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

Received for publication, September 1, 2015, and in revised form, February 6, 2016. Published, JBC Papers in Press, February 9, 2016. DOI 10.1074/jbc.M115.689026

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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 l-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 l-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 demonstrate that full-length FbiB adds multiple l-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)³) is produced by FO synthase (FbiC in mycobacteria (13) and CofG in archaea (14)). A transferase enzyme (FbiA in mycobacteria (15) and CofD in archaea (16)) subsequently catalyzes the addition of a 2-phospho-l-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 l-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 l-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 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, 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 apo-

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* 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-deazaribofla-
in; 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$_{420}$-0 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 plates, pH 8.0, containing 50 μg/ml kanamycin and were then verified using BsrGI 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 construct was performed on low salt LB agar plates, pH 8.0, containing 50 μg/ml hygromycin B. All expression constructs were verified using BsrGI digestion and sequencing. 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 HisTrap 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™(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

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**TABLE 1**

Primer sequences used in the amplification of FbiB (Rv3262) constructs.

| Construct | Primer Sequences (5’-3’) |
|-----------|--------------------------|
| Full-length FbiB | GGCACGCGCCGCGTTGACCCCCGACCAGAATGGC |
|              | GAAAGCTGAGATTCTCATGACGAAATTCC |
| FbiB N-terminal domain (FbiB(11–249)) | GGCACGCGGCGAGTACCACTGAGATCCTG |
|              | GAAAGCTGAGATTCTCATGACGAAATTCC |
| FbiB C-terminal domain (FbiB(249–448)) | GGCACGCGGCGCGCGTTGACCCCCGACCAGAATGGC |
|              | GAAAGCTGAGATTCTCATGACGAAATTCC |
The gradient profile was performed as follows: 0–25 min elution at a flow rate of 0.5 ml/min and a run time of 30 min. The wavelengths used for the diode array were 280 and 420 nm (bandwidth 20 nm) using a diode array and fluorescence detectors (Agilent Technologies).

After HPLC separation, F420 samples were analyzed using an Agilent series 1200 liquid chromatography 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 sodium acetate buffer, pH 6.0 (B). Ionization was achieved using an Agilent series 1200 liquid chromatography electrospray ionization in either negative or positive mode.

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-multiwavelength 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 FMN 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 FMN. 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-multiwavelength anomalous diffraction, the bromide sites and their occupancies were found using SHELXD (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 tiwavelength anomalous diffraction, the bromide sites and their occupancies were found using SHELXD (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
**TABLE 3**

Crystal structure refinement statistics

|                  | Apo-FbiB | FbiB-F420 | FbiB-FMN |
|------------------|----------|-----------|----------|
| Protein Data Bank code | 4XO0M    | 4X0QQ     | 4X0O     |
| Resolution range (Å) | 96.8–1.90| 48.3–2.05 | 97.0–2.10|
| R<sub>free</sub>/R<sub>work</sub> (%) | 23.3/26.7| 21.2/24.5 | 23.2/26.9 |
| No. of atoms (non-hydrogen) | 6059     | 6445      | 5996     |
| Protein | 10 | 127 | 124 |
| Ligand |
| Solvent | 240 | 398 | 50 |
| Root mean square deviation from ideality (Å) | 0.010 | 0.011 | 0.015 |
| Bonds (Å) | 1.35 | 1.46 | 1.73 |
| Angles (°) | 43.25 | 33.5 | 44.5 |
| Average B factors (Å<sup>2</sup>) | 32.5 | 33.5 | 40.1 |
| R<sub>f</sub> (r = 4) | 4.01 | 43.0 | 33.5 |
| F<sub>420</sub>:0 (n = 4) | 30.7 | 59.2 | 37.3 |
| Sulfates (n = 2) | 31.3 | 34.7 | 37.3 |
| Waters | | |
| Ramachandran statistics | | |
| Favored (%) | 97.9 | 97.6 | 96.8 |
| Allowed (%) | 100.0 | 99.5 | 100.0 |
| Outliers (%) | 0.0 | 0.50 | 0.0 |
| Molprobity score; percentile | 1.30; 100th | 1.51; 100th | 3.31; 99th |

**TABLE 4**

Small angle x-ray scattering parameters and statistics

| Data collection parameters | Data processing | Rigidity body modelling | Computation of model intensities |
|---------------------------|-----------------|------------------------|--------------------------------|
| Beamsline<sup>a</sup> | scatterBrain | SASREF | CRY SOL |
| AS SAXS/WAX | Detectors | Dectris-Pilatus 1M |
| Wavelength (Å) | 1.0322 |
| Camera length (mm) | 1600 |
| q range (Å<sup>-1</sup>) | 0.006 – 0.6 |
| Sample capillary flow rate (ml/min) | 0.5 |
| Total sample volume (µl) | 100 |
| Exposure time/image (s) | 1 |
| No. of images/sample | 20 |
| Concentration range (mg/ml) | 0.25–0.50 |
| Temperature (K) | 283 |

**Results**

**FbiB Expression and Purification**—The full-length, N-terminal, 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 expressed in *E. coli* were used for functional studies to alleviate the complications arising from the presence of co-purified F<sub>420</sub>-0 in the proteins expressed in *M. smegmatis* (Fig. 1B). The γ-glutamyl ligase activity of FbiB constructs was investigated by monitoring the addition of γ-glutamyl residues to F<sub>420</sub>-0 using HPLC, as described previously for the CofE protein (16). The F<sub>420</sub>-0 substrate was prepared by enzymatic hydrolysis of the poly-γ-glutamate tail of F<sub>420</sub> using carboxypeptidase G (17), and the resulting F<sub>420</sub>-0 was used as a substrate for FbiB activity experiments and also for crystallographic binding studies.

The intrinsic fluorescence of F<sub>420</sub> was used to monitor the addition of γ-glutamyl residues to F<sub>420</sub>-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<sup>+</sup> and K<sup>+</sup>) and divalent cation (Mg<sup>2+</sup> and Mn<sup>2+</sup>) composition, nucleotides (GTP, dGTP, ATP, and dATP), and also various time points and temperatures. FbiB showed the highest activity at pH 8.5, the same pH dependence displayed by the CoFe protein (17). A combination of Na<sup>+</sup> and Mn<sup>2+</sup> produced the highest activity in FbiB, in contrast to the previously reported dependence on K<sup>+</sup> and appropriate proximity, effectively linked. Numerous possible models were assessed against the SANS scattering profile using the CRY SOL software (40) before, eventually, the best fit was discovered as a 50:50 combination of two complete full-length models, as described fully below.
M. tuberculosis γ-Glutamyl Ligase FbiB

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 MnCl₂, 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 residues in the poly-γ-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-γ-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 (Kₐ) (Fig. 3). The results show that the full-length (two-site model; Kₐ₁ = 0.2 ± 0.4 μM and Kₐ₂ = 3.0 ± 0.9 μM), N-terminal (one-site model; Kₐ = 1.4 ± 0.1 μM), and C-terminal domains (one-site model; Kₐ = 1.47 ± 0.07 μM) 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; Kₐ = 14.7 ± 0.9 μM). 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 P₄₁2₁₂ 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 $\beta$-sheet, made up of four antiparallel $\beta$-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 $\beta$-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, top), 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.

F420-0 Binding—Four F420-0 molecules were located unambiguously from the electron density maps (Fig. 4B, bottom). A number of residues within the binding site are conserved in the multiple sequence alignment in Fig. 4C, in which the FMN binding regions are indicated by black stars. The binding site is formed in a pocket at the dimer interface (Fig. 4A) and comprises a relatively non-polar pocket that binds the isoalloxazine chromophore, with a collection of basic residues and hydrogen bond donors arrayed about the ribitol chain (Fig. 5, A and B).

An overlay of the apo-FbiB structure with the FMN-bound structure shows generally very small differences, with an overall root mean square deviation in 768 aligned Cα atomic positions of 0.43 Å. However, there are some obvious local changes seen upon FMN binding. The FMN isoalloxazine ring system inserts between the polypeptide segments Cys396-Trp397-Ile398 and Ala288-Pro289-His290 and forces these elements apart by ~1 Å. The side chain of Trp397 of the apo-structure swings 3.5 Å (at its tip) clear of the pocket to allow binding of and provide π-stacking with the FMN ring system, as illustrated in Fig. 5C. The side chain of Ile398 also provides a backstop to this binding site with close contacts to the FMN ring (~3.8 Å) and also moves noticeably.

FIGURE 2. Analysis of FbiB reaction products in vitro. The HPLC traces of the reactions performed using different FbiB constructs (fluorescence versus elution time). In the presence of the full-length construct (A), F420 molecules with various lengths of poly-γ-glutamate tail appear over time, whereas the N-terminal domain alone (B) shows only the production of F420-1. The C-terminal domain alone does not catalyze the reaction (C). The reactions using a combination of separate N- and C-terminal domains produce only F420-1 (D). The HPLC traces are shown for a time course up to 24 h as indicated. Selected peaks are labeled with the corresponding $m/z$ values and F420 species.
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 β2-α5 loop and α4 helix produces the F420 binding site; the β5-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-
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

![M. tuberculosis γ-Glutamyl Ligase FbiB](image-url)
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 CoE 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

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**FIGURE 5.** Ligand binding in the C-terminal domain of FbiB. **A**, surface drawing of the FMN-binding pocket with FMN shown as a ball-and-stick model and hydrogen bonds indicated by dashed yellow lines. Whereas part of the FMN isaloxazine ring system is buried deeply in a hydrophobic pocket, sandwiched between Trp<sup>B397</sup> and Pro<sup>A289</sup>, the remainder of the molecule remains relatively solvent-exposed. **B**, schematic diagram of protein-FMN contacts. Hydrogen bonds are shown as dashed lines, and extensive non-polar interactions are shown by an annotated, solid green line. **C**, FMN binding “pushes” Trp<sup>B397</sup> aside; if F420 were already bound (yellow structure), then an irreconcilable steric clash (yellow clash symbol) between the FMN and the tryptophan side chain would exist. **D**, F420 binding pocket; the ring system is fully buried within the protein, with only the tail exposed to solvent space. **E**, schematic of F420 binding showing hydrogen bonds (dashed lines), extensive non-polar interactions (solid green lines), and a π-stacking interaction as two connected green dots. F420 hydrogen bonding to the protein is almost exclusively indirect and mediated through water molecules. **F**, F420 binding into an FMN-bound structure (blue structure) would also be precluded due to steric clash at both the front and the back of the binding site.
protein does produce F₄₂₀ molecules with multiple L-glutamate residues in the poly-g-glutamate 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 poly-g-glutamate tails to appear. When F₄₂₀ is purified from mycobacterial cells, the major species found contain between 5 and 7 L-glutamate residues in their poly-g-glutamate 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 poly-g-glutamate 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 g-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<sub>420</sub> and FMN Binding—Given our observed binding of F<sub>420</sub> 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<sub>420</sub> and FMN explains why “crossover” is not seen in the ligand binding. Although both binding sites have similar electrostatic surface potentials, the F<sub>420</sub> chromophore extends a hydroxyl group toward Asp<sup>320</sup>, 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<sup>320</sup> as seen for F<sub>420</sub>. In contrast, in its own site, FMN projects this same nonpolar dimethyl structure into a larger nonpolar pocket (Fig. 5B); F<sub>420</sub> 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<sub>420</sub> and FMN in the FbiB C-terminal domain is an interesting feature but not a unique occurrence. A 2012 study of F<sub>420</sub>-dependent reductases from <i>M. smegmatis</i> demonstrated cofactor promiscuity in three F<sub>420</sub>-dependent reductases, which can utilize both F<sub>420</sub> and FMN to catalyze different chemistries (oxidation and reduction) of the same substrate (4). The F<sub>420</sub>-dependent reductases catalyze the reduction of aflatoxins and plant-derived furanocoumarins in the presence of F<sub>420</sub>H<sub>2</sub>. When FMN replaces F<sub>420</sub> three of the F<sub>420</sub>-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<sub>420</sub> binding is provided by FprA, a di-iron flavo-protein F<sub>420</sub>H<sub>2</sub> oxidase found in methanogenic archaea, which catalyzes the four-electron reduction of O<sub>2</sub> to 2H<sub>2</sub>O with 2 molar equivalents of reduced F<sub>420</sub> (45).

A recent systematic study of the mycobacteria oxidoreductase superfamily (flavin/deazaflavin oxidoreductases) shows some degree of promiscuity within single proteins, binding F<sub>420</sub>, 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<sub>420</sub> 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<sub>420</sub> K<sub>d</sub> = 1.47 versus FMN K<sub>d</sub> = 14.7).

Our crystal structure analysis of FMN and F<sub>420</sub> 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<sub>420</sub> 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<sub>420</sub> 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-glutamylation reaction. A more compelling explanation is that the ancestral nitroreductase appropriated by FbiB through recombination was already a promiscuous enzyme, binding both F<sub>420</sub> and FMN for oxidoreductase activity potentially toward multiple substrates. Again, this hypothesis does not explain the retention of distinct F<sub>420</sub> and FMN binding sites in the C-terminal domain of FbiB nor the potential roles these redox molecules play in the poly-γ-glutamylation 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<sub>420</sub>-2 as the largest product. The homolog structure from <i>A. fulgidus</i> (Protein Data Bank entry 2PHN), equivalent to the FbiB N-terminal domain, displays metal and GDP binding; glutamate and F<sub>420</sub> 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 CoE 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<sub>420</sub> 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 <i>M. tuberculosis</i> 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 A. fulgidus structure from which the model was produced. If, as we contend, the N-terminal domain contains all of the catalytic machinery for F_{420} elongation, then this basic surface groove connected to the postulated active site cavity (Fig. 7), could bind to the growing poly-γ-glutamate tail with its negatively charged carboxylate groups and direct the growing chain toward solvent space or toward the adjacent C-terminal domain.

**Elongation Mechanism; Insertion or Extension?**—Poly-γ-glutamate tails are found on a limited number of biomolecules, including cofactor F_{420} and folate derivatives. Poly-γ-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). FbiB (CoE in archaea), folypolyglutamate synthase, and the poly-γ-glutamic acid synthetase complex, catalyze the poly-γ-glutamylation reactions 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 F_{420} and ATP in folates and poly-γ-glutamic acid), formation of an acyl phosphate intermediate, and finally nucleophilic attack by the incoming L-glutamate (17, 46).

Our functional characterization described in the present work demonstrates the addition of multiple L-glutamate residues to the growing poly-γ-glutamate tail of F_{420} carried out by FbiB. It is not clear, however, whether each L-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 L-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-γ-glutamic acid synthetase enzymes that also carry out poly-γ-glutamylation 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 study. A. M. R. carried out most experiments, and 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 study. 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. G. B., E. N. B., and C. J. S. Wrote the paper, and all authors analyzed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Ehab Jirgis for technical assistance and Martin Middleditch for mass spectrometry, performed at the Centre for Genomics, Proteomics, and Metabolomics at the University of Auckland. This research was undertaken on the MX1, MX2, and SAXS/WAXS beamlines at the Australian Synchrotron (Victoria, Australia). We thank Drs. Neil Patterson, Jeremy Keown, David Goldstone, and Shaun Lott for synchrotron data collection.

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