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Characterization of the N5-dimethylallyl-FMN Intermediate in the Biosynthesis of Prenylated-FMN Catalyzed by UbiX

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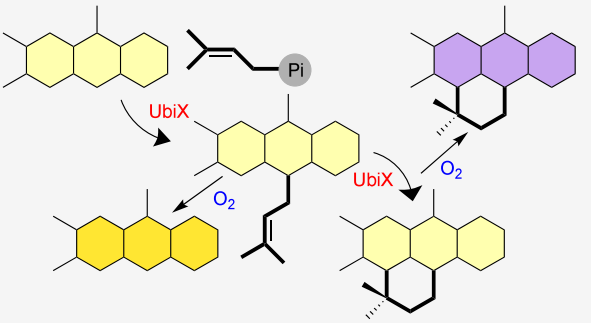
ABSTRACT: Prenylated-FMN (prFMN) is the cofactor used by the UbiD-like family of decarboxylases that catalyzes the decarboxylation of various aromatic and unsaturated carboxylic acids. prFMN is synthesized from reduced FMN and dimethylallyl phosphate (DMAP) by a specialized prenyl transferase, UbiX. UbiX catalyzes the sequential formation of two bonds, the first between N5 of the flavin and C1 of DMAP, and the second between C6 of the flavin and C3 of DMAP. We have examined the reaction of UbiX with both FMN and riboflavin. Although UbiX converts FMN to prFMN, we show that significant amounts of the N5-dimethylallyl-FMN intermediate are released from the enzyme during catalysis. With riboflavin as the substrate, UbiX catalyzes only a partial reaction, resulting in only N5-dimethylallyl-riboflavin being formed. Purification of the N5-dimethylallyl-FMN adduct allowed its structure to be verified by ^{1}H NMR spectroscopy and its reactivity to be investigated. Surprisingly, whereas reduced prFMN oxidizes in seconds to form the stable prFMN semiquinone radical when exposed to air, N5-dimethylallyl-FMN oxidizes much more slowly over several hours; in this case, oxidation is accompanied by spontaneous hydrolysis to regenerate FMN. These studies highlight the important contribution that cyclization of the prenyl-derived ring of prFMN makes to the cofactor's biological activity.



INTRODUCTION

Prenylated-flavin mononucleotide (prFMN) dependent decarboxylases (collectively referred to as UbiD-like decarboxylases) are microbial enzymes that catalyze (de)carboxylation reactions important in the utilization or biosynthesis of various aromatic, heterocyclic and unsaturated compounds.[1−3] Since the discovery in 2015 of prFMN[4,5] as the cofactor for ferulic acid decarboxylase[6] (FDC) (Figure 1A), over 35,000 putative UbiD-like enzymes have been annotated in the pfam database. UbiD-like decarboxylases catalyze (de)carboxylation reactions at otherwise be unreactive sp^2-hybridized carbon atoms.[7] In prFMN the flavin isoalloxazine nucleus is modified by the addition of an isoprene unit that introduces a 6-membered ring bridging N5 and C6 of FMN. This unusual modification introduces a nitrogen ylide into the cofactor, which results in substrates forming cyclic adducts with prFMN through a 1,3-dipolar cycloaddition mechanism.[8−10] Formation of the cycloadduct facilitates substrate (de)carboxylation, with the flavin ring system now acting as an electron sink.[11]

prFMN is synthesized by a specialized prenyltransferase, known as UbiX (Figure 1B). This enzyme uses dimethylallyl phosphate (DMAP) as a prenyl-donor to catalyze bond formation between N5 of reduced FMN and C1 of DMAP, and between C6 of reduced FMN and C3 of DMAP.[4,12−15] These reactions yield reduced prFMN ($prFMN_{red}$), which is subsequently oxidized to the active form of the cofactor (Figure 1C). The oxidative maturation, which remains poorly understood, occurs either after $prFMN_{red}$ is bound by the cognate decarboxylase or in some cases is catalyzed by a separate prFMN maturase.[16−18] Spontaneous aerobic oxidation leads only to the stable but nonfunctional semiquinone radical ($prFMN_{sq}$).[17]

The structures of UbiX have been solved from several organisms, revealing it to be a dodecameric enzyme.[4,12] We previously characterized the reaction catalyzed by the eukaryotic homologue of UbiX from *Saccharomyces cerevisiae*, PAD1, and demonstrated that the enzyme turns over extremely slowly with k_{cat} = 12 h^{-1}.[19] Consistent with this observation, X-ray structures of the N5-dimethylallylFMN (N5-dmaFMN) intermediate bound to the enzyme have also been solved.[4,12]

Here we focus on investigating the N5-dmaFMN intermediate formed in the UbiX-catalyzed reaction, which

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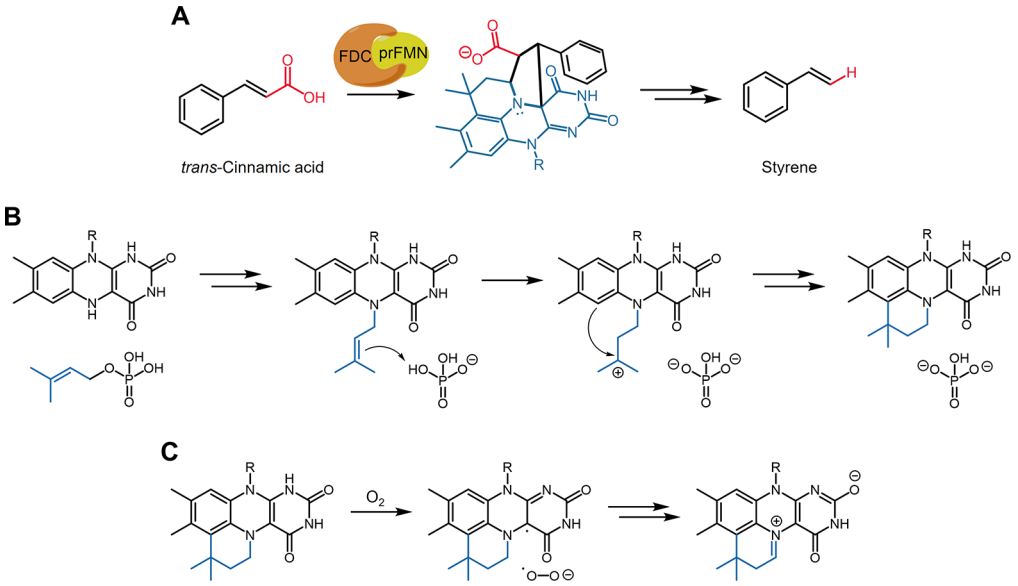

Figure 1. A) Overview of the decarboxylation reaction catalyzed by the archetypal prFMN-dependent enzyme FDC showing the cycloaddition adduct formed between the substrate and cofactor that facilitates decarboxylation. **B**) Synthesis of $prFMN_{red}$ from DMAP and FMN_{red} catalyzed by UbiX. **C**) Proposed mechanism for the oxidation of $prFMN_{red}$ to the active form of the cofactor. Oxidation may occur once bound to the UbiD-like enzyme, as is the case for FDC, or require a separate oxidase. R = ribityl phosphate.

has not been previously isolated. We show that N5-$dmaFMN_{red}$ accumulates as a significant byproduct during the reaction. This has allowed us to isolate this intermediate, validate its structure by ^1H NMR, and examine its reactivity toward oxygen and its ability to support decarboxylation reactions when bound by a cognate prFMN-dependent enzyme.

MATERIALS AND METHODS

Reagents. FMN and riboflavin were purchased from Santa Cruz Biotechnology. DMAP (3,3-dimethylallyl monophosphate ammonium salt) was purchased from Isoprenoids Co. All other materials were purchased from Sigma-Aldrich or Thermo Fisher Scientific Co.

Expression and Purification of Enzymes. Expression and purification of recombinant *Pseudomonas aeruginosa* UbiX and *Mycobacterium fortuitum* phenazine-1-carboxylate decarboxylase (PhdA) from *Escherichia coli* strains were performed as described previously.[17,20,21]

Synthesis of Prenylated Flavins by UbiX. The reaction of UbiX with DMAP and either FMN or riboflavin was studied under anaerobic conditions following previously described methods.[19,20] Typically, 500 μM FMN or riboflavin in 20 mM Bis-tris/Cl, pH 7.2, 500 mM KCl was reduced by carefully titrating it with sodium dithionite (0.6 - 1 mM final concentration). DMAP, 2 mM, and UbiX, 20 − 30 μM, were added, and the reaction was incubated at room temperature for 4 - 18 h. UbiX was removed by spin filtration (9000 g, 20 min) using Amicon Ultra-0.5 100k Centrifugal Filters (Millipore Sigma). The resulting flowthrough was used to reconstitute proteins of interest. To oxidize reduced prFMN to the C4a semiquinone radical species, the reduced prFMN solution was exposed to air for 5 min, whereupon the pale-yellow solution turned reddish-purple. For aerobic reconstitution with proteins the resulting $prFMN_{ox}$ solution was used directly. For anaerobic reconstitutions, the solution was returned to an anaerobic chamber and was allowed to degas for at least 20 min to remove residual oxygen.

HPLC Analyses. HPLC analyses were performed using a Shimadzu Prominence LC-20AT chromatography system equipped with a diode array detector. A Phenomenex kinetex C18 column (5 μm particle size, 250 × 4.6 mm) was used at a flow rate of 0.4 mL/min and a detection wavelength of 360 nm. The mobile phase consisted of 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The column was equilibrated in 5% buffer B prior to the sample injection. Samples containing flavins were eluted from the column with the following gradient: of 5% buffer B for 5 min, 5% - 95% B over 25 min and 95% B for 5 min.

Preparation and Isolation of $prFMN_{red}$ and N5-$dmaFMN_{red}$. In a 2 mL cryovial, FMN (300−400 μM) in 20 mM Tris/Cl, pH 7.2, 100 mM KCl, and 5% glycerol was reduced by titrating with $Na_2S_2O_4$. DMAP (2 mM) was added and followed by UbiX (20−30 μM) and the reaction incubated at room temperature overnight. UbiX was removed by spin filtration at 15,000 rpm for 15 min. The resulting filtrate was used for further experiments directly.

For preparative scale isolation of $prFMN_{red}$ and N5-$dmaFMN_{red}$ the filtrate from 10-enzymatic reactions (scale as above) were pooled and lyophilized to yield a purple powder. This powder was redissolved in H_2O (1 mL), filtered through a 0.22 μ syringe filter, and purified by preparative HPLC using a Vydac C18 column (L. = 250 mm, I.D. = 22 mm, 300 Å, 10 μm) on a Waters 600E system controller connected with a Waters 486 tunable absorbance detector set at 280 nm. Solvent A: 0.1% trifluoroacetic acid in water and Solvent B: 0.1% trifluoroacetic acid in 9:1 (acetonitrile: water). Fractions containing $prFMN_{sq}$ (single peak) and N5-$dmaFMN_{red}$ (eluting as two peaks) were collected and lyophilized overnight and used for further experiments. Lyophilized prFMN was isolated as a purple powder, and N5-$dmaFMN_{red}$ was isolated as a yellow powder.

Mass Spectrometry. To analyze prenylated flavin species, an Agilent 1290 series LC-MS system equipped with an Agilent 6545 quadrupole-TOF mass spectrometer and a Phenomenex kinetex C18 column (5 μm particle size, 250 ×

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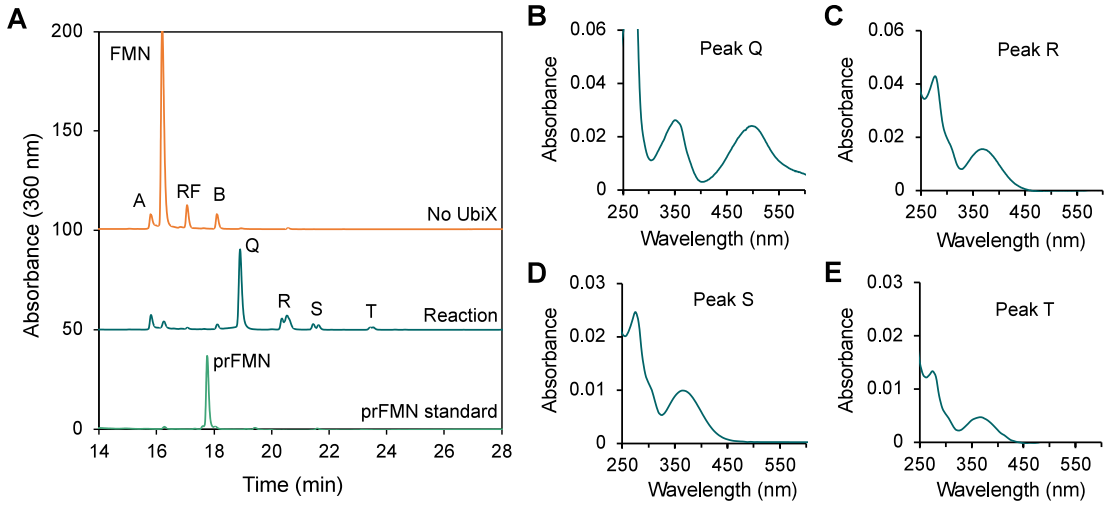

Figure 2. Prenylation of flavin species catalyzed by UbiX. **A** HPLC chromatographs of: *top trace* commercial FMN; *middle trace* products formed after reaction of reduced, commercial FMN with DMAP and UbiX; *bottom trace* oxidized prFMN standard isolated from *holo*-FDC. **B** − **E** u.v.-visible spectra associated with each of the peaks Q − T shown in panel **A**.

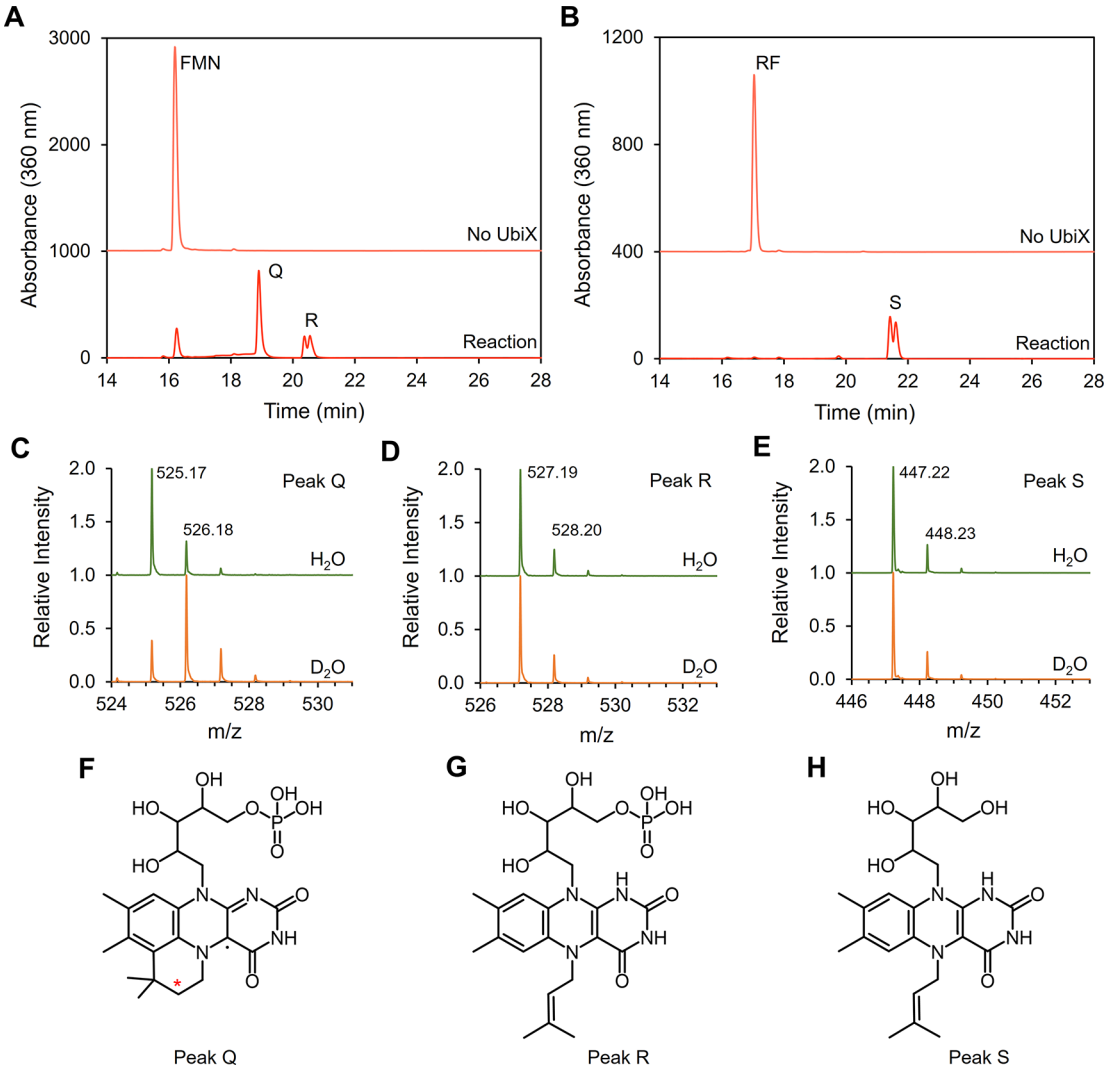

Figure 3. Characterization of the UbiX reaction products with purified FMN and riboflavin (RF). **A** and **B:** LC-MS chromatographs for UbiX-catalyzed prenylation of FMN and riboflavin, respectively. **C**, **D**, and **E:** Comparison of mass spectra for compounds Q, R, and S, respectively, for the reactions performed in H_2O or ∼80% D_2O. **F**, **G**, and **H:** Proposed structures for compounds Q, R, and S based on these data: * denotes the site of solvent deuterium incorporation.

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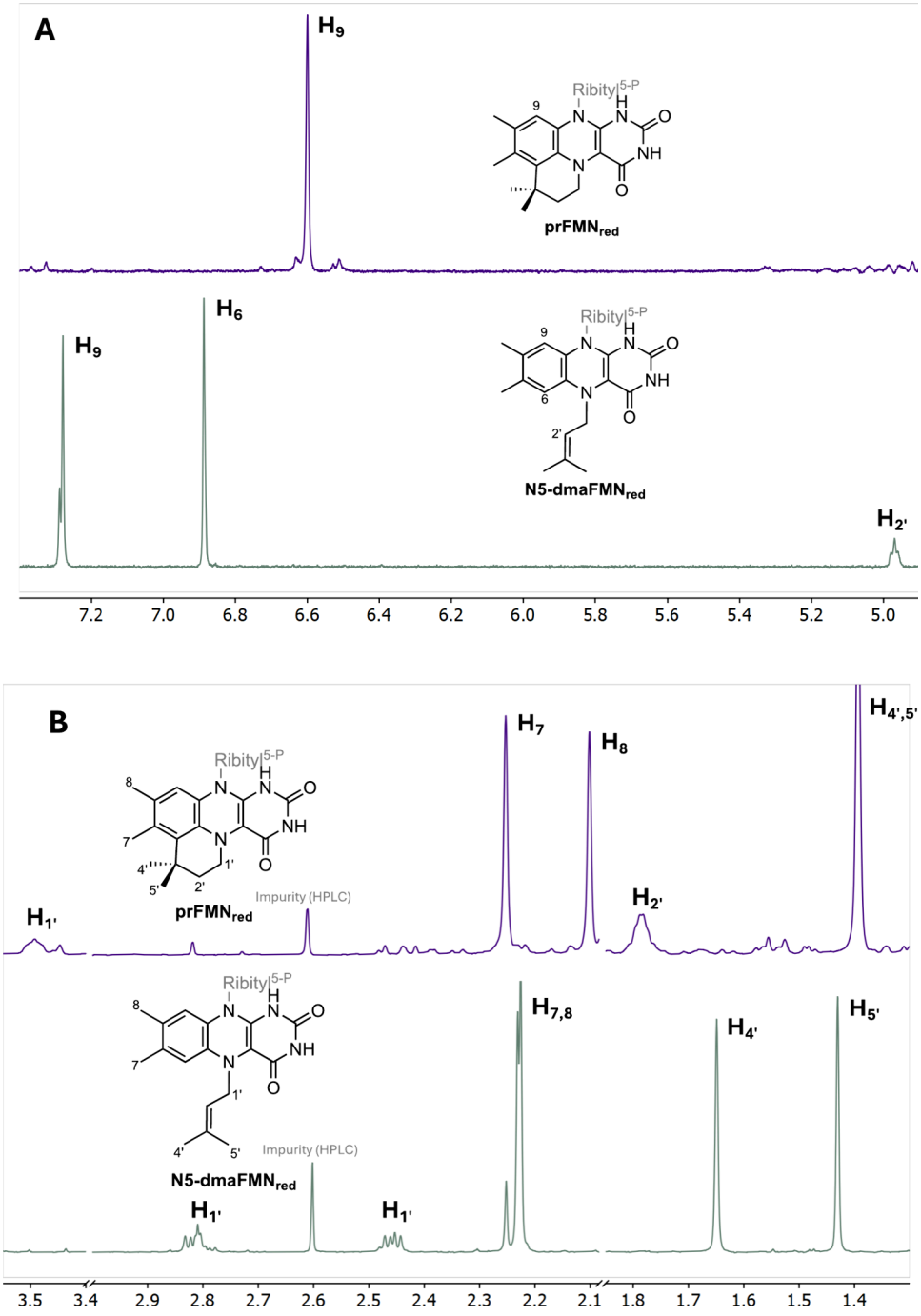

Figure 4. ^{1}H NMR spectra of prFMN$_{red}$ and N5-dmaFMN$_{red}$. **A**: low field region; **B**: high field region. The full spectra are available in the Supporting Information.

4.6 mm) was used with the same HPLC method as that described above. Analytes were eluted with 0.1% formic acid in water (buffer A) and 0.1% formic acid in 95% acetonitrile and 5% water (buffer B). Mass acquisition was carried out in positive ion mode from 50−1200 *m*/*z*.

NMR Spectroscopy. NMR spectra were acquired on either: (i) Varian Vnmrs 600 MHz instrument (14.1 T, premium shielded magnet) with a 5 mm PFF AutoX Dual Broadband probe or (ii) Bruker 800 MHz instrument with a cryoprobe (Ascend magnet with active shield, NEO console) with a 5 mm Triple resonance inverse detection TCI probe. All ^{1}H NMR spectra were: (i) recorded with highest lock efficiency and at room temperature (298 K) and, (ii) referenced to the solvent lock (D_2O or $CDCl_3$) and chemical shifts are reported in parts per million (ppm). All spectra were acquired with Wilmad NMR tubes (5 mm diameter, 7 in. length, suitable for 600 MHz) and T1 relaxation delay (D1) of 30 s. Solvent suppression was performed with gradient-based 1D-excitation sculpting (*zgesgp*, *cosygpppqf*, *roesyphpp* pulse programs from Bruker) with o1p at 4.67 ppm.

RESULTS

UbiX Reaction with Commercial FMN. Commercially available FMN that is commonly used as a substrate for UbiX

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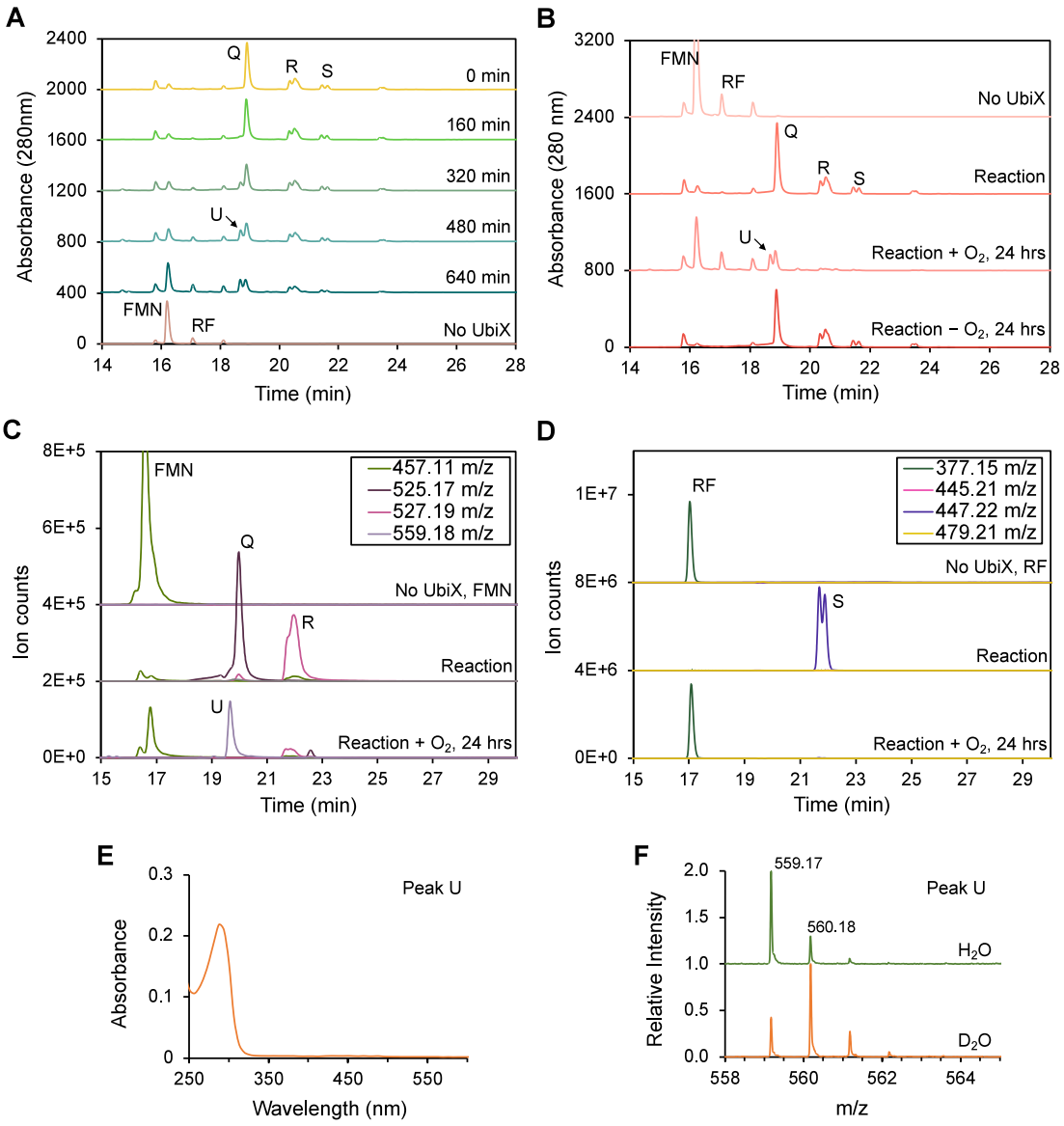

Figure 5. Reaction of the UbiX products with oxygen. **A:** Decomposition of reaction products Q, R and S as a function of time (indicated on each trace) after exposure to air monitored by HPLC. **B:** Comparison of a UBiX reaction sample exposed to air for 24 h with a sample stored anaerobically. **C, D:** Comparison of the reaction products formed by UbiX with FMN (**C**) and riboflavin (**D**). Whereas FMN is converted to both cyclized (Q) and linear products, (R) riboflavin forms only the linear 5N-alkyl flavin, which decomposes to FMN upon oxidation. **E:** u.v.-visible spectrum of peak U. **F:** Mass spectra of peak U for reactions carried out in buffers prepared in H_2O and D_2O.

is typically only 70 − 80% pure.[22] HPLC analysis of the FMN used in these experiments revealed 4 compounds with UV−visible spectra characteristic of oxidized flavins (Figure 2A). The first, minor compound (Peak A in Figure 2A) to elute (m/z = 457.11) appears to be an inactive isomer of FMN. FMN (m/z = 457.11) elutes next and comprises ∼ 75% of the sample, based on peak area. This followed by riboflavin (RF; m/z = 377.14), comprising ∼ 15% of the sample. The last flavin-like compound to elute (Peak B in Figure 2A) has m/z = 359.13 that suggests it is riboflavin from which a molecule of H_2O has been eliminated.

Using the commercially supplied FMN without further purification, we examined the UbiX-catalyzed prenylation of the flavin species present in the sample. UbiX has been described from several organisms;[12,15,19] here we used recombinantly produced enzyme from *Pseudomonas aeruginosa*. Reactions typically contained 20 − 30 μM UbiX, 500 μM FMN_{red} and 2 mM DMAP and were set up anaerobically as described in the Materials and Methods section. Reactions were incubated at room temperature for 4 − 18 h before admitting air to the tube and analyzing the products. LC-MS analysis of the products showed that, with the exception of the FMN isomer A, all of the flavins are processed by UbiX, leading to the formation of four products, here designated Q, R, S and T, (Figure 2A). The mass spectra indicate that compounds Q (m/z = 525.17) and R (m/z = 527.19) are prenylated forms of FMN, compound S (m/z = 447.22) is prenylated RF and compound T (m/z = 429.21) is a prenylated form of compound B. We note that compounds R, S, and T each appear as two closely eluting peaks that, as discussed further below, may represent conformational isomers. The UV−visible (Figure 2B) and mass spectra of peak Q (m/z = 525.17) are indicative of the prenylated-FMN semiquinone radical ($prFMN_{sq}$) species. The UV−visible

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spectra of compounds R, S and T are very similar (Figure 2 C-E), suggesting similar modifications to the isoalloxazine moiety, whereas their mass spectra indicate that the flavin remains in the reduced form.

Reaction with Purified FMN. To better characterize the reaction products formed by UbiX, we conducted further experiments using either FMN that had been purified by reverse phase HPLC or riboflavin, which was chromatographically pure as purchased. When purified FMN was reduced and reacted with UbiX, under similar experimental conditions to those described above, two reaction products were observed by HPLC corresponding to compounds Q and R (Figure 3A). Furthermore, when the reaction was performed in ∼ 80% D_2O the mass of compound Q (but not R) increased by 1 amu, indicating the incorporation of a proton (deuteration) from the solvent. These results are consistent with Q and R representing cyclized, $prFMN_{sq}$ and uncyclized, $N5\text{-}dmaFMN_{red}$ respectively.

Compounds Q and R were purified from large-scale reactions using preparative scale reverse phase HPLC, which allowed them to be characterized by ^1H NMR, with the added benefit that the two components of peak R could be separated from each other. Each of the compounds was characterized by 1D ^1H NMR, and 2D ^1H-^1H COESY and ROSEY experiments, with spectra recorded in D_2O. To our knowledge, NMR assignments have not previously been reported for any prenylated FMN species; the fully annotated spectra are provided in the supporting material. Compound Q, which spontaneously oxidizes to the semiquinone radical, was reduced with dithionite prior to recording spectra.

The NMR spectrum of $prFMN_{red}$ (Figure 4) exhibits a single broad resonance at 1.39 ppm assigned to the two methyl groups of the isoprene unit and further broad resonance at 1.80 ppm assigned to the C2' protons, respectively, of the isoprene unit. A broad signal due to the C1' protons occurs at ∼ 3.5 ppm. The C9 proton resonance of the isoalloxazine nucleus is shifted upfield, δ = 6.60 ppm, relative to the uncyclized intermediate. The C6 resonance is missing, confirming the C-C bond between C6 of the flavin and C3′ of DMAP.

The NMR spectra for both isomers of compound R proved to be very similar: the resonances for C6 and C9 protons occur at 6.88 and 7.28 ppm; a triplet at 4.97 ppm is due to the C2' proton, whereas the C1' protons occur as multiplets at 2.46 and 2.80 ppm; the *E*- and *Z*- methyl groups of the isoprene unit give rise to signals at 1.65 and 1.43 ppm respectively. The NMR data confirm the identity of both 'compound R' fractions as reduced $N5\text{-}dmaFMN_{red}$. Lastly, we note that for all three compounds the resonances for the ribityl protons overlap extensively and could not be assigned.

It is unclear from their NMR spectra how the structures of the two isomers of $N5\text{-}dmaFMN_{red}$ differ from each other, giving rise to their slightly different chromatographic properties. We speculate that the two isomers may differ in their configuration at N5, which is sp^3-hybridized and likely to be protonated under HPLC conditions. One configuration would orient the N5-prenyl group and the N1-ribityl group above the same face of the flavin ring system, whereas the other would dispose them on opposite faces. Assuming their interconversion is slow, this might give rise to different chromatographic properties.

Reaction with Riboflavin. We also examined the reaction of UbiX with pure riboflavin. Riboflavin was reduced and reacted with DMAP in the presence of UbiX, under conditions similar to those described above. In this case, HPLC analysis revealed that the riboflavin is converted to two closely related species that elute at ∼ 21.5 min (Figure 3B). The UV−visible and mass spectra were identical with those of compound S described above; repeating the reaction in D_2O did not result in deuterium being incorporated (Figure 3E). Taken together, the MS and UV−visible spectral data indicate that S represents the N5-dimethylallyl form of reduced riboflavin, hereafter referred to as $N5\text{-}dmaRF_{red}$. Notably, and somewhat surprisingly, there was no evidence for the formation of the cyclized form of pr-riboflavin, indicating that the phosphate group of FMN is important for UbiX to catalyze the C-C bond-forming step of the reaction.

Reaction of N5-dimethylallyl-flavins with Oxygen. Reduced, N5-alkyl-flavins are known to be oxygen sensitive and form stable semiquinone radicals.[23] In air, $prFMN_{red}$ undergoes rapid 1-electron oxidation to give the stable $prFMN_{sq}$ radical species. However, the oxygen reactivity of the linear N5-prenylated-flavin adducts has not been investigated.

Initially, we observed that when samples of commercial FMN were reacted anaerobically with DMAP and UbiX and then stored for HPLC analysis in septum-sealed vials (which may be considered microaerobic) the reaction products gradually decomposed on standing. Analyses of samples that had been exposed to air for increasing amounts of time (Figure 5A) showed that FMN and riboflavin were regenerated concomitant with the gradual disappearance of the species R, S and T. This observation suggests that upon oxidation these N5-prenylated flavins undergo hydrolysis of the prenyl moiety to regenerate the parent flavin. In contrast, $prFMN_{sq}$ (peak Q) did not decompose in this way, but was slowly converted to a new species, peak U in Figure 5A.

The oxygen sensitivity was confirmed by comparing a sample that was exposed to air and allowed to oxidize overnight to a control sample stored anaerobically. HPLC analysis (Figure 5B) demonstrated that whereas the control sample showed no change in product composition, the N5-prenylated flavin adducts in the sample exposed to air had decomposed and reverted to the starting flavins. We repeated this experiment with either pure FMN or pure riboflavin and monitored the products of the reaction by LC-MS, which allowed the molecular ions associated with each species to be determined (Figure 5C,D). These experiments unambiguously established that $N5\text{-}dmaRF_{red}$ cleanly reverts back to riboflavin upon air oxidation and that, similarly, $N5\text{-}dmaFMN_{red}$ reverts to FMN. The byproduct in these reactions is presumably prenal. In contrast, $prFMN_{sq}$ (peak Q) was still present, although at lower concentrations, together with increased amounts of compound U. Compound U lacks any features in the 300 − 600 nm region (Figure 5E), suggesting that the delocalized π system of the flavin has been disrupted. Compound U was found to have a molecular mass of 559.18 amu (Figure 5F) that corresponds to the addition of 2 oxygen atoms to $prFMN_{red}$ and is consistent with the formation of the prFMN-C4a-hydroperoxide.

Activity of N5-dimethylallyl-FMN. The preparative scale isolation of $prFMN_{red}$ and $N5\text{-}dmaFMN_{red}$ allowed us to investigate whether the latter compound was recognized by a cognate UbiD enzyme and, if oxidized on the enzyme, whether it might support activity. As a representative UbiD-like enzyme we chose phenazine-1-carboxylate decarboxylase (PhdA), which we have previously characterized.[20] We reconstituted

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PhdA with an excess (200−400 μM) of either prFMN$_{red}$ or N5-dmaFMN$_{red}$ under anaerobic conditions in the presence of sodium dithionite to maintain reducing conditions using the procedures we described previously.[20] After unbound cofactor was removed through a desalting column, the samples were exposed to air and their cofactor content was analyzed by HPLC (Figure 6). The chromatograph revealed that although

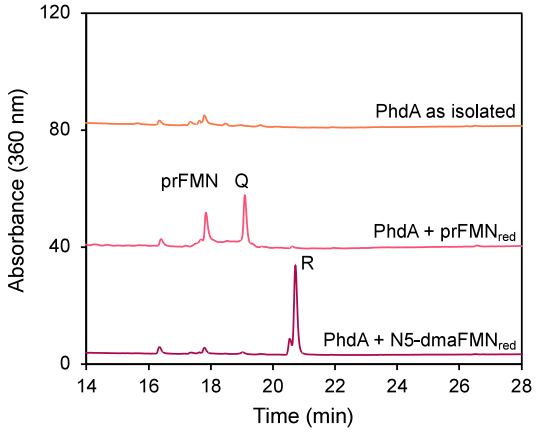

Figure 6. Reconstitution of PhdA with purified prFMN$_{red}$ or N5-dmaFMN$_{red}$. HPLC chromatographs showing cofactor content of PhdA as isolated *(top trace)*; after incubation with prFMN$_{red}$ followed by desalting to remove unbound cofactor *(middle trace)* showing that some prFMN$_{red}$ is converted to active prFMN on the enzyme; after incubation with N5-dmaFMN$_{red}$ followed by desalting to remove unbound cofactor *(bottom trace)* Although N5-dmaFMN$_{red}$ is bound by PhdA, no oxidation occurs.

PhdA binds to N5-dmaFMN$_{red}$, no new flavin species were observed, indicating that the enzyme does not oxidize or cyclize N5-dmaFMN$_{red}$. Consistent with this observation, PhdA reconstituted with N5-dmaFMN$_{red}$ showed no catalytic activity. In contrast, incubation of PhdA with prFMN$_{red}$ followed by air oxidation resulted in formation of a mixture of prFMN and prFMN$_{sq}$ on the enzyme, as has been observed previously.[20]

DISCUSSION

Since their discovery, a number of prFMN-dependent (de)carboxylases − collectively known as UbiD-like enzymes − have been structurally and mechanistically characterized.[2] Many more prospective UbiD-like enzymes are predicted to exist based on analysis of genomic sequences.[7] In contrast, UbiX, the enzyme that synthesizes the cofactor used by all UbiD-like enzymes, has received less attention. Here we have focused on characterizing the N5-prenylated FMN intermediate, which we have not previously been isolated.

The first step in the UbiX-catalyzed reaction involves the nucleophilic displacement of phosphate at C1 of DMAP by N5 of reduced flavin. In our studies we observed that even after extended reaction times N5-dmaFMN$_{red}$ accumulates in significant amounts under conditions of the reaction. The large amounts of the intermediate generated in the reaction could arise only if UbiX releases it to the solvent during a significant proportion of enzyme turnovers. This indicates that the second, C-C bond-forming step leading to the formation of prFMN$_{red}$ is not efficiently catalyzed by the enzyme.

The second step most plausibly occurs through a Friedel−Crafts alkylation involving protonation of the C2'-C3' double bond of N5-dmaFMN$_{red}$. The phosphate group liberated from DMAP is implicated as the general acid in the reaction and it has been shown that exogenous phosphate can also function in this role.[12] Therefore, in our studies, we avoided phosphate buffers to remove this complicating factor. We hypothesize that the release of N5-dmaFMN$_{red}$ and phosphate from the enzyme is effectively irreversible due the dilution of the products and therefore the ratio of N5-dmaFMN$_{red}$ to prFMN$_{red}$, reflects the partitioning of the intermediate between C-C bond formation and release from the active site.

Surprisingly, although we found that UbiX efficiently catalyzes N5-prenylation of riboflavin, under the conditions of the reaction, this compound is not further cyclized by the enzyme. FMN only differs from riboflavin by the addition of a phosphate group at the 5′-carbon of the ribose moiety and the X-ray structure of UbiX with FMN bound (PDB ID: 4ZAV) shows that this phosphate group is positioned far from the site of reaction.[4] Therefore, it is unclear why the second step in the reaction did not proceed in this case. The phosphate group of FMN makes numerous hydrogen bonds to residues in the protein and is stabilized by electrostatic interactions with a protein-bound sodium ion. Therefore, we speculate that removing these interactions may result in riboflavin being bound much less tightly than FMN, so that the intermediate is released before the much slower C-C bond-forming step has time to occur.

A striking difference between prFMN$_{red}$ and N5-dmaFMN$_{red}$ is observed in their reactions with oxygen. prFMN$_{red}$ rapidly oxidizes in air saturated buffer, $k_{obs} \sim 0.7\ s^{-1}$, to form the stable prFMN$_{sq}$ radical species, as we recently described.[17] Indeed, the stability of N5-alkyl-flavin semiquinone radicals has been known for a long time.[23] In contrast, N5-dmaFMN$_{red}$ and N5-dmaRF$_{red}$ oxidize much more slowly on the time scale of hours. This is puzzling, as *a priori* one might expect these N5-alkyl flavins to form the C4a-semiquinone radical as easily as prFMN$_{red}$ or other synthetic N5-alkyl flavins.

It is unclear to us why N5-dmaFMN$_{red}$ is so much more stable toward oxidation. However, the C2'-C3' double bond of N5-dmaFMN$_{red}$ appears to be integral to this change in reactivity because we previously found that the saturated analog, N5-isopentyl-FMN, readily forms the semiquinone radical.[17] In this study, we found no evidence for the accumulation of a semiquinone radical; instead, we observed the slow conversion of N5-dmaFMN$_{red}$ and its riboflavin analogs back to the parent flavins. We tentatively assume that the decomposition of N5-dmaFMN$_{red}$ arises from 2-electron oxidation of the molecule to form the N5-iminium ion; this compound would be expected to rapidly hydrolyze to form FMN and prenal. *In vivo*, the high barrier to oxidation of the N5−dmaFMN$_{red}$ should prevent unwanted and potentially dangerous formation of reactive radical species, and allow time for the intermediate to rebound by UbiX and cyclized to prFMN$_{red}$ Alternatively, slow oxidation and hydrolysis of N5-dmaFMN$_{red}$ would allow the liberated FMN to re-enter the cellular flavin pool.

CONCLUSIONS

We have examined the reaction of reduced FMN and riboflavin with DMAP catalyzed by UbiX. Under the conditions of the reaction, a significant proportion of the N5-dma-flavin intermediate is released during turnover, which has allowed us to isolate both prFMN$_{red}$ and N5-dmaFMN$_{red}$ in sufficient quantities to allow their ^{1}H NMR spectra to be recorded and

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assigned. The phosphate group of FMN is revealed to be a key recognition element for the second C-C bond-forming step as riboflavin is only converted to the N5-prenylated form. A striking difference in the reactivity toward oxygen is observed between the N5-prenylated intermediate and the cyclized product, $prFMN_{red}$. Whereas $prFMN_{red}$ rapidly oxidizes to the semiquinone, $prFMN_{sq}$, N5-$dmaFMN_{red}$ is unusually stable to oxygen. These observations highlight the importance of the cyclization step in generating the active cofactor: formation of the ring seems to be essential to support the generation of the ylide functionality that is central to the catalytic activity of mature, oxidized prFMN.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.4c00410

1D and 2D $^1H - ^1H$ COSY and ROESY NMR spectra recorded for reduced prFMN and reduced N5-dmaFMN.(PDF)

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Author Contributions

all the authors contributed to preparing the manuscript. P.M.D., A.M., and P.R. conducted the experiments and analyzed the data. P.M.D., P.R. and E.N.G.M wrote the manuscript. P.M.D. and E.N.G.M. conceptualized the idea for the manuscript.

Author Contributions

#These authors contributed equally to this study

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DMAP, dimethylallyl-phosphate; prFMN, prenylated-flavin mononucleotide; N5-dmaFMN, N5-dimethylallyl-FMN; N5-dmaRF, N5-dimethylallyl-riboflavin; PhdA, phenazine-1-carboxylate decarboxylase

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