_{420}$ is a flavin derivative that is sporadically distributed among microorganisms, mainly archaea and actinobacteria (including mycobacteria). F$_{420}$ has been emerging as a new player in the biology of mycobacteria (1), with increasing numbers of F$_{420}$-utilizing proteins characterized from different mycobacterial species (2–8). This cofactor has been suggested to protect Mycobacterium tuberculosis, the causative agent of tuberculosis, against oxidative and nitrosative stress during pathogenesis (9–11). At the biochemical level, cofactor F$_{420}$ functions as a hydride transfer agent in oxidoreductive reactions with a lower redox potential than that of NAD(P)⁺ (12).

The biosynthesis pathway of cofactor F$_{420}$ has been investigated in both archaeal and mycobacterial species. In the current view of the proposed pathway, the first intermediate with the complete chromophore (7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO)⁴) is produced by FO synthase (FbiC in mycobacteria (13) and CofGH in archaea (14)). A transferase enzyme (FbiA in mycobacteria (15) and CoFD in archaea (16)) subsequently catalyzes the addition of a 2-phospho-1,1-lactate moiety to FO to produce F$_{420}$-0 (F$_{420}$ with no poly-γ-glutamate tail). The final step of the pathway is performed by a γ-glutamyl ligase (FbiB in mycobacteria (15) and CofE in archaea (17)) that catalyzes successive additions of ω-glutamate residues to F$_{420}$-0 (Fig. 1A).

The length of the poly-γ-glutamate tail varies between archaeal and mycobacterial species; in archaea, two ω-glutamate residues are seen (18), whereas in mycobacteria, up to nine residues are present (3, 19). There exists an intriguing difference between the enzymes responsible for this reaction in these microorganisms, with CoF/E having only one domain, whereas FbiB is a two-domain protein. The N-terminal domain of FbiB is annotated as a γ-glutamyl ligase with sequence similarities to CoF/E, whereas the C-terminal domain has sequence similarity to an FMN-dependent family of nitroreductase enzymes. Functional homology to nitroreductases, however, seems unlikely, and it is reasonable to hypothesize that the C-terminal domain of FbiB facilitates elongation of the poly-γ-glutamate tail of cofactor F$_{420}$ in mycobacterial species.

Here we describe the structural and functional characterization of the FbiB protein from M. tuberculosis, demonstrating that the full-length enzyme is capable of adding multiple ω-glutamate residues to F$_{420}$-0 in vitro. Our results also indicate that communication between the two domains of FbiB is critical for full γ-glutamyl ligase activity, because the N-terminal domain is capable of producing only F$_{420}$-1. We have also determined the crystal structure of the C-terminal domain of FbiB in apo-, treatment would be beneficial for these patients.

8 This work was supported by the Health Research Council of New Zealand, and access to the Australian Synchrotron was supported by the New Zealand Synchrotron Group Ltd. The authors declare that they have no conflicts of interest with the contents of this article.

The atomic coordinates and structure factors (codes 4XOM, 4XOQ, and 4XOO) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: FO, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; SAXS, small angle x-ray scattering; WAXS, wide angle x-ray scattering; TEV, tobacco etch virus.
F$_{420}$-0-, and FMN-bound states (1.9, 2.05, and 2.1 Å resolution, respectively). These structures indicate specific and distinct binding sites for F$_{420}$ and FMN ligands that are adjacent and partially overlapped. Despite the presence of a conserved FMN binding site in this domain, FMN does not appear to have a role in the reaction catalyzed by FbiB and is probably a remnant of the ancestral FMN-dependent nitroreductase enzyme. A full-length structural model of FbiB produced by small angle x-ray scattering (SAXS), using our x-ray crystal structure of the C-terminal domain and an N-terminal homology model, shows that the two domains are separated in space and are linked by a flexible α-helical segment. We discuss the implications of this full-length model for our functional understanding of the C-terminal domain.

**Experimental Procedures**

**PCR Amplification and Cloning**—The open reading frame encoding the full-length FbiB protein (Rv3262) (20) was amplified from *M. tuberculosis* H37Rv genomic DNA using the primers outlined in Table 1. The cloning was conducted using the Gateway® cloning system (21). The amplified PCR product was used to produce entry clones by performing a BP reaction. Positive entry clones were selected on LB agar medium supplemented with 50 μg/ml kanamycin and were then verified using BsrG I digestion and sequencing. The resulting entry clones were used to clone the full-length construct into pDEST17 (21) and pDESTsmg (22) vectors using an LR reaction. The expression construct for pDEST17 was selected on LB agar plates containing 100 μg/ml ampicillin. Selection of pDESTsmg constructs was performed on low salt LB agar plates, pH 8.0, containing 50 μg/ml hygromycin B. All expression constructs were subsequently verified using BsrG I digestion. Two additional constructs were also prepared, using the same protocol, to express N-terminal (FbiB[11–249]) and C-terminal (FbiB[249–448]) domains of FbiB in pDESTsmg vector (Table 1).

**Expression and Purification**—FbiB constructs were expressed in *Escherichia coli* BL21(DE3)pRP and *Mycobacterium smegmatis* mc² 4517 (23) cells. In both cases, protein expression was performed in autoinduction medium as described previously (24). Protein expression in *E. coli* was started at 37 °C for 4 h, followed by overnight incubation at 18 °C. *M. smegmatis* cultures were supplemented with 0.05% (v/v) Tween 80, and protein expression was carried out for 3–4 days at 37 °C (24).

All three FbiB constructs were cloned with an N-terminal His$_6$ tag to facilitate the subsequent purification steps. The His$_6$ tag on both pDEST vectors is cleavable using tobacco etch virus (TEV) protease. All constructs were purified from *E. coli* and *M. smegmatis* cells using the same procedure, as described below. The cells were harvested and resuspended in 20 mM HEPES, pH 7.0, 150 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol. The cells were then lysed using a cell disruptor (Microfluidizer M-110P) in the presence of Complete protease inhibitor mixture mini EDTA-free tablets (Roche Applied Science). The lysate was centrifuged at 20,000 × g to separate the insoluble material. The recombinant proteins were first purified using an immobilized metal affinity chromatography step by loading the supernatant onto a HiTrap FF 5-ml nickel-affinity column (GE Healthcare) that had been pre-equilibrated in the lysis buffer. The column was washed with the lysis buffer, and the protein was subsequently eluted using a gradient of imidazole in the buffer. Appropriate protein fractions were pooled and dialyzed at 4 °C in the presence of rTEV protease (25) to remove the N-terminal His$_6$ tag. The His$_6$-tagged rTEV protease, encoded in a pProEX HTa expression vector, was produced earlier from *E. coli* Rosetta™(TM)(DE3)pLysS cells (Novagen), as described previously (25). After overnight incubation of the purified FbiB proteins with rTEV protease, a subtractive immobilized metal affinity chromatography step was performed to remove the cleaved protein from uncut protein and rTEV protease. The resulting protein fraction was concentrated and then injected onto a size exclusion Superdex 200 10/300 column (GE Healthcare) pre-equilibrated in 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM β-mercaptoethanol.

**Binding Assays**—Fluorescence spectroscopy was performed using an EnSpire® multimode plate reader (PerkinElmer Life Sciences). The fluorescence intensity was measured using a black 96-well plate with total reaction volumes of 100 μl in triplicates. Excitation and emission wavelengths of 420 and 480 nm, respectively, were used to monitor the intrinsic F$_{420}$ fluorescence. For FMN binding assays, the excitation wavelength was set at 445 nm, and emission wavelength was set at 525 nm. To determine the dissociation constant, the protein samples (0.1 μM) were incubated with either F$_{420}$ (0.001–20 μM) or FMN (0.01–100 μM) and left for 30 min at ambient temperature before fluorescence measurements. The binding reactions contained 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM β-mercaptoethanol and were corrected against a control lacking the FbiB protein. Ligand binding data were fitted using one- or two-site binding models (SigmaPlot version 12.5).

**Activity Assays**—The γ-glutamyl ligase activity (17) was measured in 50-μl reactions containing different FbiB constructs (1
M. tuberculosis γ-Glutamyl Ligase FbiB

TABLE 2
Data collection and processing statistics

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<th>Br-FbiB</th>
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<td>90, 90, 90</td>
<td>90, 90, 90</td>
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<td>96.8–2.60 (2.74–2.60)</td>
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<td>0.179 (0.879)</td>
<td>0.194 (0.911)</td>
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<td>0.221 (0.380)</td>
<td>0.224 (0.380)</td>
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<td>2,469,097 (363,254)</td>
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<td>19.2 (4.2)</td>
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<td>99.8 (100.0)</td>
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<td>0.0 (0.0)</td>
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<td>0.54/0.13</td>
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* Values in parentheses are for the outermost resolution shell.
* Pearson correlation coefficient.
* Map correlation coefficient.

µg). The optimized reaction mixture included 50 mM HEPES, pH 8.5, 100 mM NaCl, 5 mM MnCl₂, 10 mM L-glutamate, 5 mM GTP, and 2 µM F₄₂₀-0. The F₄₂₀-0 substrate was prepared by enzymatic hydrolysis of the poly-γ-glutamate tail of F₄₂₀ using carboxypeptidase G (in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.2 mM ZnSO₄) (17). The possible effect of FNN on the polyglutamylation reaction was also tested by including a range of FNN concentrations (0–1 mM) in duplicate activity assays. The reactions were incubated at 37 °C and stopped using 20 mM EDTA after various time points.

**HPLC Analysis**—Separation of F₄₂₀ species was performed on an Agilent HP 1100 HPLC system equipped with a photodiode array and fluorescence detectors (Agilent Technologies). Samples were kept at 4 °C, and the injection volume was 20 µl. Samples were separated on a Phenomenon Luna C18 column (150 × 3 mm, 5 µm) with a 0.2-µm in-line filter that was maintained at 30 °C. The mobile phase consisted of 100% methanol (A) and 25 mM sodium acetate buffer, pH 6.0 (B), with a gradient elution at a flow rate of 0.5 ml/min and a run time of 30 min. The gradient profile was performed as follows: 0–25 min 95–80% B, 25–26 min 80% B, 26–27 min 95% B, 27–30 min 95% B, and a post-run of 2 min. The wavelengths used for photodiode array were 280 and 420 nm (bandwidth 20 nm) using a diode array were 280 and 420 nm (bandwidth 20 nm) using a diode array detector (emission).

**LC-MS Analysis**—After HPLC separation, F₄₂₀ samples were analyzed using an Agilent series 1200 liquid chromatography instrument coupled with an Agilent 6460 jetstream triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). The injection volume and sample separation were performed as described above, with the mobile phase consisting of 100% methanol with 0.02% ammonia (A) and 25 mM ammonium acetate buffer, pH 6.0 (B). Ionization was achieved using electrospray ionization in either negative or positive mode.

**Crystallization and Data Collection**—The C-terminal domain of FbiB (fbiB(249–448)) was crystallized as described previously (26). The best diffracting crystals were obtained in 25% (w/v) PEG 3350, 0.35 M LiSO₄. The crystals were cryo-protected in 70% N-paratone and 30% mineral oil (v/v) before flash-freezing in liquid nitrogen.

For experimental phasing, preformed apo-crystals were soaked for 10 min in 0.5 M KBr solution that was prepared in the cryo-solution (25% PEG 3350 (w/v), 0.35 M LiSO₄, 25% glycerol (v/v)) before being flash-cooled in liquid nitrogen. Bromide-multil wavelength anomalous diffraction data sets were collected at three wavelengths at the Australian Synchrotron. Data collection statistics are summarized in Table 2.

Crystals of the C-terminal domain of FbiB in complex with ligands were obtained by soaking preformed apo crystals in precipitant solutions containing the ligands. The FNN complex was obtained by overnight soaking of apo-crystals in the cryo-solution (25% PEG 3350 (w/v), 0.35 M LiSO₄, 25% glycerol (v/v)) containing 5 mM FNN. For preparation of crystals in complex with F₄₂₀-0, apo-crystals were soaked with F₄₂₀-0 that was prepared for activity assays as mentioned earlier. F₄₂₀-0 solution in water was concentrated to near dryness and redissolved in the cryo-solution (25% PEG 3350 (w/v), 0.35 M LiSO₄, 25% glycerol (v/v)) to a final concentration of 4.5 mM, after which the crystals were soaked for 2 h.

**Structure Determination and Refinement**—All data sets were indexed and processed using XDS (27), re-indexed using POINTLESS, and scaled with SCALA from the CCP4 program suite (28, 29). For structure determination using bromide-multil wavelength anomalous diffraction, the bromide sites and their occupancies were found using SHELXL (30), as implemented in the autoSHARP program suite (31). SHARP was then used for substructure refinement and phasing (32), followed by phase improvements by the SOLOMON density modification program (33). Cycles of automatic model building by ARP/WARP (34) resulted in a partial protein model that was then used for further automated model building with the application of non-crystallographic symmetry restraints on the four molecules of the asymmetric unit. The final model was completed manually using COOT (35). Water molecules were identified by their spherical electron density and appropriate hydrogen bond geometry with the surrounding structure. Following each round of manual model building, the model was refined using...
REFMAC5 (36), against the data to 1.9 Å resolution. The auto-
BUSTER refinement program (37) was used in the final stages of
refinement, both with and without TLS refinement param-
ters. Full refinement statistics are shown in Table 3.

Structures of the C-terminal domain of FbiB in complex with
FMN and F_{420}-0 were solved by molecular replacement using
PHASER (38) with the apo-FbiB structure as a search model. All
structures were refined by cycles of manual building using
COOT (35) and refinement using REFMAC5 (36) and BUSTER
(37). Full refinement statistics are shown in Table 3.

**Full-length Model of FbiB from SAXS and Homology
Modeling**—Protein samples were extensively dialyzed against
20 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol (v/v), and 1
mM tris(2-carboxyethyl)phosphine. This buffer was also used as
a buffer control and to dilute protein samples. SAXS data
were collected on the Australian Synchrotron SAXS/WAXS
beamline and processed using the scatterBrain software pack-
age (39). In brief, protein samples and buffer controls were
diluted and aliquoted into 96-well plates, and rubber lids placed
on the wells to prevent evaporation. The 96-well plates were
mounted on the SAXS/WAXS beamline on a temperature-con-
trolled mount at 283 K for autosampling and capillary flow data
acquisition. Solutions were flowed through thin walled quartz
capillaries, and data acquisition comprised consecutive 1-s
x-ray exposures of the flowing samples to minimize radiation
damage. Appropriate images were combined, and buffer scatter-
ing was carefully subtracted using the scatterBrain software
(39). Data quality and derived parameters were calculated using
the ATSAS software package (40). Details of SAXS data collec-
tion parameters and statistics are shown in Table 4.

A structure of the N-terminal domain of FbiB was produced
by homology modeling using the Phyre2 software (41) and the
Protein Data Bank coordinate set 2PHN. The N-terminal ho-
mology model and the C-terminal crystal structure were com-
bined to produce a full-length model using the SAXS data and a
rigid body minimization protocol using SASREF (40) with a
distance restraint to keep the termini of the two domains in
appropriate proximity, effectively linked. Numerous possible
models were assessed against the SAXS scattering profile using
the CRYSOL software (40) before, eventually, the best fit was
discovered as a 50:50 combination of two complete full-length
models, as described fully below.

**Results**

**FbiB Expression and Purification**—The full-length, N-termi-
nal, and C-terminal constructs of FbiB were expressed as soluble
proteins in both *E. coli* and *M. smegmatis* cells using an
autoinduction protocol. All three constructs were purified
using immobilized metal affinity chromatography and size
exclusion chromatography steps.

**Functional Characterization**—The FbiB constructs ex-
pressed in *E. coli* were used for functional studies to alleviate
the complications arising from the presence of co-purified F_{420}
in the proteins expressed in *M. smegmatis* (Fig. 1B). The γ-glut-
amyl ligase activity of *FbiB* constructs was investigated by
monitoring the addition of L-glutamate residues to F_{420}-0 during
enzymatic reactions. A range of different conditions was optimized for the
γ-glutamyl ligase activity of the full-length FbiB protein,
including pH (6.0–9.0), monovalent (Na\(^+\) and K\(^+\)) and divalent
cation (Mg\(^2+\) and Mn\(^2+\)) composition, nucleotides (GTP,
dGTP, ATP, and dATP), and also various time points and tem-
peratures. FbiB showed the highest activity at pH 8.5, the same
pH dependence displayed by the CoF protein (17). A combi-
nation of Na\(^+\) and Mn\(^2+\) produced the highest activity in FbiB,
in contrast to the previously reported dependence on K\(^+\) and

### Table 3

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<th>Crystal structure refinement statistics</th>
<th>Apo-FbiB</th>
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<th>FbiB-FMNN</th>
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### Table 4

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### M. tuberculosis γ-Glutamyl Ligase FbiB

M. tuberculosis γ-Glutamyl Ligase FbiB
Mg$^{2+}$ for CofE (17). The enzyme was only active in the presence of GTP, with no observed activity for dGTP, ATP, and dATP nucleotides. The enzymatic assays for all FbiB constructs were subsequently conducted using the optimized condition (50 mM HEPES, pH 8.5, 100 mM NaCl, 5 mM MnCl$_2$, 10 mM l-glutamate, 5 mM GTP, and 2 μM F420-0) and were incubated for periods of up to 1 week at 37 °C.

The functional assays show that full-length FbiB can convert F420-0 to F420 molecules with varying numbers of l-glutamate residues in the poly-l-glutamate tail (Fig. 2A). F420-2 production could be detected within 1 h, with no higher order F420 molecules appearing within 2 h. Incubation for 24 h showed species as large as F420-5, and by 72 h (data not shown), the products resolved into two species with much earlier retention time than that of F420-5; although attempted, mass spectrometry analysis of these two large species was unsuccessful. This result, however, is not achieved by either the N- or C-terminal domains alone (Fig. 2, B and C, respectively), nor by a mixture of these two separate domains (Fig. 2D). Our results show that the N-terminal domain of FbiB adds one l-glutamate residue (to produce F420-1), albeit in a very slow manner compared with that of the full-length protein.

Ligand Binding—Expression of FbiB constructs in M. smegmatis host cells resulted in co-purification of F420 with a distinct coloration of the full-length and N-terminal domain constructs. Mass spectrometry analysis confirmed that F420 species containing up to 11 l-glutamate residues in the poly-l-glutamate tail co-purify with the full-length protein (Fig. 1B). Given that E. coli does not produce F420, the proteins expressed in E. coli cells were not expected to have any ligands co-purified. The C-terminal domain, however, showed a faint yellow color upon purification suggesting co-purification of flavin-like ligands from E. coli.

Fluorescence spectroscopy was used to investigate binding of F420 and other flavins (e.g. FMN) to FbiB constructs and to determine their dissociation constant ($K_d$) (Fig. 3). The results show that the full-length (two-site model; $K_{d1} = 0.2 \pm 0.4 \mu M$ and $K_{d2} = 3.0 \pm 0.9 \mu M$), N-terminal (one-site model; $K_d = 1.4 \pm 0.1 \mu M$), and C-terminal domains (one-site model; $K_d = 1.47 \pm 0.07 \mu M$) could each bind F420 in solution. Surprisingly, the full-length and C-terminal constructs could also bind FMN although with a lower affinity (one-site model; $K_d = 14.7 \pm 0.9 \mu M$). Although binding with 10-fold lower affinity to the C-terminal domain of FbiB, FMN was subsequently included in the functional studies of the different domain constructs to assess a possible role in catalytic function or inhibition/regulation.

Structure Determination—The requirement of the C-terminal domain for full activity and its binding to both F420 and FMN prompted us to pursue x-ray crystal structures of three FbiB constructs. Neither full-length FbiB nor its N-terminal domain could be crystallized. The C-terminal domain of FbiB (FbiB(249–448)), expressed in M. smegmatis cells, was successfully crystallized (26), however, and its structure was determined at 1.9 Å resolution by a multiwavelength anomalous dispersion method using crystals soaked in KBr solution (42) (Table 1). The unliganded FbiB C-terminal domain was crystallized in tetragonal space group $P_4_12_12$ and contains four molecules in the asymmetric unit. F420-0- and FMN-bound structures were produced by soaking ligand into preformed crystals and were solved using the unliganded structure for molecular replacement (Table 3).
Overall Structure—The four molecules in the asymmetric unit are organized as two sets of dimers that are related by a 2-fold non-crystallographic symmetry axis. The more extensive chain A-chain B dimer interface buries an average solvent-accessible surface area of 3247 Å²/monomer, representing 27% of each monomer’s surface, as assessed by the PDBePISA server (43), and contains 33 hydrogen bonds, 15 salt bridges, and 204 non-bonded contacts. The protein displays a fold that is typical of the family of FMN-dependent nitroreductases (Pfam PF00881). This fold is based around a central five-stranded β-sheet, made up of four antiparallel β-strands together with a fifth one (parallel to the first) that is contributed by the C-terminal residues of the opposing monomer (Fig. 4A). The β-sheet is flanked by two helices on the internal side, involved in dimerization, and three helices on the external (surface-exposed) side.

FMN Binding—Although FMN is not a previously known ligand for the reaction catalyzed by FbiB, our results show FMN binding to FbiB in solution. Four molecules of FMN are located unambiguously from the electron density maps (Fig. 4B, bottom), one in each protomer and binding in a conserved mode. Only two F420-0 molecules (bound to chains A and B), however, have well defined electron density beyond the phosphate group, enabling two atoms of a lactyl moiety to be modeled. Superimposition of the unliganded and F420-bound structures of the C-terminal domain shows little change in the protein on ligand binding, with an average root mean square deviation of 0.31 Å over 782 aligned Cα atomic positions.
The F420-0 molecule binds to the C-terminal domain in a pocket near the dimer interface (Fig. 4A) with the 8-hydroxy-5-deazaalloxazine ring system buried deeply in the protein, whereas the tail of the molecule binds in a solvent-exposed channel (Fig. 5, D and E). The 8-hydroxy-5-deazaalloxazine chromophore is sandwiched between hydrophobic elements, at the back primarily by the side chain of Phe403 and at the front by a π-stacking interaction with the side chain of Trp317 (as illustrated in Fig. 5F). Intriguingly, only a single hydrogen bond is formed directly to the protein with most other polar/charged elements of the ligand binding indirectly to the protein through water-mediated hydrogen bonding.

Structural Comparisons—A search using the PDBeFold server (44) for structural homologs using one FbiB C-terminal dimer produces numerous matches, two of which are shown in multiple sequence alignment in Fig. 4C. The putative nitroreductase from Clostridium difficile in complex with FMN (Protein Data Bank entry 3GFA) shows only 23% sequence identity but a root mean square deviation of 2.1 Å over 338 Ca atoms and a nitroreductase family protein from Agrobacterium tumefaciens (Protein Data Bank entry 3K6H), also in complex with FMN, that displays no detectable sequence similarity by BLAST but has a root mean square deviation of 2.4 Å over 291 Ca atoms. Intriguingly, 3GFA shows a very similar dimer interface to that of the FbiB C-terminal domain with an average solvent-accessible surface area of 3538 Å² (31% of each monomer’s surface). The most obvious difference between the FbiB and the homologous structures is in regions illustrated in Fig. 4D. Movement of the 2-5 loop and α4 helix produces the F420 binding site; the 5-β6 loop shift opens up the bottom of the FbiB structure near the FMN binding site.

Building a Full-length Model Using SAXS—The full-length FbiB protein was subjected to SAXS at the Australian Synchrotron to characterize its structure in solution. Analysis of the SAXS data (Table 4 and Fig. 6A), including the calcula-

FIGURE 3. Ligand binding by various FbiB constructs. The intrinsic fluorescence signal of F420 and FMN were used to monitor ligand binding and also to calculate dissociation constants (Kd) against the full-length protein and both of the individual domains, N- and C-terminal, as indicated. The F420 dissociation constants derived from these binding curves are Kd1 = 0.2 ± 0.4 µM and Kd2 = 3.0 ± 0.9 µM (full-length, two-site model), Kd = 1.4 ± 0.1 µM (N-terminal), and Kd = 1.47 ± 0.07 µM (C-terminal). FMN binding to the C-terminal domain of FbiB is also included, as labeled, and gives a dissociation constant of 14.7 ± 0.9 µM.
tion of various analytical curves, $R_g$ and $I(0)/c$ values from a concentration series, shows that the scattering profile is not concentration-dependent and, with Guinier plots displaying linearity at low angle (Fig. 6A, inset), is indicative of no inter-particle interference or aggregation. A $P(r)$ function (not shown) suggests a maximum scattering particle dimension of 130 Å, and Kratky analysis shows a well folded protein.

A full-length model of FbiB was produced via rigid body modeling by combining our structure of the C-terminal domain of FbiB with an N-terminal homology model and using the

**FIGURE 4.** Crystal structure of the dimeric C-terminal domain of FbiB. A, overlay of $F_{420}$-bound (yellow) and FMN-bound (blue) structures. $F_{420}$ and FMN molecules are shown as ball-and-stick models with carbon atoms colored in yellow and cyan, respectively. Selected secondary structure elements are labeled. B, electron density ($2F_o - F_c$ omit map) surrounding $F_{420}$ and FMN molecules in their respective structures. C, multiple sequence alignment of FbiB (C-terminal domain), the putative FMN-dependent nitroreductase from *C. difficile* (Protein Data Bank entry 3GFA), and a nitroreductase from *A. tumefaciens* (Protein Data Bank entry 3K6H). Secondary structure elements are shown for the FbiB structure, and residues involved in $F_{420}$ and FMN binding are indicated by open circles and solid stars, respectively. Many residues involved in FMN binding appear conserved across the three structures, whereas those involved in $F_{420}$ binding are not conserved. D, overlay of enlarged view of FbiB (yellow) and Protein Data Bank entry 3GFA (white). Incorporation of an $F_{420}$ binding site (left) produces a large shift in the top of helix α4 and in the β2-α5 loop compared with the *C. difficile* homolog structure. Another large difference occurs at the bottom of the molecule (right), involving the β5-β6 loop, that opens up in FbiB to produce the binding site for the phosphate group of FMN.
SAXS data for minimization. The homology model is based on the structure of an F420-glutamyl ligase from *Archaeoglobus fulgidus* that shares 40% identity and 55% similarity with the FbiB N-terminal domain over 215 residues. The full-length model shows the two domains as separated and linked by an α-helical segment (Figs. 6B and 7). The SAXS profile (Fig. 6A) is best fit by a combination of two models of the full-length protein, one “straight” (Fig. 6B, top) and one “bent” (Fig. 6B, bottom) and in equal proportions (50:50). A surface representation of the straight model (Fig. 7) shows the relative locations of the putative N-terminal active site cavity and the C-terminal FMN/F420 ligand binding sites. Directly adjacent to the N-terminal active site cavity is a surface groove lined with numerous basic residues that extends toward the C-terminal domain and into solvent space. The implications of this surface groove and the other features of this full two-domain model are discussed below.

**Discussion**

**FbiB Function**—The reaction catalyzed by CofE adds two L-glutamate residues to F420-0 to produce F420-2 in archaea (17). The presence of an additional domain in the mycobacterial FbiB protein suggested that this domain could assist the N-terminal domain in producing F420 molecules with longer poly-γ-glutamate tails in mycobacterial species. Our experimental results indeed demonstrate that the full-length FbiB...
protein does produce F420 molecules with multiple L-glutamate residues in the polyglutamate tail (Fig. 1B). The addition of further L-glutamate residues to F420-2 is evidently the rate-limiting step of the process because it takes a much longer time for the F420 species with longer polyglutamate tails to appear. When F420 is purified from mycobacterial cells, the major species found contain between 5 and 7 L-glutamate residues in their polyglutamate tails (3, 19). In addition, the full-length FbiB protein expressed in M. smegmatis shows co-purification of F420 molecules with up to 11 L-glutamate residues incorporated, with the major species having 6–9 residues. After 72 h in our in vitro experiments, we observe two major products presumably containing a larger number of L-glutamate residues in the polyglutamate tail than F420-5, but our inability to characterize these species by mass spectrometry does not allow us to comment further on the identity of these reaction products.

Investigation of the N-terminal and C-terminal domains of FbiB shows that these domains, by themselves, are unable to add L-glutamate residues to F420-0, although the N-terminal domain shows production of F420-1 in a very slow manner. This is a surprising observation because the N-terminal domain is homologous with the CofE protein of archaea (17), and we would have expected it to show D-glutamyl ligase activity on its own. Our observation that both F420-0 and FMN bind to the C-terminal domain suggests a regulatory role for this domain in the reaction catalyzed by FbiB, perhaps both as a requirement for normal catalysis and as a sensor for the relative concentrations of F420 and FMN, in a feedback regulatory role. We could not, however, observe any effect of FMN on the activity of full-length FbiB over a wide range of concentrations, up to 500 times that of F420-0 in the reaction mixture. It has been suggested that the intracellular concentration of FMN in M. smegmatis is ~10-fold higher than that of F420 (4).
Comparison of F_420 and FMN Binding—Given our observed binding of F_420 and FMN to FbiB in solution, we speculated that they would share the same binding site in the C-terminal domain. Surprisingly, this was not the case, as apparent in our crystal structure, although the two binding sites are closely adjacent in the protein structure.

A comparison of the two binding sites and the chemical structures of F_420 and FMN explains why “crossover” is not seen in the ligand binding. Although both binding sites have similar electrostatic surface potentials, the F_420 chromophore extends a hydroxyl group toward Asp^{320}, forming a hydrogen bond (Fig. 5E). If FMN were placed in this site, the bulk of its dimethyl ring substituents could not be accommodated in the cavity available and additionally would not hydrogen-bond with Asp^{320} as seen for F_420. In contrast, in its own site, FMN projects this same nonpolar dimethyl structure into a larger nonpolar pocket (Fig. 5B); F_420 would, if placed in the FMN pocket, extend its hydroxyl group into the nonpolar pocket with no hydrogen bond partner available, effectively producing a buried and unsatisfied hydrogen bonding potential.

The binding of both F_420 and FMN in the FbiB C-terminal domain is an interesting feature but not a unique occurrence. A 2012 study of F_420-dependent reductases from M. smegmatis demonstrated cofactor promiscuity in three F_420-dependent reductases, which can utilize both F_420 and FMN to catalyze different chemistries (oxidation and reduction) of the same substrate (4). The F_420-dependent reductases catalyze the reduction of aflatoxins and plant-derived furanocoumarins in the presence of F_420H_2. When FMN replaces F_420 three of the F_420-dependent reductases tested were found to catalyze FMN-mediated oxidation of two major aflatoxins, AFG1 and AFG2, via dehydrogenation. In another context, an example of competitive FMN/F_420 binding is provided by FprA, a di-iron flavo-protein F_420H_2 oxidase found in methanogenic archaea, which catalyzes the four-electron reduction of O_2 to 2H_2O with 2 molar equivalents of reduced F_420 (45). A recent systematic study of the mycobacteria oxidoreductase superfamily (flavin/deazaflavin oxidoreductases) shows some degree of promiscuity within single proteins, binding F_420, FMN, and FAD with micromolar affinity (8). However, whereas the proteins appear promiscuous, they also show selectivity for a single cofactor (~5–10-fold). Similarly, F_420 binding in the C-terminal (oxidoreductase-derived) domain of FbiB shows a 10-fold selectivity over FMN, and both bind at low micromolar affinities (F_420 K_d = 1.47 versus FMN K_d = 14.7).

Our crystal structure analysis of FMN and F_420 binding, illustrated in Fig. 5, C and F, suggests that although these ligands do not occupy the same site, they cannot bind simultaneously because steric clashes would result; competitive binding in the closely adjacent site cannot, however, be precluded, and we have not examined this. Although it is not entirely unexpected that the C-terminal domain binds F_420 potentially in a feedback regulation role, there is no suggestion of why FMN also binds to this domain; could FMN binding in the FbiB C-terminal domain merely represent an evolutionary relic from an ancestral FMN-dependent nitroreductase domain? In this hypothesis, F_420 binding evolved in the C-terminal domain after being acquired through a recombination event; the FMN binding site has been retained, although we might have expected it to mutate over an evolutionary time scale to abolish binding unless FMN plays some role in the poly-γ-glutamyl reaction.

A more compelling explanation is that the ancestral nitroreductase appropriated by FbiB through recombination was already a promiscuous enzyme, binding both F_420 and FMN for oxidoreductase activity potentially toward multiple substrates. Again, this hypothesis does not explain the retention of distinct F_420 and FMN binding sites in the C-terminal domain of FbiB nor the potential roles these redox molecules play in the poly-γ-glutamyl reaction.

Reaction Mechanism and the Role of the C-terminal Domain—Our biochemical evidence implies that the catalytic machinery of the FbiB protein is located in the N-terminal domain and functions similarly to the single domain homologs that produce F_420-2 as the largest product. The homolog structure from A. fulgidus (Protein Data Bank entry 2PHN), equivalent to the FbiB N-terminal domain, displays metal and GDP binding; glutamate and F_420 binding is not observed, but the locations of their binding sites and the catalytic residues involved in glutamate addition have been postulated (46). In relation to the C-terminal domain of FbiB, our biochemical analysis suggests that this domain only provides support for the catalytic activity of the N-terminal domain; both domains are required for catalysis, and either domain alone does not show catalytic activity. We have produced a composite model of the whole FbiB protein by combining our crystal structure of the C-terminal domain with a homology model of the N-terminal domain and by rigid body refinement of these two half-structures against SAXS data (Fig. 6). The two domains are linked via an α-helical segment that we infer is contiguous between the domains; the C terminus of the homolog structure CoEl is α-helical, as is the N terminus of our crystal structure. Two models of full-length protein in combination show best agreement with the SAXS data. The first model can be referred to as “straight” with the C-terminal domain located directly over the N-terminal domain with both domains together showing 2-fold symmetry; the two domains are separated by ~14 Å and, outside of the linker segment, do not interact. Our second model, best referred to as “bent,” shows the C-terminal domain bending ~50°, toward the N-terminal domain (Fig. 6B). This model brings the putative N-terminal catalytic site and the FMN/F_420 sites of the C-terminal domain to within ~40 Å of each other, still relatively distant.

Either in a catalytic enhancement or a regulatory role, the C-terminal domain may produce an allosteric signal from one or the other of the ligand binding sites to influence catalysis in the N-terminal domain (although we have not observed any effect). We can also hypothesize that the C-terminal domain and perhaps its dynamic behavior in solution, implied by our two-structure model of the SAXS data, apply some mechanical force through the connecting linker sequence to promote or regulate the N-terminal domain function; this is a hypothesis that we have previously explored for another M. tuberculosis enzyme, 2-isopropylmalate synthase, where its C-terminal domain is required for both feedback regulation and catalytic activity in the separate and distant N-terminal domain (47).
One very interesting feature of our N-terminal homology model is the presence of a positively charged surface groove lined by 11 arginine and lysine residues; this is not a feature of the homologous \textit{A. fulgidus} structure from which the model was produced. If, as we contend, the N-terminal domain contains all of the catalytic machinery for \textit{F}420\textsubscript{2} elongation, then this basic surface groove connected to the postulated active site cavity (Fig. 7), could bind to the growing poly-\(\gamma\)-glutamate tail with its negatively charged carboxylate groups and direct the growing chain toward solvent space or toward the adjacent C-terminal domain.

\textit{Elongation Mechanism; Insertion or Extension?—Poly-\(\gamma\)-glutamate tails are found on a limited number of biomolecules, including cofactor \textit{F}420\textsubscript{2} and folate derivatives. Poly-\(\gamma\)-glutamic acid is also a polymer that is produced by a number of microorganisms, with roles from virulence to promising potential for medical and industrial applications (48). \textit{FbiB} (CoE in archaea), folypolyglutamate synthase, and the poly-\(\gamma\)-glutamic acid synthetase complex, catalyze the poly-\(\gamma\)-glutamyl reaction to produce these molecules. The chemical mechanism has been generally assumed to be similar for all of these enzymes, involving activation of the carboxylic acid on the elongated substrate in a nucleotide-dependent manner (GTP in \textit{F}420\textsubscript{2} and ATP in folates and poly-\(\gamma\)-glutamic acid), formation of an acyl phosphate intermediate, and finally nucleophilic attack by the incoming \(\gamma\)-glutamate (17, 46).

Our functional characterization described in the present work demonstrates the addition of multiple \(\gamma\)-glutamate residues to the growing poly-\(\gamma\)-glutamate tail of \textit{F}420\textsubscript{2} carried out by \textit{FbiB}. It is not clear, however, whether each \(\gamma\)-glutamate is added to the terminal residue of the growing chain (an extension mechanism) or inserted somewhere into the middle of the chain, perhaps between the phospholactate moiety and the first \(\gamma\)-glutamate residue (an insertion mechanism). Elucidation of the full mechanistic details of the elongation mechanism will also have implications for further understanding of the function and mechanism of the folypolyglutamate synthase and poly-\(\gamma\)-glutamic acid synthetase enzymes that also carry out poly-\(\gamma\)-glutamyl reactions.

Author Contributions—G. B., E. N. B., and C. J. S. designed the study. G. B. and A. M. R. performed the majority of experiments, including protein production and characterization. A. M. R. carried out protein crystallization. G. B. and S. S. performed the functional experiments. Final structure refinement and deposition was carried out by H. M. B. and C. J. S. wrote the paper, and all authors analyzed the results and approved the final version of the manuscript.

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M. tuberculosis γ-Glutamyl Ligase FbiB


Elongation of the Poly-γ-glutamate Tail of F_{420} Requires Both Domains of the F_{420}:γ-Glutamyl Ligase (FbiB) of Mycobacterium tuberculosis

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