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Biosynthetic Versatility and Coordinated Action of 5′-Deoxyadenosyl Radicals in Deazaflavin Biosynthesis

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Supporting Information

ABSTRACT: Coenzyme F420 is a redox cofactor found in methanogens and in various actinobacteria. Despite the major biological importance of this cofactor, the biosynthesis of its deazaflavin core (8-hydroxy-5-deazaflavin, Fo) is still poorly understood. Fo synthase, the enzyme involved, is an unusual multidomain radical SAM enzyme that uses two separate 5′-deoxyadenosyl radicals to catalyze Fo formation. In this paper, we report a detailed mechanistic study on this complex enzyme that led us to identify (1) the hydrogen atoms abstracted from the substrate by the two radical SAM domains, (2) the second tyrosine-derived product, (3) the reaction product of the CofH-catalyzed reaction, (4) the demonstration that this product is a substrate for CofG, and (5) a stereochemical study that is consistent with the formation of a p-hydroxybenzyl radical at the CofH active site. These results enable us to propose a mechanism for Fo synthase and uncover a new catalytic motif in radical SAM enzymology involving the use of two 5′-deoxyadenosyl radicals to mediate the formation of a complex heterocycle.

INTRODUCTION

Coenzyme F420 (4) is a redox cofactor found in methanogens and in various actinobacteria, while its biosynthetic precursor Fo (8-hydroxy-5-deazaflavin, 3) can also be found in certain cyanobacteria and eukaryotes.1−4 F420 was first isolated from Methanobacterium strain M.o.H. as a fluorescent cofactor involved in hydrogen metabolism and has subsequently been shown to be a key cofactor in methanogenesis.5−7 F420 is required for the breakdown of aflatoxin in Mycobacterium smegmatis.8 In addition, M. tuberculosis, the etiologic agent of tuberculosis, is predicted to contain a large, yet unexplored, number of F420-dependent enzymes, some implicated in nitrosative stress resistance.9,10 F420 is biosynthesized in Methanocaldococcus jannaschii by the action of eight enzymes with the formation of the deazaflavin chromophore (Fo) as the remaining unsolved step (Figure 1).11−17

Despite the major biological importance of this cofactor, the biosynthesis of its deazaflavin core (Fo) remains only partially understood. The formation of FO is mediated by two separate radical SAM active sites, one each in the CofG and CofH enzymes or both in the FbiC enzyme. These two radical SAM domains constitute the functional domains of Fo synthase as we recently demonstrated.18 While two [4Fe-4S] clusters have been found in other systems (MoaA, AlbA, HydG),19−24 Fo synthase is an unusual multidomain radical SAM enzyme in that it uses two separate 5′-deoxyadenosyl radicals to catalyze Fo formation.18

We recently reconstituted the Fo synthase and identified diaminouracil (5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidine-dione) 2 and tyrosine 1 as the enzyme substrates.18 The detection of diaminouracil 2 bound to freshly purified CofH suggested that CofH catalyzes the early steps in deazaflavin formation. The tyrosine lyase activity of Fo synthase is likely to be mechanistically analogous to the tyrosine lyase activity of HydG (involved in [FeFe]-hydrogenase maturation)22 and ThiH.
involved in anaerobic thiamin thiazole biosynthesis). 25 A recent structure of tryptophan lyase (NosL) demonstrated that the initial hydrogen atom abstraction was occurring from the amine NH,26 suggesting that the corresponding tyrosine lyases are also likely to occur by hydrogen atom abstraction from the amino group rather than the previously assumed phenolic hydroxyl. Based on these data, we elaborated the mechanism shown in Figure 2 as a starting point for our mechanistic investigations on Fo synthase.

In this paper, we report a detailed mechanistic study on this complex enzyme that led us to identify (1) the hydrogen atoms abstracted from the substrate by the two radical SAM domains, (2) the second tyrosine-derived product, (3) the reaction product of the CofH-catalyzed reaction, (4) the demonstration that this product is a substrate for CofG, and (5) a stereochemical study that is consistent with the formation of a \( \alpha \)-hydroxybenzyl radical at the CofH active site. Our study elucidates a new catalytic motif in radical SAM enzymology involving the use of two \( 5' \)-deoxyadenosyl radicals to mediate the formation of a complex heterocycle.

## RESULTS

### Identification of the Two Sites of Hydrogen Atom Abstraction.

Reconstitution of the CofG/CofH-catalyzed reaction in the presence of D\(_7\)-tyrosine resulted in the incorporation of a single deuterium atom into 5\( ' \)-deoxyadenosine as judged by mass spectrometry (MS) analysis, which showed an increase in the intensity of the 253.1 peak relative to the 252.1 peak (48.6% vs 13.4% in the reaction performed with D\(_7\)-tyrosine compared with unlabeled substrate, respectively, Figure 3a,b, left panel). MS analysis of the \( F_e \) formed from D\(_7\)-Tyr showed that it contained four deuteriums (Figure 3b, right panel). Reconstitution of the CofG/CofH reaction with other isotopologues of tyrosine (\([2-D_1]\)-, \([3,3-D_2]\)-, \([2',6'-D_2]\)-, and \([3',5'-D_2]\)-Tyr) localized the site of deuterium abstraction to the \( \beta \)-carbon of Tyr since deuterium incorporation in 5\( ' \)-
deoxyadenosine was detected only when using D7- or 3,3-D2-Tyr as substrate (Figure 3c).

MS analysis of the F3o formed in the reaction with [3,3-D2]-Tyr demonstrated that the second β-deuteron was retained in F3o. Using [3′,5′-D2]-Tyr led to the incorporation of both deuterium atoms into F3o, while only one was incorporated when using [2′,6′-D2]-Tyr as substrate (Supplementary Figure 1). Conversely, no deuterium incorporation into the products was observed with [2-D1]-Tyr (Supplementary Figure 1). This confirmed that, in addition to one deuterium on the 3-position, a deuterium from a second nonexchangeable site (i.e., 2′/6′ position) is removed. Similar results were obtained using the full length FbiC enzyme demonstrating that both types of F3o synthase use an identical mechanism.

The observation that deuterium transfer from a second site on stably labeled tyrosine to the S′-deoxyadenosyl radical was not occurring suggested that the second hydrogen atom transfer was from an exchangeable site. This was confirmed by demonstrating the incorporation of deuterium from solvent into S′-deoxyadenosine when the CoFH reaction was run in a buffer containing 80% D2O. In addition, noticeable peaks corresponding to [S′,S−2′]-S′-deoxyadenosine and [S′,S−2′]-S′-deoxyadenosine were observed in the mass spectrum (Figure 4). The residual 252.1 peak in spectrum a is most likely due to the uncoupled formation of S′-deoxyadenosine and to the presence of H2O in the reaction mixture.

Figure 4. CoFH-catalyzed deuterium incorporation into S′-deoxyadenosine in reactions run in 80% D2O containing SAM, diaminouracil and methyl viologen. (a) Reaction run in the presence of tyrosine showing an enhanced 253.1 peak (calculated to be 69.4, 21.5 and 16.7% for the [M + D + H]+, [M + D2 + H]+, and [M + D3 + H]+, respectively, in panel a and 30.8% for the [M + D + H]+ in panel b). (b) Reaction run in the absence of tyrosine showing the uncoupled production of S′-deoxyadenosine. S′-deoxyadenosine has an expected [M + H]+ of 252.1 m/z with the 253.1 peak calculated to be 12.9 ± 3%.

Characterization of the Second Tyrosine-Derived Product. Glyoxylate, derived from the hydrolysis of the glycine imine 6, was the most likely second product resulting from the fragmentation of the tyrosyl radical 5. Our initial attempts to trap glyoxylate as the 2-quinoxalinol 17, by treating the reaction mixture with o-phenylenediamine, failed because the glycrol used to stabilize the enzyme contained relatively large quantities of glyoxylate as an impurity. We therefore performed the reaction using [15N,13C9]-Tyr, which allowed for the selective LC-MS detection and quantitation of enzymatically produced glyoxylate (Figure S). Comparison to standard curves showed that the glyoxylate:F3o ratio was 1.1:1. We were able to detect glyoxylate only in the presence of CoFH (Figure S), further supporting the proposal that CoFH is the enzyme responsible for the tyrosine cleavage reaction.

Identification of the CoFH Reaction Product. HPLC analysis of the CoFH reaction mixture revealed the formation of a peak eluting at 11.2 min. This peak appeared only in reaction mixtures containing CoFH, I, 2, SAM and reduced methyl viologen (or dithionite) (Figure 6). LC-MS analysis yielded a protonated molecular ion at 383.1573 m/z when the reaction was reconstituted in the presence of tyrosine and 390.1775 m/z when the reaction was reconstituted in the presence of [15N,13C9]-Tyr (Figure 7). This demonstrated that CoFH catalyzed the formation of a product containing seven tyrosine-derived carbons and suggested a molecular formula of C16H22N4O7 ([M+H]+ calc 383.1561, 3.3 ppm error). CID fragmentation of the unlabeled product resulted in the formation of product ions at m/z 107.1 and 277.1, while the labeled product showed product ions at m/z 114.1 and 277.1 (Figure 7). This suggested that the smaller fragment contained all the tyrosine-derived carbon atoms (i.e., 7 carbon atoms) while the 277.1 fragment originated from diaminouracil.

The CoFH reaction product was then produced on a larger scale, purified by HPLC and analyzed by nuclear magnetic resonance (NMR) spectroscopy. The 1H NMR spectrum had multiple signals in the 3–3.8 ppm region consistent with protons attached to oxygen-bound carbons, and two doublets at 2.87 ppm suggestive of a benzylic methylene group. Using 2D NMR techniques (1H−1H COSY, 1H−13C HSQC, and 1H−15C HMBD), the CoFH reaction product was unambiguously identified as compound 9 (Supplementary Figures 2–10).

To confirm that 9 was an intermediate and not a shunt or a decomposition product, we produced D7-9 on a large scale using CoFH and [3,3-D2]-Tyr. Treatment of the HPLC purified compound with reduced CoG resulted in the formation of F3o. MS analysis of the reaction products demonstrated deuteration incorporation into S′-deoxyadenosine and F3o (Figure 8). The mixture of m/z 364/365 in the mass spectrum of F3o, is due to the nonenzymatic reduction of [5-D2]−F3o by dithionite followed by nonenzymatic aerobic oxidation after removal of the reaction mixture from the anaerobic chamber as was previously observed during the initial characterization of F420/F3o.

Identification of the Reaction Product of a FbiC Variant. Disruption of the conserved CXXXXXCC motifs of FbiC individually resulted in the variants FbiC-C1 (cluster is disrupted in the CoG homologous domain) and FbiC-C2 (cluster is disrupted in the CoFH homologous domain). Both
protein variants had all three cysteine residues substituted with alanine residues, thereby abolishing the [4Fe-4S] cluster. Incubation of each variant with tyrosine, diaminouracil, SAM, and flavodoxin/flavodoxin reductase led to the identification of a new peak only in the FbiC-C1 reaction (Figure 9). The intermediate was subjected to LC-MS/MS analysis and had an identical retention time, protonated ion and MS2 fragmentation pattern to intermediate 9 generated by CofH.

Mixing of FbiC-C1 and FbiC-C2 resulted in the formation of Fo (Figure 10) demonstrating that both variants contain a successfully reconstituted [4Fe-4S] cluster and are fully active. Interestingly, this confirmed that a stable diffusible reaction intermediate is released from one radical SAM domain (CofH or the C-terminal part of FbiC) to the other one (CofG or the N-terminal part of FbiC). Surprisingly, the rate of product formation for the wild-type enzyme (25 μM enzyme = 50 μM [4Fe-4S]) was similar to the rate of product formation for the mixture of the two mutated enzymes (50 μM each = 50 μM [4Fe-4S]). This suggests that FbiC catalyzes two independent reactions where intermediate 9 is not transferred directly from the C-terminal domain to the N-terminal domain but most likely diffuses from one active site to the other.

Stereochemistry of the C3 Hydrogen Atom Abstraction from Tyrosine. MS analysis of the product generated by treating [2,3-D2,3S]-Tyr or [3-D,3R]-Tyr with reduced CofG/CofH demonstrated the formation of deazaflavin as a 1:1 mixture of Fo and D1-Fo. This product ratio was independent of the stereochemistry at C3 of the starting tyrosine (Figure 11b,c) demonstrating that the stereochemical information at this carbon is lost during the formation of Fo. A control experiment using [3,3-D2]-Tyr demonstrated that Fo reduction/oxidation was not occurring under the reaction conditions (Figure 11a).

**DISCUSSION**

Fo synthase catalyzes the reductive condensation of tyrosine 1 and ribityl-diaminouracil 2 in a reaction catalyzed by two radical SAM enzymes (CofG and CofH) or the two-domain enzyme FbiC. The central mechanistic question for this reaction is how two highly reactive 5′-deoxyadenosyl radicals cooperate to form the deazaflavin chromophore. A mechanistic proposal is outlined in Figure 2. In this paper, we report the isolation and characterization of the CoH reaction product and describe a series of experiments that enable us to test this mechanistic hypothesis.
Most radical SAM enzymes use the S′-deoxyadenosyl radical to abstract a hydrogen atom from the substrate (Dph229 and MqnE30 are exceptions). Since Fbi synthase uses two S′-deoxyadenosyl radicals, two hydrogen atom abstractions are likely to occur during Fo formation. We determined that one of these takes place at the C3 position of tyrosine by characterizing the S′-deoxyadenosine produced in the CoG/CofH reaction using various deuterated tyrosine isotopologues (Figure 3). This experiment also demonstrated the loss of a single deuterium in the Fo produced from [2′,6′-D2]-Tyr (Supplementary Figure 1d) as expected due to C–N bond formation at C2′/6′ of tyrosine.

Failure to observe a second hydrogen atom transfer from the stable deuterated tyrosine isotopologues suggested that the second hydrogen atom abstraction was occurring from an exchangeable position on tyrosine 1 or dianimouracil 2. Consistent with this prediction, treatment of unlabeled substrates 1 and 2 with CofH in D2O buffer resulted in deuteration transfer to S′-deoxyadenosine (Figure 4). Phylogenetic analysis of HydG, ThiH, CoF, and CoG with Clustal Omega33 shows that CoF clusters with ThiH and HydG with CofG present as an out-group (Supplementary Figure 11). This sequence similarity between CofH, HydG, and ThiH suggested that these enzymes might catalyze a similar tyrosine fragmentation; hence we initially proposed that the second hydrogen atom abstraction is occurring from either the phenolic hydroxyl or the amino group of tyrosine (Figure 12). Since CoF catalyzes the formation of intermediate 9, generation of the radical at the exchangeable site must precede generation of the radical at the nonexchangeable site. This is consistent with the proposed order of the hydrogen atom abstraction events shown in Figure 2. We also observed significant [M + 2] and [M + 3] peaks for the S′-deoxyadenosine produced in the CoF reaction (Figure 4). This observation suggests that the hydrogen atom abstraction from the exchangeable site is reversible (1 to 5 in Figure 2) and establishes that the rate of the back hydrogen atom transfer is competitive with the rate of the β-scission reaction. The relevant bond dissociation energies of phenol, methylamine and S′-deoxyadenosine are 362, 425, and 433 kJ mol−1, respectively.33 These thermochemical data support hydrogen atom abstraction from the amine NH rather than from the phenolic OH because the phenoxy radical is not sufficiently reactive to abstract a hydrogen atom from S′-deoxyadenosine. The reversibility also eliminates the possibility of a protein glycyl radical at the CofH active site because the bond dissociation energy of glycine is 358 kJ mol−1, demonstrating that the glycyl radical is also insufficiently reactive to abstract a hydrogen atom from S′-deoxyadenosine.33

Our mechanism predicts that the tyrosine radical 5 will fragment to form glycine imine 6 which should then undergo hydrolysis to glyoxylate 15 (Figure 5a). Our search strategy for this putative product involved its conversion to a stable chromatographic 2-quinoxalinol 17 by derivatization with o-phenylenediamine 16 followed by detection and quantitation using LC-MS. The enzymatic reaction was run using [15N,13C9]-Tyr, and the resulting [2,3-13C]-2-quinoxalinol could be unambiguously differentiated from the unlabeled contaminant. In this way, we demonstrated that the amount of...
glyoxylate formed averaged 1.1 times the amount of F₀ formed in two separate experiments. A similar amount of glyoxylate was present in the CofG/CofH coupled reaction and the CofH only reaction (Figure 5), demonstrating that glycine imine formation is catalyzed by CofH.

We previously demonstrated that CofH generated a product that served as a substrate for CofG.18 Here we characterized this product (Figure 6) and determined its structure as compound 9. To demonstrate that this compound was an intermediate, it was enzymatically synthesized on a large scale using [3,3-D₂]-Tyr, purified by HPLC and then treated with dithionite reduced CofG and SAM. Analysis of the reaction by LC-MS showed that CofG-dependent production of F₀ and also showed the incorporation of deuterium into enzymatically generated S′-deoxyadenosine and F₀ (Figure 8). In this experiment we also observed washout of deuterium from [D₁]-F₀ presumably due to its reduction by dithionite followed by aerobic oxidation with loss of deuterium.

In a complementary experiment, we created two variants of FbiC in which either the first or second CXXXCXXC motif was disrupted by alanine substitution (named FbiC-C1 or FbiC-C2, respectively). When the two variants were incubated with substrates, we observed a new peak only in the reaction mixture containing FbiC-C1 (Figure 9). This was identified as compound 9 by co-elution with an authentic standard, molecular mass determination and MS² fragmentation. We then demonstrated that FbiC-C2 could also convert 9 to F₀ and that the rate of F₀ production by wild-type FbiC was approximately equal to the rate of F₀ production by a FbiC-C1/FbiC-C2 mixture (Figure 10). This suggests that FbiC catalyzes two independent reactions where intermediate 9 is not transferred directly from the C-terminal domain to the N-terminal domain but instead follows diffusion from one active site to the other.

Our mechanism is in line with recent EPR results obtained with HydG, which show evidence for the formation of a dehydroglycine and p-hydroxybenzyl radical during tyrosine Ca-Cβ bond cleavage.34 Our initial attempts to trap the proposed p-hydroxybenzyl radical 7 were unsuccessful because CofH did not catalyze the tyrosine lyase reaction in the absence of diaminouracil 2. Analysis of the stereochemistry of the C-C bond formation leading to 9, using tyrosine made chiral at C3 by deuterium substitution, suggested an alternative approach for the detection of 7 (Figure 13).

In this approach, 3-deutério tyrosine, chiral at C2 and C3, would generate the protein-bound deuterated p-hydroxybenzyl radical 7a. We propose that this radical intermediate could scramble the stereochemical information originally present at C3 of tyrosine by C-C bond rotation or by flipping of the entire radical. It is well established that the ring of tyrosine and phenylalanine can undergo rapid flipping in the interior of a protein.35,36 Radical addition to 2 followed by oxidation would then give a mixture of 9a and 9b, the deuterated epimers of 9. Stereospecific hydrogen atom abstraction by the S′-deoxyadenosyl radical at the active site of CofG would give 11 and 11a which would be converted to a mixture of 3 and 3a as shown in Figure 2. If 7 is not an intermediate, this mechanism of stereochemical scrambling could not operate, and the deazafavin formed would be exclusively protonated or deuterated at C5 depending on the stereochemistry of the starting tyrosine. To test this, [2,3-D₂]- and [3(R)-D₁]-Tyr were synthesized37 and subjected to the enzymatic reaction. LC-MS analysis demonstrated that the F₀ produced was a 1:1 mixture of 3 and 3a consistent with the intermediacy of the p-hydroxybenzyl radical 7.

In this paper we describe a set of experiments that explain how two S′-deoxyadenosyl radicals cooperate to assemble the deazafavin chromophore of the F₄₃₀ cofactor. These experiments support the mechanistic proposal outlined in Figure 2. In

Figure 12. Comparison of the reaction catalyzed by ThiH (thiamin biosynthesis) HydG (FeFe hydrogenase biosynthesis), NosL (Nosiheptide biosynthesis), and the proposed reaction catalyzed by CofH (F₄₃₀ biosynthesis). Reversible abstraction of the exchangeable hydrogen atom has been observed for ThiH, (Begley, T. P. and Mehta A, unpublished) HydG,32 and CofH and structural studies on NosL.19 clearly demonstrates abstraction of the amino hydrogen.

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this mechanism, abstraction of the tyrosine amine hydrogen atom by the CofH $S'$-deoxyadenosyl radical gives 5, which then undergoes fragmentation leading to the formation of the $p$-hydroxybenzyl radical 7. Addition of this radical to diaminouracil 2 followed by oxidation gives intermediate 9. This intermediate diffuses to the CofG active site where a second hydrogen atom abstraction generates 11. Cyclization to 13 followed by oxidation and elimination of ammonia completes the formation of the deazaflavin 3.

This mechanism is consistent with the copurification of 2 with CofH, with the reversibility of the first hydrogen atom transfer (1 to 5), with the second hydrogen atom abstraction occurring from C3 of tyrosine (10 to 11) and with the formation of the glycine imine 6 as a byproduct. The scrambling of stereochemistry at the C3 of tyrosine during Fo formation also supports the formation of the $p$-hydroxybenzyl radical 7. Finally, it was possible to trap 9, the product of the CofH-catalyzed reaction, and to demonstrate that it was a substrate for CofG.

Finally, this paper reveals how a major cofactor of the prokaryotic world is assembled by an unprecedented mechanism and provides yet another example of the remarkably complex chemistry that nature uses in the assembly of the heterocyclic intermediates used in cofactor biosynthesis.35

# ASSOCIATED CONTENT

## Supporting Information

Methods and materials, MS and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org

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