Diversification by CofC and Control by CofD Govern Biosynthesis and Evolution of Coenzyme F$_{420}$ and Its Derivative 3PG-F$_{420}$

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ABSTRACT Coenzyme F$_{420}$ is a microbial redox cofactor that mediates diverse physiological functions and is increasingly used for biocatalytic applications. Recently, diversified biosynthetic routes to F$_{420}$ and the discovery of a derivative, 3PG-F$_{420}$, were reported. 3PG-F$_{420}$ is formed via activation of 3-phospho-D-glycerate (3-PG) by CofC, but the structural basis of substrate binding, its evolution, as well as the role of CofD in substrate selection remained elusive. Here, we present a crystal structure of the 3-PG-activating CofC from *Mycetohabitans* sp. B3 and define amino acids governing substrate specificity. Site-directed mutagenesis enabled bidirectional switching of specificity and thereby revealed the short evolutionary trajectory to 3PG-F$_{420}$ formation. Furthermore, CofC stabilized its product, thus confirming the structure of the unstable molecule and revealing its binding mode. The CofD enzyme was shown to significantly contribute to the selection of related intermediates to control the specificity of the combined biosynthetic CofC/D step. These results imply the need to change the design of combined CofC/D activity assays. Taken together, this work presents novel mechanistic and structural insights into 3PG-F$_{420}$ biosynthesis and evolution and opens perspectives for the discovery and enhanced biotechnological production of coenzyme F$_{420}$ derivatives in the future.

IMPORTANCE The microbial cofactor F$_{420}$ is crucial for processes like methanogenesis, antibiotics biosynthesis, drug resistance, and biocatalysis. Recently, a novel derivative of F$_{420}$ (3PG-F$_{420}$) was discovered, enabling the production and use of F$_{420}$ in heterologous hosts. By analyzing the crystal structure of a CofC homolog whose substrate choice leads to formation of 3PG-F$_{420}$, we defined amino acid residues governing the special substrate selectivity. A diagnostic residue enabled reprogramming of the substrate specificity, thus mimicking the evolution of the novel cofactor derivative. Furthermore, a labile reaction product of CofC was revealed that has not been directly detected so far. CofD was shown to provide another layer of specificity of the combined CofC/D reaction, thus controlling the initial substrate choice of CofC. The latter finding resolves a current debate in the literature about the starting point of F$_{420}$ biosynthesis in various organisms.

KEYWORDS bacterial metabolism, biosynthesis, coenzyme, enzyme catalysis, substrate specificity, X-ray crystallography

Organic cofactors like thiamin pyrophosphate (TPP), flavins (FAD, FMN), or nicotinamides (NAD$,^+$, NAD$^{\text{P}}$) are small molecules other than amino acids that are required for the catalytic activity of enzymes (1). Knowledge about cofactor structure, function, and biosynthesis is therefore crucial to understand the biochemical and physiological processes their
dependent enzymes are involved in and facilitates the biotechnological exploitation of these enzymes. Coenzyme F₄₂₀ is a specialized redox cofactor that was so far mainly identified in archaea and some actinobacteria (2). In archaea, F₄₂₀ is a key coenzyme of methanogenesis (3). In mycobacteria, F₄₂₀ plays a vital role in respiration (4, 5), cell wall biosynthesis (6, 7), as well as the activation of medicinally relevant antimycobacterial (pro-)drugs. For instance, the novel anti-tubercular drug pretomanid is activated by Ddn, an F₄₂₀-dependent nitroreductase (8, 9). In streptomycetes, F₄₂₀H₂ is used for reduction steps during the biosynthesis of antibiotics like thiopепtins (10), lanthipeptides (11), or oxytetracycline (12, 13). Increasing interest in F₄₂₀ is also driven by the utilization of F₄₂₀H₂-dependent reductases in biocatalysis, for example, for asymmetric ene reductions (14–18).

Intriguingly, F₄₂₀ also occurs in a few Gram-negative bacteria where it has been acquired most likely by horizontal transfer of its biosynthetic genes from actinobacteria (19, 20). Initial studies have revealed that F₄₂₀ is indeed produced by some of these organisms but their physiological role remains unknown (20, 21). We have recently identified F₄₂₀ biosynthetic genes in the genome of Mycetohabitans (synonym: Paraburkholderia) rhizoxinica (22), a symbiont that inhabits the hyphae and spores of the phytopathogenic mold Rhizopus microsporus (23–26). Surprisingly, we discovered that the symbiont produced a novel derivative of F₄₂₀, which we termed 3PG-F₄₂₀ (22). The cofactor activity of 3PG-F₄₂₀ was comparable to classical F₄₂₀ and could serve as a substitute for the latter in biocatalysis (22). Although this congener has not been described in any other organism, it could also be detected in the microbiota of a biogas production plant, thus demonstrating that it is not restricted to endofungal bacteria (22). The producers of 3PG-F₄₂₀ in these habitats, however, are unknown. We hypothesize that analysis of organisms that have evolved a derivative of an otherwise conserved cofactor may also harbor unusual enzyme families that utilize this cofactor derivative. These enzymes could have novel activities or substrate specificities and are therefore of potential interest for biocatalysis.

The biosynthesis of 3PG-F₄₂₀ (Fig. 1) is generally similar to the biosynthesis of classical F₄₂₀ (27). The pathway starts with the formation of the redox-active core moiety 7,8-dide-methyl-8-hydroxy-5-deazariboflavin (F₀) from L-tyrosine and 5-amino-6-ribitylamino-uracil, a reactive metabolite of the riboflavin biosynthesis pathway. The F₀ core is then elongated by a chemical group that can formally be described as 2-phospho-L-lactate (2-PL) before an oligoglutamate tail is added. The biosynthesis of the 2-PL moiety has been the subject of several studies. Seminal work on archaea suggested that it is directly formed from 2-phospho-L-lactate: Incubation of cell extracts of Methanosarcina thermophila or Methanocaldococcus jannaschii with F₀, 2-PL, and GTP led to the formation of F₄₂₀ (28). Biochemical assays with purified CofC and CofD finally corroborated the model that the guanylyltransferase CofC catalyzes the reaction of 2-PL and GTP to lactyl-2-phospho-guanosine (LPPG) (29), which is then passed on to CofD to transfer the activated 2-PL moiety onto the precursor F₀. However, the unstable nature of LPPG has prevented confirmation of its structure by NMR or mass spectrometry so far. The last biosynthetic step leading to the mature coenzyme F₄₂₀ is catalyzed by the F₄₂₀-glutamyl ligase CofE (30), which is responsible for the addition of the γ-linked oligoglutamate moiety to the F₀ core, thus forming F₄₂₀ with n indicating the number of glutamate residues.

In mycobacteria, CofE is not a free-standing enzyme but constitutes the N-terminal domain of the FbiB protein (31). It was shown recently that mycobacteria utilize phosphoenolpyruvate (PEP), but not 2-PL, to form F₄₂₀. Instead of LPPG, EPPG is formed, which is converted into dehydro-F₄₂₀ by the action of FbiA, the mycobacterial CofD homolog. DF₄₂₀ is then reduced to classical F₄₂₀ by the C-terminal domain of FbiB, which belongs to the nitroreductase superfamily (32). We have shown that a similar pathway is present in the thermophilic bacterium Thermomicrobium roseum and related species (33). The formation of 3PG-F₄₂₀, however, does not require any reduction step. Instead, enzyme assays revealed that 3-phospho-D-glycerate (3-PG) is activated by CofC, presumably forming the short-lived intermediate 3-(guanosine-5′-diphospho)-D-glycerate (GPPG), which is further transferred to the F₀ core by the action of CofD.
However, it remained elusive, which amino acid residues within the CofC protein conferred the specificity switch toward 3-PG and how genetic mutation might have led to the evolution of 3PG-F420 biosynthesis. Furthermore, the question persisted, why the CofC/CofD reaction only proceeds as a combined reaction and how reactive intermediates like LPPG are stabilized. Another open question concerned the role of 2-PL in the biosynthesis of F420 in archaea. While our previous data (22) matched seminal observations (29) of a substantial turnover of 2-PL by CofC enzymes of archaeal origin, other studies raised doubts that 2-PL is a genuine substrate of archaeal CofC homologs (32).

Here, we present a crystal structure of the 3-PG activating CofC from *Mycetohabitans* sp. B3 and revealed the amino acid residues governing 3-PG activation. By site-directed mutagenesis, we shed light on the evolution of 3PG-F₄₂₀ biosynthesis. Furthermore, we bring to attention that CofC strongly binds its product GPPG and collaborates closely with its partner CofD to control the flux of intermediates into the F₄₂₀ biosynthesis pathway.

**RESULTS**

**Assessment of substrate specificities of CofC enzymes from several organisms.**

To gain a better understanding of CofC substrate specificities, we set out to identify more homologs of CofC accepting 3-PG as a substrate. We reasoned that related bacteria, harboring CofC homologs highly similar to the *M. rhizoxinica* enzyme (*Mrhiz*-CofC),...
would have a similar substrate preference. Indeed, using LC-MS we detected 3PG-F₄₂₀(Fig. 2A/B) in cell extracts of *Mycetohabitans* sp. B3, a close relative of *M. rhizoxinica* that shares the same lifestyle as a symbiont of a phytopathogenic *Rhizopus microsporus* strain. Neither classical F₄₂₀ nor DF₄₂₀ was detectable.

Next, we produced CofC of *Mycetohabitans* sp. B3 (*Myc* B3-CofC, accession number KQH55_09515) as a hexahistidine fusion-protein in *E. coli*, purified the enzyme by Ni-NTA affinity chromatography, and investigated its activity in a combined CofC/D assay using CofD from *Methanocaldococcus jannaschii* (*Mjan*-CofD). When the substrates were provided in equal concentrations in a competitive assay, *Myc*B3-CofC (Fig. 2C) accepted 3-PG (65%), 2-PL (26.5%), and PEP (8.5%), a profile that was similar to the one obtained previously for the *Mrhiz*-CofC (22).

To obtain an overview of the substrate specificities of CofC we re-assessed CofC enzymes from well-studied F₄₂₀ producing organisms such as *Mycolicibacterium smegmatis*, *Thermomicrobium roseum*, and *Methanosarcina mazei* (22) and assayed CofCs from further Gram-negative bacteria like *Paracoccus denitrificans*, *Oligotropha carboxidovorans*, as well as the uncultivable *Candidatus* (*Ca*.) *Entotheonella factor TSY1* that is rich in genes encoding F₄₂₀-dependent enzymes (21). For all CofC-related enzymes analyzed, 2-PL was used most efficiently from all substrates compared. We observed PEP turnover in the range of 3.5% to 30% (Fig. 2C). Generally, it can be concluded that CofC assays cannot discriminate whether 2-PL or PEP is the relevant substrate *in vivo*. The only CofC that accepted 3-PG to a certain extent (8%) was the enzyme from *Ca*. E. factor. However, compared to the *Mycetohabitans* enzyme there was no significant preference of 3-PG over PEP.

**Identification of 3-PG-binding residues of CofC.** Next, we aligned primary amino acid sequences of CofC homologs to identify the residues that might be responsible for the altered substrate preference (Fig. 3A). A crystal structure of FbiD from *Mycobacterium tuberculosis* (*Mtb*-FbiD) in complex with PEP (PDB: 6BWH) showed eight amino acid residues to be in close contact with PEP suggesting a role in conferring the substrate specificity (32). Three of them are aspartate residues (D116, D188, D190) that complex two Mg²⁺ ions which in turn interact with the phosphate group of PEP. The remaining residues were supposed to bind the PEP molecule via side chain atoms (K17, L92, S166) or backbone amino groups (T148 and G163). Although most of these residues were highly conserved, two alignment positions showed a deviation in those residues, namely, L92 and G163 of *Mtb*-FbiD. While L92 is replaced by methionine (M91), the residue corresponding to FbiD-G163 was replaced by serine (S162) in *Mrhiz*-CofC and *Myc* B3-CofC. Homology modeling further suggested...
H145 to be a potentially critical residue for 3-PG binding and C95 to be involved in the correct positioning of M91 (Fig. 3D, Fig. S1 in the supplemental material). Mutagenesis of CofC reveals S162 to be crucial for 3-PG activation. To probe the role of the suggested residues, we performed site-directed mutagenesis in Mrhiz-CofC (Fig. 3B). Especially S162 turned out to be critical for 3-PG activation. While the least

**FIG 3** Residues determining substrate specificities of CofC homologs. (A) Multiple sequence alignment of CofC proteins from selected source organisms. Amino acids of Mtb-FbiD suggested previously to be involved in PEP binding residues (32) are indicated above the identity graph. Residues tested by mutagenesis are shown below sequences. Asterisks: crucial for 3-PG activation in Mrhiz-CofC, double asterisk: enabled 3-PG activation by Msmeg-FbiD. (B) Substrate specificity of Mrhiz-CofC after site-directed mutagenesis. Substitution of S162, C95, and M91 by residues occurring in 2-PL/PEP activating enzymes led to reduction or abolishment of 3-PG activation. (C) Substrate specificity of Msmeg-FbiD after site-directed mutagenesis. Gly169, Leu98, and Thr152 were exchanged by amino acids found in 3-PG activating homologs. While single mutations did not result in 3-PG activation, the combination G169S;T152H enabled 3-PG turnover. Error bars represent the standard deviation of three biological replicates. (D) Homology model of Mrhiz-CofC in complex with GTP (placed by molecular docking) and 3-PG (placed manually). For details, see Fig. S1 in the supplemental material.

H145 to be a potentially critical residue for 3-PG binding and C95 to be involved in the correct positioning of M91 (Fig. 3D, Fig. S1 in the supplemental material). **Mutagenesis of CofC reveals S162 to be crucial for 3-PG activation.** To probe the role of the suggested residues, we performed site-directed mutagenesis in Mrhiz-CofC (Fig. 3B). Especially S162 turned out to be critical for 3-PG activation. While the least
invasive mutation, S162T, retained most of the activity of wild-type CofC toward 3-PG, all other mutants of this residue preferentially turned over 2-PL and, to a lesser extent, PEP. This finding suggested that the hydroxy group present in S162 of WT and S162T might support the recruitment of 3-PG to the active site. M91L displayed reduced activation of 3-PG, while M91A was not impaired in 3-PG activation. Possibly, M91 controls the size of the substrate-binding pocket thus hindering (M91L) or facilitating (M91A) access of the larger substrate 3-PG to the active site. The C95A mutant approximately retained wild-type activity toward 3-PG, while C95L strongly reduced 3-PG activation. This was an indication that C95 might indeed affect the orientation of M91 and as a consequence 3-PG binding. Finally, the proposed interaction of 3-PG with H145 was not reflected in altered specificity profiles of H145A and H145T mutants of Mrhiz-CofC.

**Engineering *M. smegmatis* FbiD into a 3-PG activating enzyme.** Inspired by the finding that S162 of the Mrhiz-CofC is necessary for 3-PG activation we wondered if mutation of the corresponding residue G169 of FbiD to serine (Fig. 3C) could turn FbiD from *M. smegmatis* (*Msme*)-FbiD into a 3-PG activating enzyme, thereby imitating the molecular processes underlying the evolution of 3PG-F420 biosynthesis. The G169S mutant, however, did not accept any 3-PG as substrate. We also mutated L98 to facilitate the entry of 3-PG into the active site. However, neither the single mutant L98C, nor the double mutant G169S;L98C enabled 3-PG binding. Based on the homology model we suspected a residue corresponding to H145 of the *Mrhiz*-CofC might facilitate 3-PG binding. Indeed, while the single mutant T152H did not show any significant effect, the double mutant G169S;T152H successfully turned over 3-PG (19.6%). The triple mutant (G169S;T152H;L98C) resulted in insoluble protein. Overall, these results showed that substrate specificity of *Msme*-FbiD can be readily switched by changing only two residues and again supported a prime role of the critical serine residue for 3-PG recruitment.

**Structural insights into 3-PG activation by CofC.** After several attempts had failed to crystallize recombinant Mrhiz-CofC we turned to MycB3-CofC that was more soluble despite only minor differences in the amino acid sequence (96.8% sequence identity). From diffraction data collected to 2.4 Å the crystal structure could be solved by molecular replacement with a model of two superimposed structures (*Mtb*-FbiD and *Methanosarcina mazei* CofC, *Mmaz*-CofC). The overall structure was similar to the known homologs with the core of the single-domain protein being a six-stranded mixed β-sheet (Fig. 4).

Intriguingly, after initial refinement both molecules in the asymmetric unit independently showed unambiguous difference density for GPPG, the reaction product of GTP with 3-PG, completely immersed into the active site pocket. Each building block of GPPG (i.e., guanine, ribose, phosphate, and glycerate) is bound by several interactions (Fig. 4B, Table S1). Guanine is distinguished from adenine by two H-bond donors to its oxygen (main-chain nitrogen of V65 and P86) and two H-bond acceptors to its amino group (main-chain carbonyl group of E89 and G90). The α- and β-phosphates are bound by two Mg²⁺ ions, which in turn are positioned by three aspartates (D116, D193, D191) similarly as had been shown for FbiD before (32). In the homologous structures the binding site for guanosine and ribose is almost completely conserved. Hence, GPPG is tightly bound, in fact, it turned out to be still quantitatively bound after purification of the protein from *E. coli* cell lysate, where both substrates were present.

The 3-PG moiety is primarily bound by H-bonds with the side chain of S165 to the carboxy group, apparently a highly conserved binding site of the carboxylate of the C3-acid as known from *Mtb*-FbiD (32). More interactions with the carboxy group arise from H-bonds with main-chain nitrogen atoms of T147 and S162, the latter being critical for selective 3-PG activation. Although the mild effect of S162T suggested otherwise, S162 did not interact via its hydroxy group with the ligand. Furthermore, there are H-bonds from the main-chain carbonyl groups of T147 and N148 to the 2-hydroxy group of 3-PG. Surprisingly, these binding partners of the 3-PG hydroxy group are structurally very well conserved and thus not likely to be involved in discrimination between 3-PG and 2-PL/PEP. The residue corresponding to *Mtb*-FbiD-K17 (*Mrhiz*-CofC-K20) did not interact with the substrate however, instead, K26 has taken over its role.
Taken together, the majority of the residues forming the binding pocket residues of the PEP-binding FbiD (32) were also found to be involved in 3-PG binding. Although all C3-acids form the same hydrogen bonds, the carboxylate rotates by 36° about the axis defined by the oxygens. This moves the phosphate by 2–3 Å.

The hydrogen bonds for PEP have a more favorable geometry (average out-of-plane distortion 0.74 Å) than those for 3-PG (1.38 Å) but the phosphate group will not be positioned properly anymore to attack the α-phosphate of GTP. 3-PG with one more bond (carboxylate-C2-C3-O-P compared to carboxylate-C2-O-P in PEP or 2PL) can compensate for the new orientation of the carboxylate. The reason why the 3-PG adopts a new orientation is the main-chain rotation of S162, the CA and CB of which then squeeze the 3-PG into the productive conformation.

Based on the position of GPPG, the binding site for GTP is evident for the GMP moiety. Differential scanning fluorimetry (nano-DSF) measurements further corroborated the direct binding of GTP by CoC (K<sub>d</sub> ~20 μM) (see Text S1, Fig. S3, Table S5 in the supplemental material). The β-phosphate can either bind in the same position as the second phosphate of GPPG or point outward into the solvent close to R28. The latter conformation allows the second substrate 3-PG to bind like PEP in FbiD. GTP and 3-PG are then positioned well for the reaction, the nucleophilic attack of the 3-PG phosphate on the α-phosphate of GTP (Fig. 4D).

Identification of further 3-PG accepting enzymes. After establishing that serine or threonine in the position corresponding to <i>M. hiru-CoFC-S162</i> are linked to 3-PG formation, we hypothesized that the residue might be exploited as a diagnostic residue to identify further 3-PG activating enzymes. Going beyond highly related <i>Mycetohabitans</i> species, which...
can be expected to be 3PG-F₄₂₀ producers, database searches revealed candidate proteins from as-yet uncultivated archaeal species (Fig. S2A in the supplemental material) that contained serine or threonine at the critical alignment position. Since their source organisms were not accessible, we obtained the coding sequences of three of these candidate enzymes as synthetic genes and tested their substrate specificities (Fig. S2B). Interestingly, all of those enzymes accepted 3-PG as substrates. The circumstance that 2-PL was the best substrate of all three enzymes does not rule out the possibility that these enzymes are involved in 3PG-F₄₂₀ formation given the fact that 2-PL is the default case for many enzymes examined in our assay system even if PEP is the natural substrate. Notably, two of the enzymes tested did not accept PEP as a substrate, a rather unusual finding. In the absence of 2-PL, this profile would result in the production of 3PG-F₄₂₀. Taken together, S162 represents a diagnostic residue correlated with specificity or tolerance of CofC toward 3-PG.

Evolution of 3-PG accepting enzymes. To answer the question how 3-PG accepting enzymes might have evolved, we constructed a phylogenetic tree of CofC/FbiD enzymes examined in this study (Fig. 5). The *Mycetohabitans* CofC clade branched off early in the evolution of bacterial CofC enzymes and is neither closely related to nor derived from actinobacterial CofC/FbiD nor to other CofC enzymes found in Gram-negative bacteria. The archaeal 3-PG tolerating enzymes represent a monophyletic clade within the archaeal proteins. Taken together, we conclude that 3-PG preference evolved once in evolution while 3-PG tolerance originated at least twice from an ancestral 2-PL/PEP activating enzyme.

Role of CofD in substrate specificity of F₄₂₀ side chain biosynthesis. After gaining insights into the substrate specificity of CofC a few questions remained. For instance, in almost all CofC homologs tested, 2-PL was the preferred substrate. This finding contrasted previous results for *Mtb*-FbiD and CofC from *M. jannaschii* (*Mjan*-CofC), which was reported to accept exclusively PEP (32). Furthermore, the residual activity of *Mrhiz*-CofC and *MycB3*-CofC toward PEP suggested the PEP-derived DF₄₂₀ to be formed as a side product, while DF₄₂₀ was not found in their source organisms (22). We therefore assumed that the choice of the CofD homolog used in the combined CofC/D assay might have an impact on the overall outcome of the assay.
To test this hypothesis, we produced CofD homologs of several model species as hexahistidine-fusion proteins and performed CofC/D assays using several combinations of CofC and CofD. Strikingly, the choice of CofD homologs had a significant influence on the product spectrum of the CofC/D pair (Fig. 6). For instance, when Mrhiz-CofC and MycB3-CofC were combined with their cognate CofD, the apparent substrate specificity shifted almost completely toward 3-PG. The PEP- and 2-PL-derived products were only produced in traces by the CofC/D reaction. Similarly, when Msmeg-FbiD was combined with its natural partner Msmeg-FbiA instead of the homolog encoded in M. jannaschii (Mjan-CofD), the apparent activity toward 2-PL was almost entirely abolished. Obviously, CofD homologs have the ability to select between the pathway intermediates LPPG (2-PL-derived), EPPG (PEP-derived), and GPPG (3-PG-derived). In bacterial systems, CofD/FbiA appears to favor the intermediate that is known to be relevant in their source organisms, i.e., EPPG for Mycobacteria or GPPG for M. rhizoxinica/Mycetohabitans sp. B3. The archaeal Mjan-CofD, however, appears to prefer its natural substrate LPPG but displays relaxed specificity toward EPPG and GPPG.

To clarify, whether CofCs that tolerate 3-PG to a certain extent could be involved in 3PG-F420 biosynthesis, we reassessed CofCs from Ca. E. factor, Ca. H. archaeon, and Archaeon GBE54128 together with their cognate CofDs (Fig. 6 D–F). However, the combined CofC/D pairs did not turn over any 3-PG. The archaeal CofC/D pairs were highly specific for 2-PL. Intriguingly, the Ca. E. factor CofC/D pair gained significantly higher preference for PEP than shown in the standard assay, suggesting that Ca. E. factor might produce F_{420} via DF_{420}.

**DISCUSSION**

**Structural basis of CofC specificity.** Extensive characterization of various CofC homologs, mutagenesis studies, and crystallography enabled us to spot residues responsible for the unusual substrate choice of CofC from Mycetohabitans. The crystal structure obtained from MycB3-CofC revealed that most of the amino acid positions described for PEP-binding Mtb-FbiD play a role in 3-PG binding as well. However, rather than specific interactions with the free 2-hydroxy moiety, it is the conformation of S162 that forces the substrate into a position from which only the larger substrate 3-PG can undergo productive reaction of its phosphate group with GTP. The effect of M91 and C95 on substrate specificity shows that indirect influences on the overall conformation of the active site can be crucial for the correct positioning of the substrate. Nevertheless, the S162 residue proved as diagnostic residue correlated with tolerance against 3-PG and even enabled engineering of Msmeg-FbiD into a 3-PG activating enzyme. Notably, a bidirectional change of the substrate specificity, i.e., from 3-PG to 2-PL/PEP in Mrhiz-CofC as well as from 2-PL/PEP toward 3-PG in Msmeg-FbiD FbiD as described here is a rather exceptional achievement.

**Evolution and occurrence of 3PG-F_{420} in nature.** Interestingly, our mutagenesis study answers the question of how 3PG-F_{420} might have originated via mutation of 2-PL/PEP activating CofC on a molecular level. The phylogenetic tree suggests that 3-PG activating enzymes have evolved from an ancestral 2-PL/PEP activating CofC. Since DF_{420} is less stable than saturated forms, we suppose that the metabolic switching event has occurred to enable the formation of a stable F_{420}-derivative in a metabolic background that lacked 2-PL or the DF_{420} reductase that is present in Actinobacteria (32) and Thermomicrobia (33). Here, we showed that the exchange of two amino acids in CofC/FbiD is mainly affecting substrate specificity and is thus sufficient to mimic this evolutionary process in the laboratory. Considering that 3PG-F_{420} was detectable in biogas-producing sludge (22), there must be microorganisms outside the monophyletic clade of endofungal bacteria (Mycetohabitans) that produce 3PG-F_{420}. Efforts to isolate further 3PG-F_{420} producers remain ongoing.

**CofD influences substrate specificity of the CofC/D pair in vivo and in vitro.** Another important key finding of this study is that CofC and CofD together contribute to the substrate specificity of the combined reaction, where CofD of some species seems to represent a restrictive filter that acts after the more promiscuous CofC. This finding can explain the before-mentioned inconsistencies between results obtained in vivo and in vitro.
Even more importantly, we can now resolve the discrepancies concerning the results of CofC/D assays performed with CofC from archaea existing in the literature. Seminal work identified 2-PL to be a suitable substrate of archaeal CofC enzymes and thus proposed the original biosynthetic pathway to start from 2-PL (29). In contrast, a more recent study did not observe any turnover of 2-PL neither using the archaeal CofC from *M. jannaschii*, nor using FbiD from *M. tuberculosis* and suggested a biosynthetic route starting from PEP via EPPG, even for archaea (32). A follow-up study delivered further evidence supporting the

FIG 6 Influence of CofD/FbiA on the biosynthesis of F_{420} derivatives. (A) CofC from *M. rhizoxinica*, (B) CofC from *Mycetohabitans* sp. B3, and (C) FbiD from *M. smegmatis*, as well as (D) Ca. E. factor, (E) Ca. H. archaeon, and (F) Archaeon GBE54128 were tested with either *M. jannaschii* CofD (standard assay) or together with their cognate CofD enzyme from the same source organism. (G) Schematic drawing illustrating the channeling of the labile metabolites LPPG, EPPG, GPPG from CofC to CofD. While CofC selects primary substrates, CofD is able to select between these intermediates. For abbreviations see Fig. 1.
biosynthetic route via DF$_{420}$ in mycobacteria. The authors showed the formation of DF$_{420}$ from PEP in cell extracts of *M. smegmatis* and could reveal strong binding of DF$_{420}$ to the active site of FbiA (CofD) by X-ray crystallographic studies of the enzyme (34).

Our previous study (22), however, found 2-PL to be the best substrate of the *M. jannaschii* CofC enzyme, a result that we could reproduce here using CofC from *M. mazei*. It is also confusing that even the *M. smegmatis* FbiD tested in this study preferred 2-PL as substrate, again challenging the hypothesis that PEP is the preferred substrate of FbiD.

The solution to this perplexing situation comes with the herein defined influence of CofD/FbiA on the overall specificity of the CofC/D pair. CofD/FbiA selects between the unstable pathway intermediates LPPG, EPPG, and GPPG (27). According to our results, *Msmeg*-FbiA exclusively accepted EPPG to form DF$_{420}$. Bashiri et al. used the closely related *Mtb*-FbiA to perform CofC/D assays (32). Since *Mjan*-CofC can accept PEP as a minor substrate when combined with its cognate CofD (22), the assay resulted exclusively in DF$_{420}$ formation when combined with FbiA, thus erroneously suggesting that 2-PL was not accepted by CofC. Similarly, the activation of 2-PL by FbiD remained undetected when assays were carried out with FbiA as a partner enzyme. Conversely, it is plausible that the unexpectedly high turnover of 2-PL observed in all our assays performed with *Mjan*-CofD might be an artifact caused by the choice of a CofD homolog that might preferably turn over its natural substrate LPPG. For future studies toward the biosynthesis of novel F$_{420}$ derivatives we, therefore, suggest that only a combination of CofC and its cognate CofD is suitable to reflect the *in vivo* situation. We also conclude that combining compatible CofC/D pairs will be beneficial for biotechnological production of F$_{420}$.

The combined CofC and CofD reaction. The X-ray structure of *Mycetohabitans* B3-CofC presented here included the reaction product GPPG, while the previous crystal structure of FbiD was obtained in the presence of PEP only (32). This is the first direct analytical evidence for the labile reaction product GPPG. So far, the existence of its congener LPPG was confirmed by chemical synthesis followed by successful turnover by CofD (35). The fact that GPPG remains tightly bound to the enzyme might point to a substrate-channeling mechanism where the GPPG molecule is directly transferred to CofD to avoid degradation of the labile intermediate in the absence of F$_{420}$. Product inhibition could also explain why any attempt to measure the activity of CofC in the absence of CofD remained unsuccessful (27, 32) and why direct detection of GPPG or the related LPPG and EPPG from solution has failed so far.

The binding mode of GPPG also clearly revealed the GTP binding site of CofC. Notably, no evidence for GTP binding could be obtained experimentally for *Mtb*-FbiD and it was even speculated that GTP binding might require the presence of FbiA (32). This mechanism could be disproved for CofC of *M. rhizoxinia*.

Conclusion. Taken together, this study represents a significant advance in understanding the flexibility of substrate specificity in CofC homologs and offers a molecular model for the evolution of 3PG-F$_{420}$. By direct detection of the unstable reaction product GPPG via X-ray crystallography we gained insights into the structural basis of the combined CofC/D reaction. The demonstration that CofC and CofD cooperate closely to control the entry of central carbon metabolites into the biosynthetic route to F$_{420}$ derivatives also solved an ongoing debate in the literature and thereby reestablished 2-PL as the most likely starting point of F$_{420}$ biosynthesis in archaea. One important practical conclusion of this work is the suggestion that CofC/D assays should always be performed using homologs from the same source organism to better reflect the *in vivo* situation. Future perspectives are opened up to investigate the cooperation of biosynthetic enzymes on a molecular level and to exploit this knowledge gained here for enhanced biotechnological production of coenzyme F$_{420}$ and potentially novel derivatives.

**MATERIALS AND METHODS**

**Chemicals and microbial strains and metabolite extraction.** Chemicals and media components were purchased from Acros Organics, Sigma-Aldrich, Alfa Aesar, Carl Roth, and VWR. The *E. coli* strains BL21(DE3), LOBSTR-BL21 (Kerafast), ETH101 and Top10 were grown routinely in Lysogeny Broth (LB). Axenic *Mycetohabitans* sp. B3 (NCBI genome accession JAHLKN000000000) was grown in 50 ml MGY media (22) at 30°C at 110 rpm for 1 week. Cultures (OD$_{600}$ = 2.5) were lyophilized, extracted with 10 ml...
ice-cold methanol, sonicated for 20 min and incubated at 250 rpm for 60 min. Cell debris was removed by centrifugation (4°C, 10 min, 10000 rpm) and the supernatant was filtered into a round-bottom flask. The extract was dried in a vacuum rotary evaporator at 40°C and re-dissolved in 1 ml LC-MS-grade water. Samples were analyzed using LC-MS as described before (22).

**Construction of expression vectors.** Unless stated otherwise, primers (Table S2A in the supplemental material) were designed using the software tool Geneious (36) and cloning was based on DNA recombination following the Fast Cloning protocol (37). *E. coli Top10* strain was used to propagate plasmids. PCRs were carried out using Q5 High-Fidelity polymerase (New England Biolabs) and oligomers used for amplifications listed in Table S1. Constructed plasmids (Table S2B) were confirmed by Sanger sequencing (Eurofins Genomics). CoFC and CoFD encoding plasmids (pMH04, 05, 10, 18, 19, 20, 43, 56, 57, 58, 59, 60, 89, 90 and 91) were purchased from BioCat as codon-optimized synthetic gene constructs cloned into pET28a + between BamHI and HindIII restriction sites. Plasmids pMH43 (pACYC-Duet backbone), pMH59 (pET28), and pMH60 (pET28) were obtained by gene synthesis (BioCat), sequences are provided in the Supplemental Material (Text S2).

**Site-directed mutagenesis.** Putative substrate-binding residues of CoFC were subjected to site-directed mutagenesis on DNA level using PCR (38). Amino acid numbering corresponds to the original residue position in the native CoFC protein.

To mutate CoFC from *M. rhizoxinica* (pFS03) at position 162 (S162), primer pairs oMH03/34 or (oMH37/38) or (oMH45/46) or oMH47/48 were used to obtain G169S (pMH32), G169Y (pMH33), G169A (pMH34) or G169E (pMH35), respectively. Primer pairs oMH108/109 were used to substitute leucine (L98) with cysteine (L98C, pMH70). The double mutants G169S; L98C were obtained from plasmid pMH24 by amplification with primer pairs oMH110/111 and oMH124/125. The triple mutation was introduced in the pMH69 plasmid using primer pairs oMH116/117 (pMH74) or oMH118/119 (pMH75). Methionine at position 91 was substituted with alanine (M91A) or leucine (M91L) to yield pMH76 and pMH77.

**Heterologous protein production and purification.** Production condition and purification of all N-terminal hexahistidine (N-His6) tagged proteins (CoFC and CoFD) were similar as described before (22). Accession numbers of native CoFC/FbiD and CoFD (accession numbers of native CoFC/FbiD and CoFD) were listed in Table S3. Primers (Table S2A in the supplemental material) were used for amplification of histidine at position 145 either to alanine (H145A) or threonine (H145T) was obtained using primer pairs oMH116/117 (pMH74) or oMH118/119 (pMH75). Methionine at position 91 was substituted with alanine (M91A) or leucine (M91L) to yield pMH76 and pMH77.

**Combined CoFC/D assay.** Distinct derivatives of F$_420$-0 were produced via biochemical reaction of purified CoFC and CoFD proteins in a combined assay (22, 29). In *vivo* reaction conditions were analogous to Braga et al. (22) and 50 µl reaction consisted of 100 mM HEPES buffer (pH 7.4), 2 mM GTP, 2 mM MgCl$_2$, 0.14 mM F$_420$, 34 µM CoFD, and 0.5 µM of substrates (3-phospho-D-glyceric acid, phosphoenolpyruvic acid, and 2-phospho-L-lactate). The reactions were initiated upon the addition of 26 µM CoFC. Reactions were quenched with one volume of acetonitrile and formic acid (20%). Production of F$_420$-0 derivatives was monitored in LC-MS. Technical set up, method, conditions for LC-MS analysis were similar as described before (22). Data analysis followed extraction of ion chromatograms (XICs), calculation of area under the curve (AUC), normalization of AUC, plotting area against time, and product formation was calculated for a linear time range (0 to 20 min). Quantification of relative protein formation was determined from three biological replicates (n = 3) and plotted as bar charts. Standard deviations (SD) were used as error bars.

**CoFC sequence alignment and phylogenetic tree inference.** Multiple protein sequences of CoFC from different F$_420$ producing organisms (Table S3) were retrieved from the NCBI database and primary sequences were aligned based on their predicted structure using Expresso (T-Coffee) (39). For phylogenetic tree inference as implemented in Geneious Prime (36), the MUSCLE algorithm (40) was used to align sequences and a maximum Likelihood tree was inferred using PhyML 3.0 (41) with the LG model for protein evolution and a gamma distribution of rates. Support values (Shimodaira-Hasegawa-like branch test) were computed and are shown above branches. Trees were visualized in Geneious Prime.

**Structural modeling.** The Phyre2.0 web portal was used to obtain a structural model for *M. rhizoxinica* CoFC (PDB: 6BWW) (42). This enabled the identification of three enzymes (PDB: 3GKX, PDB: 2I5E, PDB: 6BWH) that were used as a template for structural modeling resulting in models with 100% conformational identity. The Phyre2.0 web portal was used to obtain a structural model for *M. rhizoxinica* FbiD (PDB: 6BWW) (42) that were used as a template for structural modeling resulting in models with 100% conformational identity. The Phyre2.0 web portal was used to obtain a structural model for *M. rhizoxinica* FbiD (PDB: 6BWW) (42). This superimposed a total of 189 residues with an RMSD of 1.2 Å in the backbone.
1.687 Å. FbiD binds to two Mg$^{2+}$ ions that are important for catalysis and PEP binding (32). To place the Mg$^{2+}$ ions in CoF, the CoF model was superposed on FbiD using residues surrounding the Mg$^{2+}$ binding site. This model was used as a template for molecular docking of GTP into the CoF model using AutoDoc Vina. The input PDBQT files for AutoDoc Vina were generated with AutoDoc Tools V1.5.6 (46). The PRODRG server was used to generate the three-dimensional coordinates for GPPG from two-dimensional coordinates (47). The 3-PG was manually modeled in CoF-GTP using COOT (48). Representations of structures were prepared using PyMOL Molecular Graphics System (Schrödinger, LCC). The Adaptive Poisson-Boltzmann Solver (APBS) electrostatics plugin in PyMOL was used for the electrostatic surface representation (49).

Crystallography and data collection. *Mycetohabitans* B3 CoF was further purified on a size exclusion column (Superdex75, 16/600, Cytiva) and concentrated in 50 mM Tris pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$, 2 mM mercaptoethanol (SEC buffer) to 8.2 mg/ml. Sitting drop crystallization trials were set up with screens Wizard I and II (Rigaku), PEG/Ion (Hampton Research), and JBScreen (Jena Bioscience) using 0.3 M glucose solution and 0.3 M reservoir. Crystals appeared after 2 weeks with reservoir 10% PEG 3000, 200 mM MgCl$_2$, 100 mM sodium cacodylate pH 6.5. After briefly soaking in a reservoir with 20% glucose added crystals were cryocooled in liquid nitrogen. Diffraction data were collected at BESSY, beamline 14.1. Data collection parameters are given in Table S4 in the supplemental material. Data sets were processed with XDSAPP (50).

Structure solution and refinement. Programs used for this part were all used as provided by CCP4 (45). Sequence search against the PDB revealed structures of two related proteins, FbiD from *Methanosarcina mazei* with sequence identities of 30%. Molecular replacement by Phaser with 6BWG (Mtb-FbiD) or 215E (Mmaz-CoF) did not solve in the automatic mode, an assembly of both structures superimposed and truncated (residues 9–82, 91–173, 178–211 of monomer A of 6BWG and aligned residues of monomer A of 215E) solved the phase problem with an LLG of 233. The 48WG structure was used as starting model for replacing the sequence with the *Mycetohabitans* sequence, one round of automatic model building with BUCANEER (51) and iterative rounds of refinement with REFMACS (52) and manual model building with COOT (48) completed the two protein chains.

Unambiguously water molecules were added when R$_{free}$ reached 0.315 and a GDP moiety with two Mg$^{2+}$ ions was built into the difference density in the active site. Pertaining difference density was connected to the $\beta$-phosphate suggesting a covalently bound PEP or 3-PG. Only the latter refined without residual difference density above $\pm 2\sigma$. For final refinement, non-crystallographic symmetry was not used. TLS refinement was applied with one group per monomer. Refinement statistics are given in Table S4.

The C3-acid substrates are fixed in the active site by three H-bonds of their carboxylate group. For H-bonds to $\pi$-systems (peptide bond, carboxylate group) the partner should lie in the same plane. To characterize deviations from favorable H-bonding geometry we calculated the average distance of the partner to the $\pi$-plane.

Data deposition. The crystal structure of CoF from *Mycetohabitans* sp. B3 was deposited at the protein database PDB (PDB code: 7P97).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.

TEXT S2, DOCX file, 0.02 MB.

FIG S1, TIF file, 2.5 MB.

FIG S2, EPS file, 2.1 MB.

FIG S3, EPS file, 1.4 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.03 MB.

TABLE S3, DOCX file, 0.02 MB.

TABLE S4, DOCX file, 0.02 MB.

TABLE S5, DOCX file, 0.02 MB.

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M.H., D.B. (cloning, CoF/D assays, mutagenesis, LC-MS analysis), and D.L. (diagnostic sequence analysis) performed research and analyzed data and contributed to writing.
the manuscript. I.R. performed research (cultivation and metabolite extraction). S.S. and L.B. performed research (crystallization, GTP binding assays). G.J.P. and M.L. analyzed data (X-ray crystallography, structural modeling, GTP binding assays) and contributed to writing the manuscript. G.L. performed research (phylogenetic reconstruction), designed the study together with M.L., and wrote the manuscript.

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