A revised biosynthetic pathway for the cofactor F₄₂₀ in bacteria

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Abstract

Cofactor F₄₂₀ plays critical roles in primary and secondary metabolism in a range of bacteria and archaea as a low-potential hydride transfer agent. It mediates a variety of important redox transformations involved in bacterial persistence, antibiotic biosynthesis, pro-drug activation and methanogenesis. However, the biosynthetic pathway for F₄₂₀ has not been fully elucidated: neither the enzyme that generates the putative intermediate 2-phospho-*L*-lactate, nor the function of the FMN-binding C-terminal domain of the γ -glutamyl ligase (FbiB) in bacteria are known. Here we show that the guanylyltransferases FbiD and CofC accept phosphoenolpyruvate, rather than 2-phospho-*L*-lactate, as their substrate, leading to the formation of the previously uncharacterized intermediate, dehydro-F₄₂₀-0. The C-terminal domain of FbiB then utilizes FMNH₂ to reduce dehydro-F₄₂₀-0, which produces mature F₄₂₀ species when combined with the γ -glutamyl ligase activity of the N-terminal domain. This new insight has allowed the heterologous expression F₄₂₀ from a recombinant F₄₂₀ biosynthetic pathway in *Escherichia coli*.

Cofactor F₄₂₀ is a deazaflavin that acts as a hydride carrier in diverse redox reactions in both bacteria and archaea^{1,2}. While F₄₂₀ structurally resembles the flavins FMN and FAD, it functions as an obligate two-electron hydride carrier and hence is functionally analogous to the nicotinamides NAD⁺ and NADP⁺³. The lower reduction potential of the F₄₂₀, relative to the flavins, results from the substitution of N5 of the isoalloxazine ring in the flavins for a carbon in F₄₂₀^{4,5}. Originally characterized from methanogenic archaea in 1972^{4,5}, F₄₂₀ is an important catabolic cofactor in methanogens and mediates key one-carbon transformations of methanogenesis⁶. F₄₂₀ has since been shown to be synthesized in a range of archaea and bacteria^{1,2,7,8}. In *Mycobacterium tuberculosis*, the causative agent of tuberculosis, F₄₂₀ has been shown to contribute to persistence^{9,10} and to activate the new clinical antitubercular prodrugs delamanid and pretomanid¹¹. There are also growing numbers of natural products that have been shown to be synthesized through F₄₂₀-dependent pathways, including tetracyclines¹², lincosamides¹³, and thiopeptides¹⁴. F₄₂₀-dependent enzymes have also been explored for bioremediation and biocatalytic applications^{15,16}.

The currently accepted F₄₂₀ biosynthetic pathway consists of two branches². In the first branch, tyrosine is condensed with 5-amino-6-ribitylamino-2,4[1*H*,3*H*]-pyrimidinedione from the flavin biosynthetic pathway to generate the deazaflavin chromophore Fo (7,8-dide-methyl-8-hydroxy-5-deazariboflavin) *via* the activity of the two-domain Fo synthase FbiC, or the CofG/H pair (where ‘Fbi’ refers to mycobacterial proteins and ‘Cof’ refers to archaeal homologs). In the second branch, it has been hypothesized that a 2-phospho-*L*-lactate guanylyltransferase (CofC in archaea and the putative enzyme FbiD in bacteria) catalyzes the guanylylation of 2-phospho-*L*-lactate (2-PL) using guanosine-5'-triphosphate (GTP), yielding *L*-lactyl-2-diphospho-5'-guanosine (LPPG)¹⁷. The two branches then merge at the reaction

catalyzed by the transferase FbiA/CofD, where the 2-phospho-*L*-lactyl moiety of LPPG is transferred to Fo, forming F₄₂₀-O^{18,19}. Finally, the γ -glutamyl ligase (FbiB/CofE) catalyzes the poly-glutamylation of F₄₂₀ to generate mature F₄₂₀, with poly- γ -glutamate tail lengths of ~2-8, depending on species^{20,21}.

There are three aspects of the F₄₂₀ biosynthetic pathway that require clarification. First, the metabolic origin of 2-PL, the proposed substrate for CofC, is unclear. It has been assumed that a hypothetical kinase (designated CofB) phosphorylates *L*-lactate to produce 2-PL²². However, no such enzyme for the production of 2-PL has been identified in bacteria or archaea, and our genomic analysis of F₄₂₀ biosynthesis operons has failed to identify any candidate enzymes with putative *L*-lactate kinase activity². Second, the existence of FbiD has only been inferred through bioinformatics and genetic knockout studies and the enzyme has not been formally characterized^{23,24}. Finally, the bacterial γ -glutamyl ligase FbiB is a two-domain protein²⁰, in which the N-terminal domain is homologous to other F₄₂₀- γ -glutamyl ligases (including the archaeal equivalent, CofE) and the C-terminal domain adopts an FMN-binding nitroreductase (NTR) fold²⁰. Although both domains are required for full γ -glutamyl ligase activity, no function has been associated with either the C-terminal domain or the FMN cofactor, given no redox reactions are known to be involved in F₄₂₀ biosynthesis.

Here we demonstrate that 2-PL is not required for F₄₂₀ biosynthesis in bacteria and instead phosphoenolpyruvate (PEP), an abundant intermediate of glycolysis and gluconeogenesis, is incorporated into F₄₂₀. We show that PEP guanylation is catalyzed by the CofC and FbiD enzymes that were previously thought to act upon 2-PL. In bacteria, the incorporation of

PEP in the pathway results in the production of the previously undetected intermediate dehydro-F₄₂₀-0, which is then reduced by the C-terminal domain of FbiB alongside poly-glutamylation. These findings result in a substantially revised pathway for F₄₂₀ biosynthesis and have allowed us to heterologously produce functional F₄₂₀ in engineered *Escherichia coli*, an organism that does not normally produce F₄₂₀, at levels comparable to some native F₄₂₀-producing organisms.

Results

FbiD/CofC use phosphoenolpyruvate, rather than 2-phospho-*L*-lactate, as a substrate

The archaeal enzyme CofC has previously been suggested to catalyze the guanylation of 2-PL to produce LPPG during F₄₂₀ biosynthesis (Fig. 1a)²⁵. Another study, using transposon mutagenesis, has shown that MSMEG_2392 of *Mycobacterium smegmatis* is essential in the biosynthesis of F₄₂₀ from Fo²³. We have recently shown that homologs of this gene have sequence homology to CofC and belong to operons with other validated F₄₂₀ biosynthetic genes in a wide variety of bacteria². In keeping with the bacterial naming system, we refer to this enzyme as FbiD. To test the function of this putative bacterial FbiD, we cloned the Rv2983 gene from *M. tuberculosis*²⁶ into a mycobacterial expression vector and purified heterologously expressed *Mtb*-FbiD from *M. smegmatis* mc²4517 host cells. We also expressed and purified *Mtb*-FbiA (the enzyme thought to transfer the 2-phospho-*L*-lactyl moiety of LPPG to Fo to produce F₄₂₀-0)¹⁸ to use in coupled HPLC-MS enzymatic assays with *Mtb*-FbiD. Surprisingly, we found that when *Mtb*-FbiD and *Mtb*-FbiA were included in an assay with 2-PL, GTP (or ATP) and Fo, no product was formed (Fig. 1b). We then tested whether *Mtb*-FbiA

and CofC from *Methanocaldococcus jannaschii* (*Mj*-CofC) could catalyze F₄₂₀-0 formation under the same conditions, which again yielded no product (Fig. 1b).

Although 2-PL is hypothesized to be an intermediate in F₄₂₀ biosynthesis, this has never been experimentally confirmed in bacteria. Additionally, no enzyme capable of phosphorylating *L*-lactate to 2-PL has been identified in F₄₂₀ producing organisms, despite considerable investigation²². 2-PL has been little studied as a metabolite and is only known to occur as a by-product of pyruvate kinase activity²⁷. 2-PL has not been implicated as a substrate in any metabolic pathway outside the proposed role in F₄₂₀ biosynthesis; rather it has been shown *in vitro* to inhibit several enzymes involved in glycolysis and amino acid biosynthesis²⁸⁻³⁰. Our inability to detect activity with 2-PL led us to consider alternative metabolites that could potentially substitute for 2-PL, namely the structurally analogous and comparatively abundant molecule phosphoenolpyruvate (PEP)³¹ (Fig. 1a).

While there was no activity when 2-PL was used in the FbiD/CofC:FbiA coupled assays, when these enzymes were incubated with PEP, GTP (or ATP) and Fo, a previously unreported intermediate in the F₄₂₀ biosynthesis pathway, which we term ‘dehydro-F₄₂₀-0’, was produced (Fig. 1b). The identity of this compound, which is identical to F₄₂₀-0 except for a methylene group in place of the terminal methyl group, was verified by tandem mass spectrometry (Fig. 1c). The only difference that we observed between *Mtb*-FbiD or *Mj*-CofC was that while *Mtb*-FbiD exclusively utilizes GTP to produce dehydro-F₄₂₀-0, *Mj*-CofC can also catalyze the reaction with ATP, albeit to a lesser extent (Fig. 1b). Interestingly, in our experiments the FbiD/CofC enzymes were only active in the presence of FbiA. This was not unexpected given that the inferred intermediate (enolpyruvyl-diphospho-5'-guanosine; EGGP) is expected to be unstable²² (Fig. 1a).

To understand the molecular basis of PEP recognition by *Mtb*-FbiD, we crystallized the protein and solved the structure by selenium single-wavelength anomalous diffraction (Se-SAD), and then used this selenomethionine-substituted structure to obtain the native FbiD structure by molecular replacement. The latter was then refined at 1.99 Å resolution ($R/R_{\text{free}}=0.19/0.22$) (SI Table 2). As expected, *Mtb*-FbiD adopts the same MobA-like nucleoside triphosphate (NTP) transferase family protein fold as CofC: a central 7-stranded β -sheet (six parallel strands and one antiparallel), with α -helices packed on either side (Fig. 2a). Superposition of CofC from *Methanosarcina mazei* (PDB code 2I5E) on to *Mtb*-FbiD gives a root mean square difference (rmsd) of 1.85 Å over 181 C α atoms, with 25.4% sequence identity, establishing clear structural homology.

We also soaked PEP into pre-formed FbiD crystals to obtain an FbiD-PEP complex (2.18 Å resolution, $R/R_{\text{free}}=0.22/0.26$). FbiD has a cone-shaped binding cleft with a groove running across the base of the cone, formed by the C-terminal end of the central β -sheet (Fig. 2a). PEP binds in the cleft with its phosphate group anchored through two Mg²⁺ ions to three acidic side chains (D116, D188 and D190) (Fig. 2b). The PEP carboxylate group is hydrogen bonded to the hydroxyl group of S166 and the main chain NH groups of T148 and G163. All PEP binding residues are conserved in the CofC protein of *M. mazei* (PDB code 2I5E) (Fig. 2c and Fig. S2), consistent with the enzymatic assays that showed PEP is the substrate for archaeal CofC, as well as FbiD. In the GTP-bound structure of *E. coli* MobA³² (PDB code 1FRW), GTP binds in a characteristic surface groove, providing a structural framework for substrate binding and catalysis. In our enzyme assays, neither FbiD nor CofC showed activity in the absence of FbiA. Moreover, we did not observe GTP binding in either our co-crystallization or differential scanning fluorimetry experiments. We speculate that the GTP binding

site is not formed until FbiD/CofC interacts with FbiA/CofD, enabling catalysis to proceed through to formation of dehydro-F₄₂₀-0. This may provide an advantage by producing EPPG/EPPA only when both proteins are available, thereby overcoming the issue of intermediate instability.

The C-terminal domain of FbiB catalyzes reduction of dehydro-F₄₂₀-0 to F₄₂₀-0

Dehydro-F₄₂₀-0 would yield F₄₂₀-0 upon reduction of the terminal methylene double bond. However, no masses corresponding to F₄₂₀-0 were identified in any of the LC-MS traces from the FbiD:FbiA coupled assays, suggesting that an enzyme other than FbiD or FbiA catalyzes dehydro-F₄₂₀-0 reduction. We have previously shown that full-length FbiB consists of two domains: an N-terminal domain that is homologous to the archaeal γ -glutamyl ligase CofE^{21,33}, and a C-terminal domain of the NTR fold³⁴ that binds to FMN and has no known function²⁰, but is essential for extending the poly- γ -glutamate tail.

We tested whether FbiB could use dehydro-F₄₂₀-0 as a substrate with a three enzyme assay in which FbiB and *L*-glutamate were added to the FbiD:FbiA coupled assay. *Mtb*-FbiB was observed to catalyze the addition of *L*-glutamate residues to dehydro-F₄₂₀-0, forming dehydro-F₄₂₀ species with one ([M+H]⁺, *m/z* of 643.40) and two ([M+H]⁺, *m/z* of 772.40) glutamate residues (Fig. S1). We then tested the hypothesis that the orphan function of the FMN-binding C-terminal domain could in fact be a dehydro-F₄₂₀-0 reductase. We used a four-enzyme *in vitro* assay where *E. coli* NAD(P)H:flavin oxidoreductase³⁵ (Fre), FMN, NADH and 10 mM dithiothreitol (to maintain reducing conditions and generate reduced FMNH₂) were added to the FbiD:FbiA:FbiB assay and the reaction was performed in anaerobic conditions (to prevent reaction of FMNH₂ with oxygen). We found that F₄₂₀-1, *i.e.* the fully reduced and

mature glutamylated cofactor, was produced; but only in the presence of both FbiB and Fre/FMNH₂ (Fig. 3a). Thus, dehydro-F₄₂₀-0 is a *bona fide* metabolic intermediate that can be converted to mature F₄₂₀ by FbiB in an FMNH₂-dependent fashion. These results demonstrate that bacterial FbiB is a bifunctional enzyme, functioning as a dehydro-F₄₂₀-0 reductase and as a γ -glutamyl ligase (Fig. 3c).

When the previously published crystal structures of *Mtb*-FbiB are analyzed in the context of these results, the molecular basis for this activity becomes clear. Crystal structures with both F₄₂₀ and FMN bound have been separately solved and when these are overlaid, it is apparent that the FMN molecule is ideally situated to transfer a hydride to the terminal methylene of dehydro-F₄₂₀-0 (assuming dehydro-F₄₂₀-0 binds in a similar fashion to F₄₂₀). Interestingly, the phospholactyl group of F₄₂₀ appears to be disordered in these crystal structures, suggesting it may adopt multiple conformations. To test this, we docked and minimized dehydro-F₄₂₀-0 and FMNH₂ simultaneously using the OPAL3a forcefield. The results show that in this ternary complex the two molecules can adopt ideal positions and orientations for the reduction of dehydro-F₄₂₀-0 (Fig. 3b). The methylene group of dehydro-F₄₂₀-0 is accommodated by a small hydrophobic pocket mostly comprised of P289 and M372 allowing it to be positioned above the N5 atom of FMNH₂, in a plausible Michaelis complex for hydride transfer. We therefore suggest that the phosphoenolpyruvyl (analogous to the phospholactyl) group of dehydro-F₄₂₀-0 most likely samples conformations within this pocket where it can be reduced.

Interestingly, the γ -glutamyl ligase CofE from archaea is a single domain enzyme; there is no homology to the C-terminal NTR-fold domain of FbiB. In an analogous situation,

F₄₂₀ synthesis is performed by two single domain enzymes in archaea, CofH and CofG², whereas in bacteria this reaction is catalyzed by a two-domain protein, FbiC (with N- and C-terminal domains homologous to CofH and CofG, respectively). Previous analysis of archaeal genomes revealed that *cofH* and *cofG* are closely associated in genomic context³⁶. We therefore investigated the genomic context of archaeal *cofE* genes to investigate whether genes with homology to the C-terminal NTR domain of FbiB were located nearby. From over 1000 archaeal genomes, we only detected 16 open reading frames (ORFs) in the neighboring context of *cofE* that could encode proteins with an NTR fold, although none of these shared substantial (>34% sequence identity) homology and all lacked the key F₄₂₀ binding residues observed in FbiB. There was one interesting exception: the unusual archaea *Lokiarchaeum* sp.³⁷ are unique among all sequenced archaea in that they alone encode an FbiB-like γ -glutamyl ligase:NTR fusion protein.

A recombinant F₄₂₀ biosynthesis operon can be heterologously expressed in *Escherichia coli* to produce mature F₄₂₀

Cofactor F₄₂₀ is only produced by certain bacterial species; the majority of bacteria, including *Escherichia coli*, lack the genes required for F₄₂₀ biosynthesis. Our *in vitro* assay results suggest that 2-PL, and by extension, the hypothesized *L*-lactate kinase CofB, are not required for heterologous production of F₄₂₀ in a non-native organism. To test our hypothesis, we generated a plasmid expressing the *Mycobacterium smegmatis* F₄₂₀ biosynthesis genes *fbiB/C/D* along with the *fbiA* homolog *cofD* from *Methanosarcina mazei*¹⁹, which was substituted as *Ms*-FbiA was found to express poorly in *E. coli*.

We generated two versions of a plasmid-encoded recombinant F₄₂₀ biosynthetic operon, both of which encode codon-optimized genes for expression in *E. coli*: one encodes the native enzymes (pF₄₂₀), and the second encodes C-terminal FLAG-tagged versions of the enzymes to allow their detection in a western blot (pF₄₂₀-FLAG) (Fig. 4a). Plasmids were designed to allow induction of F₄₂₀ biosynthesis in the presence of anhydrotetracycline. A western blot using anti-FLAG antibodies was used to detect whether the proteins were expressed in soluble form in *E. coli*, and confirmed that all were expressed to varying degrees (Fig. S3). We then tested, using HPLC with fluorescence detection, whether F₄₂₀ was heterologously produced in the cell lysate of our recombinant *E. coli* strain expressing this operon. As shown in Fig. 4b, *E. coli* expressing both the FLAG-tagged, and untagged, plasmids produced traces consistent with mature, poly-glutamylated F₄₂₀, although they differed slightly to the *M. smegmatis* produced F₄₂₀ standard in terms of the distribution of poly-γ-glutamate tail lengths.

To confirm that this was indeed mature F₄₂₀, and not dehydro-F₄₂₀ species, we purified the compound from *E. coli* lysate and performed high-resolution MS/MS analysis. Mass fragmentation did indeed show that the compound was reduced, and not dehydro-F₄₂₀ (Fig. 4d). No dehydro-F₄₂₀ species were detected. The yield of purified F₄₂₀ was approximately 27 nmol/L of culture, which is comparable to physiological levels of several F₄₂₀-producing species³⁸. UV-Vis and fluorescence spectra of the purified F₄₂₀ matched literature values (Fig. S4)^{4,5}. Finally, we confirmed that the purified cofactor was functional by measuring enzyme kinetic parameters with F₄₂₀-dependent glucose-6-phosphate dehydrogenase (FGD) from *M. smegmatis* (Fig. 4c). The apparent Michaelis constant was within error of that observed with FGD and F₄₂₀ produced from *M. smegmatis*, while the k_{cat} was approximately half that of the *M. smegmatis* F₄₂₀ (Fig. 4c), which could result from slight differences in the distribution of

tail lengths, as previously reported (Table S3)³⁹. These results confirm that the recombinant production of F₄₂₀ was achieved with a biosynthetic pathway containing only CofD (equivalent to FbiA)/FbiB/FbiC/FbiD.

Discussion

It has become widely accepted within the field that one of the essential initial steps in F₄₂₀ biosynthesis involves a hypothetical *L*-lactate kinase that produces 2-PL, which is subsequently incorporated into F₄₂₀ through the activities of CofC and CofD. However, neither bioinformatics nor genetic knockout studies have identified plausible candidate genes for a *L*-lactate kinase^{2,18,23}. Furthermore, 2-PL has been shown to inhibit several enzymes involved in central metabolism²⁸⁻³⁰. In terms of pathway flux, this makes 2-PL an unusual starting point for biosynthesis of an abundant metabolite such as F₄₂₀, which can exceed 1 μ M in some species³⁸. The results presented in this paper unequivocally demonstrate that PEP, rather than 2-PL, is the authentic starting metabolite in bacteria. These results reconcile the previously problematic assumptions that are required to include 2-PL within the biosynthetic pathway and establish a revised pathway (Fig. 5) that is directly linked to central carbon metabolism (*via* PEP) through the glycolysis and gluconeogenesis pathways.

Our observation that CofC accepts PEP (and not 2-PL) *in vitro*, appear to contradict previous studies in which *Mj*-CofC was reported to use 2-PL as substrate^{19,33}. However, this discrepancy is most likely due to the supplementation of the coupled CofC/CofD reaction in these studies with pyruvate kinase and 2 mM PEP, a strategy that was used to overcome apparent product inhibition by GMP^{19,33}. Regardless, we cannot explain how a pathway starting from PEP can produce mature (*i.e.* not dehydro-F₄₂₀) F₄₂₀ in archaea given that their equivalent of FbiB (CofE) lacks the C-terminal domain dehydro-F₄₂₀-O reductase domain seen in

FbiB. One possibility is that an unknown dehydro-F₄₂₀-0 reductase exists elsewhere in the genome (remote from CofE). Further studies are required to resolve this step in archaeal F₄₂₀ biosynthesis.

The discovery of dehydro-F₄₂₀-0 as the product of FbiD:FbiA activity in mycobacteria indicated that another enzyme must be required to reduce dehydro-F₄₂₀-0 and produce F₄₂₀-0. This allowed us to define the function of the orphan C-terminal domain of mycobacterial FbiB²⁰. The N-terminal domain is homologous with the archaeal γ -glutamyl ligase CofE³³, but is only capable of catalyzing glutamylation of F₄₂₀-1²⁰. The C-terminal domain, which binds both F₄₂₀ and FMN, is essential for extending the poly- γ -glutamate tail of F₄₂₀-0²⁰, but we could not explain the possible function of the FMN cofactor. Here, we show that FbiB can catalyze both poly-glutamylation and reduction of dehydro-F₄₂₀-0, *via* the activities of the N- and C-terminal domains, respectively.

The increasing recognition of the importance of F₄₂₀ in a variety of biotechnological, medical and ecological contexts underlines the need for making the compound more widely accessible to researchers; however our inability to produce F₄₂₀ recombinantly in common laboratory organisms has been a major barrier to wider study. Here, we confirm the results of our *in vitro* experiments by showing that recombinant expression of the four characterized F₄₂₀ biosynthesis genes allows production of F₄₂₀ in *E. coli*. This should now facilitate the use of F₄₂₀ in a variety of processes with recombinant organisms, such as biocatalysis using a bio-orthogonal cofactor, directed evolution of F₄₂₀-dependent enzymes, recombinant production of antibiotics for which F₄₂₀ is a required cofactor, and metabolic engineering.

Methods

Bacterial strains and growth conditions

Protein expression utilized either *M. smegmatis* mc²4517⁴⁰, *E. coli* BL21(DE3) or LOBSTR-BL21(DE3)⁴¹ cells. For growth of *M. smegmatis*, media were supplemented to 0.05% (v/v) Tween80. *M. smegmatis* cells were grown in ZYM-5052⁴² or modified auto-induction media TB 2.0 (2.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 0.6% glycerol, 0.05% glucose, 0.2% lactose)⁴³. For selenomethionine labelling, cells were grown in PASM-5052 media⁴². *E. coli* expressions were conducted in either the above modified auto-induction media, or Terrific Broth (TB) medium modified for auto-induction of protein expression (1.2% tryptone, 2.4% yeast extract, 72 mM K₂HPO₄, 17 mM KH₂PO₄, 2 mM MgSO₄, 0.8% glycerol, 0.015% glucose, 0.5% lactose, 0.375% aspartic acid), grown for 4 h at 37° C followed by overnight incubation at 18° C.

Protein expression and purification

Mtb-FbiD. The open reading frame encoding FbiD (Rv2983) was obtained by PCR from *M. tuberculosis* genomic DNA (Table S1). The pYUBDuet-*fbiABD* co-expression construct was then prepared by cloning *fbiD* into pYUBDuet⁴⁴ using *Bam*HI and *Hind*III restriction sites, followed by cloning the *fbiAB* operon using *Nde*I and *Pac*I restriction sites. This construct expresses FbiD with an N-terminal His₆-tag, whereas the FbiA and FbiB proteins are expressed without any tags.

The pYUBDuet-*fbiABD* vector was transformed into *M. smegmatis* mc²4517 strain⁴⁰ for expression. The cells were grown in a fermenter (BioFlo[®]415, New Brunswick Scientific)

for 4 days before harvesting. The cells were lysed in 20 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM imidazole, 1 mM β -mercaptoethanol 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 $20,000 \times g$ to separate the insoluble material. Recombinant FbiD was separated from other proteins by immobilized metal affinity chromatography (IMAC) on a HisTrap FF 5-ml Ni-NTA column (GE Healthcare), eluted with imidazole, and further purified by Size Exclusion Chromatography (SEC) on a Superdex 75 10/300 column (GE Healthcare) pre-equilibrated in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM β -mercaptoethanol.

Mtb-FbiA. The open reading frame encoding *M. tuberculosis* FbiA was commercially synthesized and cloned into pRSET-A (Invitrogen). The pYUB28b-*fbiA* construct used for expression in *M. smegmatis* mc²4517 was prepared by subcloning *fbiA* into pYUB28b⁴⁴ using an *Nde*I site introduced by overhang PCR utilizing the *Hind*III restriction site present on both vectors amplified with the T7 reverse primer (Table S1). The resulting pYUB28b-*fbiA* construct expresses FbiA with an N-terminal His₆-tag. The protein was expressed in *M. smegmatis* mc²4517 in ZYM-5052 media auto-induction media^{42,45} in a fermenter (BioFlo[®] 415, New Brunswick Scientific) for 4 days. The protein was then purified using Ni-NTA and size-exclusion chromatography steps, as described above, in 20 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM β -mercaptoethanol.

Mj-CofC. The open reading frame encoding *Methanocaldococcus jannaschii* CofC (MJ0887)²⁵ was synthesized (GenScript) and cloned into pYUB28b vector⁴⁴ using *Nde*I and *Hind*III restriction sites. The protein was expressed in TB auto-induction media and purified

using Ni-NTA and size-exclusion chromatography steps, as described above, in 20 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM β -mercaptoethanol.

Ec-Fre. The *E. coli* flavin reductase³⁵ was cloned into pProEX-HTb using *Kas*I and *Hind*III restriction sites (Table S1). Protein expression and purification was performed similar to *Mj*-CofC.

Ms-FbiD. The open reading frame encoding *M. smegmatis* FbiD (MSMEG_2392) was synthesized (Integrated DNA Technologies) and cloned into pETMCSIII by Gibson Assembly as outlined previously⁴³. The protein was expressed overnight at 30 °C in auto-induction media and purified similarly to *Mj*-CofC.

Construction of synthetic F₄₂₀ biosynthesis operon

Ribosome binding sites were individually optimized for each of the codon-optimized F₄₂₀ biosynthesis genes using the Ribosome Binding Site Calculator server^{36,46}. Multiple operon designs were analyzed using the server's operon calculator and modified to remove unwanted translation products and RNA instability elements while maintaining predicted translation initiation rates for all coding sequences within an order of magnitude. The final design placed the operon under the control of the tetracycline-inducible promoter BBa_R0040 and the artificial terminator BBa_B1006. For subsequent assembly the operon was flanked by BioBrick prefix and suffix sequences. The operon was synthesized by GenScript and cloned into pSB1C3 containing the constitutive tetracycline repressor cassette BBa_K145201 using the standard BioBrick assembly protocol with *Eco*RI and *Xba*I/*Spe*I restriction enzymes⁴⁷. This construct, hereafter referred to as pF₄₂₀, allowed for production of F₄₂₀ to be induced by the

addition of anhydrotetracycline. For western blot analysis as second version of the operon with single C-terminal FLAG tags on all four genes was likewise synthesized.

To test for *in vivo* F₄₂₀-dependent reductase/oxidase activity the open reading frame of *M. smegmatis* FGD (MSMEG_0777) was codon optimized and commercially synthesized by GenScript and cloned into multiple cloning site 1 of pCOLADuet-1 (Novagen) using *NcoI* and *HindIII* sites. Subsequently MSMEG_2027 was subcloned from pETMCSIII-MSMEG_2027⁴³ into multiple cloning site 2 using *NdeI* and *KpnI* to give pFGD2027.

***Mtb*-FbiD crystallization and structure determination**

Crystallization. Apo-FbiD (20 mg/mL in 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM β -mercaptoethanol) was crystallized using the sitting drop vapor diffusion method in 30% PEG 1500, 3% MPD, 0.2 M MgSO₄, 0.1 M sodium acetate pH 5.0. For experimental phasing, selenomethionine-substituted FbiD crystals were grown using protein produced in *M. smegmatis* host cells⁴⁵. Se-SAD anomalous diffraction data sets were collected at the Australian Synchrotron. Data collection statistics are summarized in Table S2. Crystals of *Mtb*-FbiD in complex with PEP were obtained by soaking pre-formed apo crystals in precipitant solutions containing 10 mM PEP for 30 min.

Structure determination. All data sets were indexed and processed using XDS⁴⁸, and scaled with AIMLESS⁴⁹ from the CCP4 program suite⁵⁰. The structure was solved using the SAD protocol of Auto-Rickshaw⁵¹, the EMBL-Hamburg automated crystal structure determination platform. Based on an initial analysis of the data, the maximum resolution for substructure determination and initial phase calculation was set to 2.83 Å. All three of the ex-

pected heavy atoms were found using the program SHELXD⁵². The initial phases were improved using density modification and phase extension to 2.33 Å resolution using the program RESOLVE⁵³. Cycles of automatic model building by ARP/wARP⁵⁴ and phenix.autobuild⁵⁵ resulted in a protein model that was completed manually using COOT⁵⁶. 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 REFMAC5⁵⁷, against the data to 1.99 Å resolution. The PDB_redo program⁵⁸ was used in the final stages of refinement. Full refinement statistics are shown in Table S2.

The structure of *Mtb*-FbiD in complex with PEP was solved by molecular replacement using PHASER⁵⁹ with the apo-FbiD structure as a search model. The structure was refined by cycles of manual building using COOT⁵⁶ and refinement using REFMAC5⁵⁷, against the data to 2.18 Å resolution. Full refinement statistics are shown in Table S2.

HPLC assays

FbiD/CofC-FbiA coupled activity was monitored in a reaction mixture containing 100 mM HEPES pH 7.5, 2 mM GTP, 0.1 mM Fo, 5 mM MgCl₂, 1 mM 2-PL or PEP, 1 μM FbiD and 5 μM FbiA. The reactions were incubated at 37 °C and stopped using 20 mM EDTA at various time points. Separation of F₄₂₀ species was performed on an Agilent HP 1100 HPLC system equipped with 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 Phenomenex 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 reference of 550 nm (bandwidth 50 nm). The wavelengths used for the fluorescence detector were 420 nm (excitation) and 480 nm (emission).

LC-MS characterization of dehydro-F₄₂₀ species

Enzymatic reactions were set up as described above. 10 uL aliquots were injected onto a C18 trap cartridge (LC Packings, Amsterdam, Netherlands) for desalting prior to chromatographic separation on a 0.3 × 100 mm 3.5 µm Zorbax 300SB C18 Stablebond column (Agilent Technologies, Santa Clara, CA, USA) using the following gradient at 6 uL/min: 0-3 min 10% B, 24 min 50% B, 26 min 97% B, 29 min 97% B, 30.5 min 10% B, 35 min 10% B, where A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The column eluate was ionised in the electrospray source of a QSTAR-XL Quadrupole Time-of-Flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). For IDA (Information Dependent Analysis) analyses, a TOF-MS scan from 330-1000 *m/z* was performed, followed by three rounds of MS/MS on the most intense singly or doubly charged precursors in each cycle. For targeted work, defined Product Ion Scans were created to isolate and fragment specific ions of interest with various collision energies (10-60 kV). Both positive and negative modes of ionisation were used as appropriate.

MS/MS confirmation of reduction of dehydro-F₄₂₀ species

To confirm the reduction of the PEP moiety *in vitro* assays were prepared in 50 mM HEPES pH 7.5, 100 mM KCl, 5 mM MgCl₂, 2 mM GTP, 0.1 mM Fo, 1 mM PEP, 5 μM *Mtb*-FbiA, 6.5 μM *Msg*-FbiD, 10 mM DTT, 20 μM FMN, 0.2 mM NADH, 0.1 μM *Ec*-Fre, 2 μM *Mtb*-FbiB and 1 mM *L*-glutamate. To minimize futile oxidation of FMNH₂ by oxygen the reaction mixture was repeatedly evacuated and purged with nitrogen and maintained under a nitrogen atmosphere. Samples were incubated at ambient temperature for up to 36 hours and stopped by addition of 20 mM EDTA. Samples were desalted using Bond Elut C18 tips (Agilent Technologies) and eluted in 0.1 % formic acid in acetonitrile. Samples were injected on to a Q Exactive Plus (ThermoFischer Scientific) at 100 μl/min with isocratic 50% B where A was 10 mM ammonium acetate pH 6.0 and B was 0.02% ammonia in methanol. Scans from 150-2250 *m/z* were performed and data-dependent MS/MS on targeted metabolites was done in both positive and negative mode.

Production of F₄₂₀ in *E. coli*

The pF₄₂₀ vector was transformed into *E. coli* BL21(DE3) for expression. Cells were cultivated in M9 minimal media supplemented with chloramphenicol (25 μg/mL) and tyrosine (18 μg/mL) in 1 L shake flasks with 500 ml working volume at 28 °C. At O.D. of 0.60, 200 ng/mL of tetracycline was added to induce the expression of F₄₂₀ biosynthesis pathway. After induction, cells were cultivated for at least 16 h. To test expression of the F₄₂₀ biosynthetic genes, cell pellets were lysed by resuspending to O.D. of 0.8 in buffer containing BugBuster (Novagen). Following centrifugation at 16,000 ×g proteins were resolved on a gel (Bolt™ 4-12% Bis-Tris Plus, Invitrogen) for 1 h and visualized using Coomassie Brilliant Blue. For immunoblotting, proteins were transferred to a membrane (iBlot® 2 NC Regular Stacks, Invitrogen) using iBlot Invitrogen (25 V, 6 mins). After staining and de-staining, the membrane was

blocked with 3 % skim milk solution and blotted with anti-FLAG antibodies conjugated with HRP (DYKDDDDK Tag Monoclonal Antibody, Thermofisher Scientific).

For detection of F₄₂₀ in *E. coli* lysate by HPLC-FLD, cells were grown overnight in media containing 2.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 100 ng/mL anhydrotetracycline, 34 µg/mL chloramphenicol at 30 °C. Cells from 500 µL of culture were pelleted by centrifugation at 16,000 ×g. Cells were resuspended in 500 µL of 50 mM Na₂HPO₄ pH 7.0 and lysed by boiling at 95 °C for 10 minutes. Cell debris was pelleted by centrifugation at 16,000 ×g and filtered through a 0.22 µm PVDF filter. Analysis was conducted as described previously². pSB1C3 containing only BBa_K145201 was used as a control.

Purification and analysis of *E. coli* -produced F₄₂₀

For F₄₂₀ extraction, 1 L of cell culture was centrifuged at 5,000 ×g for 15 minutes. The cell pellet was re-suspended in 30 ml of 75% ethanol and boiled at 90 °C in a water bath for 6 min for cell lysis. The cell extract was again centrifuged at 5,000 ×g for 15 minutes to remove cell debris and the supernatant was lyophilized. The lyophilized cell extract was re-dissolved in 10 ml milli Q water centrifuged at 5,000 ×g for 15 minutes and the supernatant passed through 0.45 µm syringe filter (Millex-HV). The filtered cell extract was purified for F₄₂₀ using a 5 ml HiTrap QFF column (GE Healthcare) as previously described⁴⁴. The purified F₄₂₀ solution was further desalted by passing it through C18 extract column (6 ml, HyperSep C18 Cartridges, ThermoFischer Scientific). The C18 extract column was first equilibrated by passing through 10 ml of 100% methanol and 10 ml of milli Q. Afterwards the F₄₂₀ solution was passed through the C18 extract column, F₄₂₀ was eluted in 2 ml fraction of 20% methanol.

The purified F₄₂₀ solution was further concentrated in GeneVac RapidVap and re-dissolved in 500 µl of milliQ for further analysis and assays.

UV and fluorescence spectra were collected on a Varian Cary 60 and a Varian Cary Eclipse, respectively, in a 10 mm QS quartz cell (Hellma Analytics). Samples were buffered to pH 7.5 with 50 mM HEPES and scanned from 250-600 nm. For fluorescence, the excitation wavelength was 420 nm and the emission scanned from 435 to 600 nm.

Activity assays with *M. smegmatis* and *E. coli*-derived F₄₂₀ were conducted with *M. smegmatis* FGD1 expressed and purified as described previously¹⁵. Assays were performed in 50 mM Tris pH 7.5, 300 mM NaCl, 50 nM FGD1, 5 µM F₄₂₀ and 0-1000 µM glucose-6-phosphate using a SpectraMAX e2 plate reader. Activity was measured by following loss of F₄₂₀ fluorescence at 470 nm. Apparent k_{cat} and K_m values were calculated using GraphPad Prism 7.04 (GraphPad Software, La Jolla California).

2-PL synthesis

In the absence of a commercial source, 2-PL was chemically synthesized by a slight modification of the method of Kirsch⁶⁰. Briefly, benzyl lactate was condensed with chlorodiphenyl phosphate in pyridine, with cooling, to give benzyldiphenylphosphoryl lactate. Hydrogenolysis of this material in 70% aqueous tetrahydrofuran over 10% Pd-C gave phospholactic acid as a colorless, viscous oil, which was characterized by proton, carbon and phosphorus NMR spectroscopy, and by mass spectrometry. ¹H NMR (DMSO-d₆) δ 11.68 (br, 3H), 4.53 (m, 1H), 1.36 (d, J=6.8 Hz, 3H). ¹³C NMR (DMSO-d₆) δ 172.50 (d, J_{P-C}=0.05 Hz), 69.56 (d, J_{P-C}=0.04 Hz), 19.27 (d, J_{P-C}=0.04 Hz). ³¹P NMR (DMSO-d₆) δ -1.64. APCI-MS found: [M+H]⁺=171.1, [M-H]⁻=169.1.

Fo purification

Fo was purified from *M. smegmatis* culture medium over-expressing *Mtb*-FbiC as described previously⁴⁴.

Genomic context analysis

A non-redundant CofE dataset of 4,813 sequences was collected using the Pfam identifier PF01996 and the InterPro classifications IPR008225, IPR002847, and IPR023659. Archaeal CofE sequences were extracted from this dataset, resulting in a set of 1,060 sequences that included representatives across twelve phyla: Crenarchaeota, Euryarchaeota, Thaumarchaeota, *Candidatus* Bathyarchaeota, *Candidatus* Diapherotrites, *Candidatus* Heimdallarchaeota, *Candidatus* Korarchaeota, *Candidatus* Lokiarchaeota, *Candidatus* Marsarchaeota, *Candidatus* Micrarchaeota, *Candidatus* Odinarchaeota, and *Candidatus* Thorarchaeota. The genomic context (10 upstream and 10 downstream genes) of each archaeal *CofE* was analyzed for the presence of a neighboring PF00881 domain with homology to the C-terminal domain of FbiB using the Enzyme Function Initiative - Genome Neighborhood Tool⁶¹.

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Author contribution

JA performed experiments, analysed results and cowrote the manuscript; ENMJ, MH, JC, SMS, SS, BP, MM performed experiments and analyzed results; BN conceived project, performed experiments and analyzed results; CG, ENB conceived project, designed experiments, analyzed results and cowrote the manuscript; CS designed experiments, analyzed results and cowrote the manuscript; GB, CJJ conceived project, designed experiments, performed experiments, analyzed results and cowrote the manuscript. All authors provided feedback on the manuscript.

Declaration of interests

The authors declare no competing financial interest.

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Fig. 1. Phosphoenolpyruvate (PEP) is an intermediate in the formation of dehydro-F₄₂₀-0.

0. (a) Production of F₄₂₀-0 in our revised biosynthesis pathway (left) compared to the currently accepted pathway (right). **(b)** Coupled-reaction HPLC assays showing that both *Mtb*-FbiD and *Mj*-CofC enzymes use PEP to produce dehydro-F₄₂₀-0. **(c)** Tandem mass spectral identification of dehydro-F₄₂₀-0. MS/MS fragmentation of dehydro-F₄₂₀-0, showing fragment ions with their corresponding structures. The inset displays the observed spectrum of the parent molecule (expected monoisotopic *m/z* 512.0711 [M-H]⁻).

Fig. 2. Crystal structure of *Mtb*-FbiD. (a) Cartoon representation of the *Mtb*-FbiD structure in complex with PEP, shown as a ball-and-stick model. (b) The phosphate group of PEP binds to three aspartic acid side chains through two Mg^{2+} ions (shown in cyan). PEP is shown in $2F_o - F_c$ omit density contoured at 2.0σ , and drawn as ball-and-stick model. Water molecules are shown as red spheres and hydrogen bond interactions are outlined as dashed lines. (c) Superposition of *Mtb*-FbiD (wheat ribbon) and *Mj*-CofC (green ribbon), indicating $1.85 \text{ \AA}$ rmsd over 181 superimposed Ca . The protruding hairpin in the *Mj*-CofC structure is involved in dimer formation, which is absent in *Mtb*-FbiD.

Fig. 3. *Mtb*-FbiB catalyzes reduction of dehydro- F_{420-0} . (a) F_{420-1} is produced in FbiD:FbiA:FbiB coupled assays in the presence of Fre/FMNH₂ and *L*-glutamate. MS/MS confirmation of F_{420-1} in both negative (643.12811, $[M-H]^-$) and positive (645.27094, $[M+H]^+$) modes. (b) Docking of FMNH₂ and dehydro- F_{420-0} into the crystal structure of FbiB C-terminal domain. The methylene group of the enolpyruvyl moiety sits in a pocket made up of M372 and P289 while the carboxylate hydrogen bonds with R337. The methylene double bond sits planar above the isoalloxazine ring of FMNH₂ at an appropriate distance (3.6 Å, shown by dashed line) and oriented for a hydride transfer to the *Si* face of the methylene bond, accounting for the observed (*S*)-lactyl moiety of F_{420} . (c) *Mtb*-FbiB is a bifunctional enzyme catalyzing the reduction of dehydro- F_{420-0} and its poly-glutamylation to form F_{420-n} .

Fig. 4. Heterologous expression of F₄₂₀ biosynthesis pathway in *E. coli*. (a) Schematic representation of the vector generated for expression of the F₄₂₀ biosynthesis pathway. (b) HPLC-FLD traces of *E. coli* lysates containing F₄₂₀ biosynthesis constructs as well as a purified standard from *M. smegmatis*. (c) Kinetic studies indicate an identical Michaelis constant for FGD as measured with F₄₂₀ purified from *M. smegmatis* (blue) and *E. coli* (red). (d) Fragmentation of F₄₂₀-4 and F₄₂₀-5 extracted from *E. coli* shows a mature F₄₂₀ production.

Fig. 5. The revised bacterial F₄₂₀ biosynthesis pathway. The revised pathway is a modified scheme showing that PEP acts as the substrate for the FbiD/CofC enzymes to produce EPPG or EPPA (in the case of CofC). The immediate reaction product formed from Fo and EPPG/EPPA is dehydro-F₄₂₀-0, which is reduced to F₄₂₀-0 through the newly-described reductase activity of the C-terminal domain of FbiB in mycobacteria. A separate enzyme in archaea and some bacteria is expected to catalyze this reduction step (CofX). FbiB/CofE subsequently adds a poly-γ-glutamate tail to form F₄₂₀. ‘Fbi’ refers to bacterial proteins, whereas ‘Cof’ represents archaeal ones.

Fig. S1. Addition of *L*-glutamate residues to dehydro-F₄₂₀-0. Dehydro-F₄₂₀-1 ([M+H]⁺, monoisotopic *m/z* of 643.40) and dehydro-F₄₂₀-2 ([M+H]⁺, monoisotopic *m/z* of 772.40) are formed upon the addition of one and two *L*-glutamate residues, respectively, to dehydro-F₄₂₀-0.

Fig. S2. Conservation of PEP binding residues between *Mtb*-FbiD and *Mj*-CofC.

Superposition of the *Mtb*-FbiD (wheat) on to that of *Mj*-CofC (green) indicates conservation of the residues in the PEP binding site of both proteins. The only change in the binding site, Gly>Tyr in *Mj*-CofC, is not likely to affect binding considering that the hydrogen bond interaction takes place between the backbone nitrogen atom and the oxygen atom of PEP carboxylate group. Protein side chains are shown in ball-and-stick model and labelled with the corresponding color.

Figure S3. Expression of F₄₂₀ biosynthetic genes in *E. coli*. Whole cell lysates were re-solved on SDS-PAGE, then immunoblotted with anti-FLAG antibodies to detect expression of F₄₂₀. 1) Vector-only control 28 °C; 2) pF₄₂₀-FLAG expressed at 37 °C; 3) pF₄₂₀-FLAG expressed at 28 °C; pF₄₂₀-FLAG expressed at 18 °C.

Figure S4. Spectrophotometric identification of *E. coli*-produced F₄₂₀. UV-Vis scan (red) and fluorescence emission (blue) spectra indicate the characteristic features of F₄₂₀; maximum absorption at 420 nm and maximum fluorescence emission at 480 nm.

Table S1. Primers used in the amplification of the constructs used in this study.

Construct	Primer Sequences (5'-3')		Restriction site
pYUBDuet- <i>fbiD</i>	Forward	CAGGATGGATCCAGTGAGCGGCACACCGG	<i>Bam</i> HI
	Reverse	GTCCCGGAAGCTTCAACGATGTGCGACCGC	<i>Hind</i> III
pYUBDuet- <i>fbiAB</i>	Forward	GAAGGAGATATACATATGAAGGTCACCGTTCTG	<i>Nde</i> I
	Reverse	GCCGGCCTTAATTAATCACTTCAGGATCAG	<i>Pac</i> I
pYUB28b- <i>fbiA</i>	Forward	CAATGACATATGAAAGTTACCGTTCTGGC	<i>Nde</i> I
	Reverse	GCTAGTTATTGCTCAGCG	-
pProEX- <i>fre</i>	Forward	ATTAAATAAGGCGCCATGACAACCTTAAGCTG	<i>Kas</i> I
	Reverse	TAATAAAAGCTTCAGATAAATGCAAACGC	<i>Hind</i> III
pETMCSIII- <i>fbiD</i>	Forward	GTTTAATCGGATCCTAAGGAGGTTAATATTATG	-
	Reverse	GTTAGCAGCCGGATCTATCGATGCATGCCATGG TAC	-

Table S2. Data collection, processing and refinement statistics.

	SeMet-FbiD	Apo-FbiD	FbiD-PEP
Data collection			
Wavelength (Å)	0.97929	0.95370	0.95370
Space group	C222 ₁	C222 ₁	C222 ₁
Cell dimensions			
<i>a</i> (Å)	66.56	66.54	66.17
<i>b</i> (Å)	110.79	109.05	110.44
<i>c</i> (Å)	167.69	166.49	165.91
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution range ^a (Å)	47.17-2.33 (2.41-2.33)	46.92-1.99 (2.04-1.99)	46.84-2.18 (2.25-2.18)
<i>R</i> _{merge} ^a	0.13 (0.37)	0.354 (2.71)	0.213 (2.93)
<i>R</i> _{pim} ^a	0.035 (0.1)	0.095 (0.757)	0.057 (0.827)
Observed reflections ^a	394201 (37101)	621278 (38169)	471504 (35875)
Unique reflections ^a	27060 (2607)	41955 (2818)	32157 (2724)
Multiplicity ^a	14.6 (14.2)	14.8 (13.5)	14.7 (13.2)
Mean I/ σ I ^a	21.3 (8.1)	8.8 (1.2)	14.5 (1.0)
Completeness (%) ^a	99.91 (99.3)	99.85 (96.9)	100.0 (99.7)
CC(1/2) ^b	0.995 (0.97)	0.994 (0.321)	0.998 (0.376)
R _{Cullis} anomalous	0.95		
MapCC ^c (%)	71.25		
Mean FOM	0.697		
Refinement			
PDB code		6BWG	6BWH
Resolution range (Å)		83.25-1.99	82.95-2.18
<i>R</i> _{work} / <i>R</i> _{free} (%)		19.1/22.8	22.8/26.2
Number of atoms (non-hydrogen)			
Protein		4969	4643
Ligand		-	36
Solvent		544	180
R.m.s.d. from ideality			
Bonds (Å)		0.011	0.01
Angles (°)		1.416	1.383
Average <i>B</i> factors (Å²)			
Protein		11.98	24.12
PEP (n=3)		-	48.78
Mg ²⁺ (n=6)		-	44.33
Waters		33.18	48.46
Ramachandran statistics			
Favored (%)		99.18	99.02
Allowed (%)		0.82	0.98
Outliers (%)		00	00
Molprobability score/percentile		1.07/100 th	1.35/99 th

^a Values in parentheses are for the outermost resolution shell.

^b Pearson correlation coefficient.

^c Map correlation coefficient.

Table S3. Kinetic parameters of FGD with F₄₂₀ purified from *M. smegmatis* and *E. coli*.

	$k_{\text{cat (app)}} \text{ (s}^{-1}\text{)}$	$K_{\text{m (app)}} \text{ (}\mu\text{M)}$
<i>M. smegmatis</i>	1.19 ± 0.05	79 ± 11
<i>E. coli</i>	0.46 ± 0.02	77 ± 7