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Research Article

An Enzyme Catalyzing the Oxidative Maturation of Reduced Prenylated-FMN to Form the Active Coenzyme

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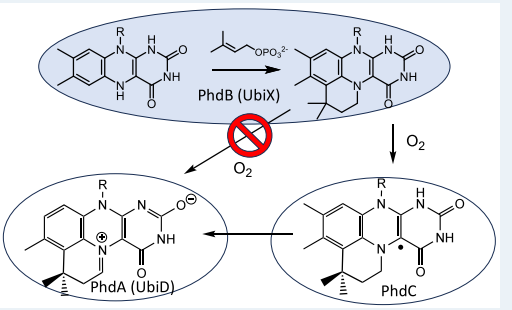
ABSTRACT: UbiD-like (de)carboxylase enzymes employ prenylated-FMN (prFMN) as a cofactor to catalyze (de)carboxylation reactions on otherwise unreactive aromatic rings and conjugated double bonds. UbiD-like enzymes are attractive for biocatalysis applications but are often difficult to obtain as active holoenzymes. Phenazine-1-carboxylic acid decarboxylase (PhdA) is one such case: even when coexpressed with its cognate prenylated-FMN synthase (PhdB), PhdA is largely obtained as inactive apoenzyme. Here, we show that a third protein, PhdC, encoded in the same operon, functions as maturase to catalyze the oxidation of reduced prFMN to the catalytically active form. Coexpression in *E. coli* of PhdA, PhdB, and PhdC allowed highly active holo-PhdA to be purified. Using purified proteins, we show that PhdC uses molecular oxygen to oxidize the prFMN semiquinone radical, formed by spontaneous air oxidation, to the active cofactor. Formation of the prFMN semiquinone radical by reaction with oxygen occurs nonenzymatically with $k_{2app} \sim 6500\ M^{-1}\ s^{-1}$ while the second, PhdC-catalyzed step to form fully oxidized prFMN occurs with $k_{app} \sim 0.35\ min^{-1}$. *In vitro* reconstitution of apo-PhdA with prFMN oxidized by PhdC gives fully active holo-PhdA. PhdC also facilitated the installation of prFMN in furan-1,4-dicarboxylate decarboxylase, HmfF, suggesting that this enzyme may have general utility in the production of active holo-UbiD-like enzymes.



KEYWORDS: *prenylated-flavin mononucleotide, decarboxylation reaction, biocatalysis, aromatic C−H activation, UbiD, UbiX, prFMN*

INTRODUCTION

The UbiD-like family of prenylated-flavin mononucleotide (prFMN)-dependent decarboxylases catalyze (de)-carboxylation reactions on a wide range of aromatic, heterocyclic, and unsaturated compounds.[1−3] A defining feature of the (de)carboxylations catalyzed by these enzymes is that they take place at an sp^2-hybridized carbon atom that would otherwise be unreactive toward (de)carboxylation. prFMN is biosynthesized from reduced FMN and dimethylallyl phosphate (DMAP) by a specialized prenyl-transferase, UbiX.[4−8] This enzyme catalyzes the formation of the C−N bond between N5 of FMN and C1 of DMAP, followed by formation of the C−C bond between C6 of FMN and C3 of DMAP. This reaction yields reduced prFMN ($prFMN_{red}$), which is biologically inactive (Figure 1A). How $prFMN_{red}$ is oxidized to the correct, active form of the cofactor remains poorly understood: spontaneous air oxidation leads only to formation of the very stable but nonfunctional semiquinone radical ($prFMN_{sq}$).

The oxidized form of prFMN ($prFMN_{ox}$) appears to be inherently unstable and undergoes tautomerization between the functional iminium and nonfunctional enamine forms.[9,10] The N5 iminium functionality readily hydrates to form hydroxy-prFMN. The cofactor is also prone to further, poorly understood oxidative reactions that result in its degradation. prFMN appears to be stabilized by binding to the cognate UbiD enzyme, which in some cases may play a role in the oxidative maturation of the cofactor after its synthesis by UbiX (Figure 1A).[9,11] This appears to be the case for ferulic acid decarboxylase (FDC), the best studied UbiD-like enzyme, but FDC is atypical in that it can efficiently be produced as the holoenzyme, either through *in vitro* reconstitution or by coexpressing UbiX with FDC in *E. coli*.[9,11] In general though, *in vitro* reconstitution of UbiD-like enzymes with prFMN is inefficient and results in enzyme preparations that contain only a small fraction of the active holoenzyme, together with significant amounts of inactive coenzyme forms.

We previously characterized the UbiD-like enzyme phenazine-1-carboxylate decarboxylase, PhdA (Figure 1B).[12] We found that even when coexpressed with PhdB, the cognate UbiX enzyme, the proportion of PhdA containing the active cofactor was extremely low.[12] To obtain active, holo-PhdA, it was necessary to reconstitute the apoenzyme with enzymati-

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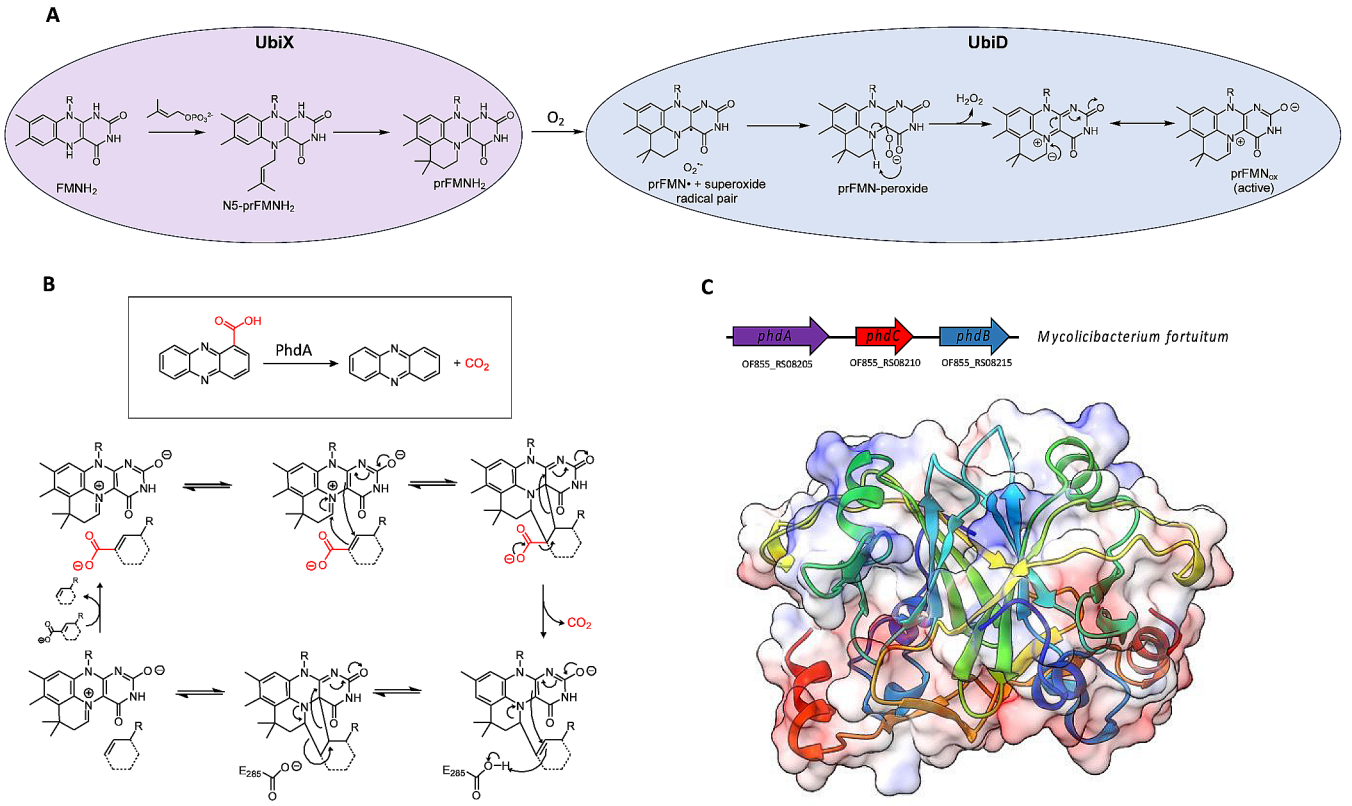

Figure 1. (A) Overview of the biosynthesis of prFMN: reduced prFNM is synthesized by a specialized prenyl-transferase, UbiX; oxidative maturation of the cofactor occurs on the binding to the decarboxylase, UbiD. (B) Reaction catalyzed by PhdA and the proposed mechanism involving cycloaddition of the substrate to prFMN. (C) The phenazine-1-carboxylate decarboxylase operon in *M. fortuitum* contains an additional gene, *phdC*. The SWISS-MODEL-generated model of the PhdC dimer.

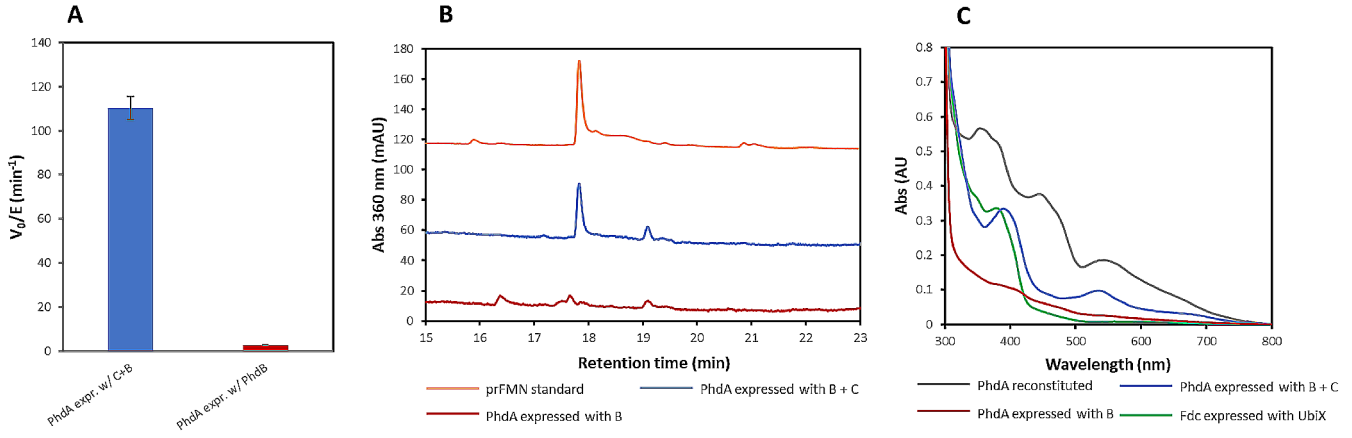

Figure 2. Coexpression of PhdA with PhdC and PhdB results in active PhdA. (A) Activity of purified PhdA coexpressed with PhdC and PhdB compared with that of PhdA coexpressed with only PhdB. (B) HPLC analysis of prFMN from purified PhdA; top trace: authentic prFMN standard obtained from holo-FDC; middle trace: cofactor content of PhdA coexpressed with PhdB and PhdC; and bottom trace: cofactor content of PhdA coexpressed with only PhdB. (C) UV−visible spectra of PhdA coexpressed with PhdB and PhdC (blue); PhdA coexpressed with only PhdB (red); PhdA reconstituted with prFMN after purification (gray); the spectrum of holo-FDC (green) is shown for reference.

cally synthesized prFMN. Even then, LC-MS analysis revealed that only a minority of the enzyme active sites contained the active form of prFMN and that other oxidized forms of prFMN were also present.[12]

Typically, the *ubiD* and cognate *ubiX* genes are adjacent on the genome and cotranscribed; however, the *phd* operon contains an additional open reading frame between the *phdA* and *phdB* (*ubiX* homologue) genes that encodes a small 17.8 kDa protein (Figure 1C), which we term *phdC*. The SWISS-MODEL-predicted[13] structure of PhdC (Figure 1C) suggested that the protein adopts the "split barrel" characteristic of many flavoproteins. These observations suggested to us that PhdC might play a role in the oxidative maturation of reduced prFMN.

Here, we have investigated the function of PhdC. We found that coexpressing PhdC with PhdA and PhdB in a recombinant *E. coli* strain results in highly active holo-PhdA. Further experiments with purified enzyme demonstrated that PhdC

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facilitates the oxidation of reduced prFMN to prFMN through the intermediacy of the prFMN semiquinone radical species. The correctly oxidized form of prFMN produced by PhdC can be transferred from PhdC to PhdA or other prFMN-dependent enzymes, such as to HmfF, to reconstitute the active holo-enzymes.

RESULTS

Initial sequence comparison of PhdC using the "BLAST" tool (NIH-NLM) indicated that it was most similar (62% sequence identity) to a putative FMN-dependent pyridoxamine oxidase from the Gram-positive bacterium *Actinoallmurus* sp. WRP9H−5. Preliminary experiments using PhdC recombinantly expressed and purified from *E. coli* established that although the protein bound FMN (Figure S1), it was unable to catalyze the oxidation of either pyridoxamine phosphate or pyridoxine phosphate. These observations suggest that this protein, and other homologues, may be misannotated in the protein database, as has been suggested by others.[14]

Coexpression of PhdC with PhdA and PhdB. The observation that PhdC binds FMN, together with the location of its gene between *phdA* and *phdB*, suggested that it may be involved in the production of active PhdA. Previously, we have shown that even when PhdA is coexpressed with its cognate prenyl-transferase, PhdB, the enzyme has very low decarboxylase activity.[12] We therefore examined the effect of coexpressing PhdC with PhdA and PhdB. Attempts to express all 3 proteins in *E. coli* as an operon, using the DNA sequence from the parent organism *M. fortuitum*, were unsuccessful. We therefore coexpressed the proteins by subcloning each gene independently into a set of compatible expression vectors, as described in the Methods section.

Coexpressing PhdA in *E. coli* with both PhdB and PhdC resulted in a dramatic increase in the activity of PhdA (Figure 2A). The specific activity of the purified enzyme, $k_{cat} \sim 110$ min^{-1} (Table 1), was comparable with the highest activities

Table 1. Decarboxylase Activity (Reported as Turnover Number: $v/[E]$) Measured for Purified PhdA and HmfF Expressed or Reconstituted under Various Different Conditions

enzyme	reconstitution conditions	$v/[E]$ (min^{-1})
PhdA	coexpressed with PhdB	2.6 ± 0.15
PhdA	coexpressed with PhdB and PhdC	110 ± 5.2
PhdA	*in vitro* reconstitution with PhdC	101 ± 2.1
HmfF	coexpressed with PhdB	1.2 ± 0.3
HmfF	coexpressed with PhdB and PhdC	15 ± 0.5
HmfF	*in vitro* reconstitution with PhdC	40 ± 0.8

obtained from PhdA preparations reconstituted with enzymatically synthesized prFMN *in vitro*, using our previously described procedure.[12] In contrast, coexpression of PhdA with either PhdB or PhdC on their own did not produce active PhdA.

The cofactor content of PhdA was examined by HPLC (Figure 2B); $prFMN_{ox}$ isolated from holo-FDC was used as an authentic standard. When coexpressed with PhdB alone, PhdA contained only a trace amount of $prFMN_{ox}$ together with similar amounts of other flavin species. However, coexpression with both PhdB and PhdC resulted in PhdA containing predominantly $prFMN_{ox}$ with a small amount of the semiquinone radical, $prFMN_{sq}$, also present (Figure 2B). The UV−visible spectra of PhdA (Figure 2C) were consistent with the HPLC analysis. The spectrum of the enzyme purified from *E. coli* coexpressing PhdB and PhdC most closely resembled the reference spectrum of holo-FDC. An additional band at 540 nm characteristic of the $prFMN_{sq}$ radical species is also observed, as would be expected based on the HPLC analysis. Presumably, under the expression conditions used, PhdC does not convert all the $prFMN_{red}$ produced by PhdB into $prFMN_{ox}$.

Mechanism of Oxidation and Holo-enzyme Formation. In considering how PhdC facilitates the formation of active PhdA, we first examined whether PhdC may form a complex with PhdA and/or PhdB that would promote the insertion of prFMN into PhdA. However, initial experiments designed to detect complex formation, including size exclusion chromatography, native-PAGE, and protein cross-linking, found no evidence that PhdC forms a stable interaction with either PhdA or PhdB (Figure S2).

Previous studies on FDC provided evidence that, after synthesis by UbiX, oxidation of reduced prFMN to form the mature, active cofactor required the reduced cofactor to first bind to FDC.[9,15] Spontaneous oxidation of $prFMN_{red}$, followed by reconstitution with FDC, resulted in an enzyme preparation with very low activity.[9,15] Drawing on these observations, we considered whether PhdC may catalyze the oxidative maturation of prFMN to its active form. $prFMN_{red}$ was synthesized from reduced FMN and DMAP using purified, recombinant *P. aeruginosa* UbiX under anaerobic conditions as previously described,[12] and the protein removed by desalting on a spin filtration column. PhdC (100 μM) was then added to the crude prFMN solution and allowed to bind the cofactor. Finally, the protein was desalted to remove any unbound flavin species.

The PhdC solution was exposed to low concentrations of oxygen by allowing air to diffuse into the reaction vial through a needle inserted through the septum cap. The solution almost immediately turned pale purple and showed a distinctive UV−visible spectrum closely resembling that of an N5-alkyl 4a-semiquinone.[16] Analysis of the reaction products by HPLC (Figure 3) showed that PhdC cleanly converts the $prFMN_{sq}$ radical species (retention time 15.6 min; m/z = 526.18) to the correctly oxidized form of prFMN (retention time 14.6 min; m/z = 525.19).

A control experiment in which PhdA was reconstituted with $prFMN_{red}$ using the same protocol demonstrated that little, if any, of the cofactor was converted by the enzyme to the correctly oxidized form of prFMN (Figure 3). Exposing the $prFMN_{red}$ formed in the UbiX-catalyzed reaction to air in the presence of UbiX (Figure 3) also did not result in the formation of correctly oxidized prFMN, in accord with previous observations. Similarly, allowing the reduced prFMN isolated from the UbiX reaction to spontaneously oxidize also failed to generate correctly oxidized prFMN.

To examine whether $prFMN_{ox}$ produced by PhdC is functional as a cofactor for PhdA, we performed the following experiment. PhdC (50 μM) was reconstituted with $prFMN_{red}$ and, after removing any unbound flavin species by desalting, a stoichiometric amount of PhdA was added (50 μM). The solution containing the two proteins was exposed to air and incubated for 10 min at 20 °C. PhdA (M_r = 310 kDa, hexamer) was separated from the much smaller PhdC (M_r = 32 kDa, dimer) by spin filtration, and the cofactor content of the two proteins was analyzed by HPLC. A control experiment was also performed in which PhdA was omitted. In the absence of

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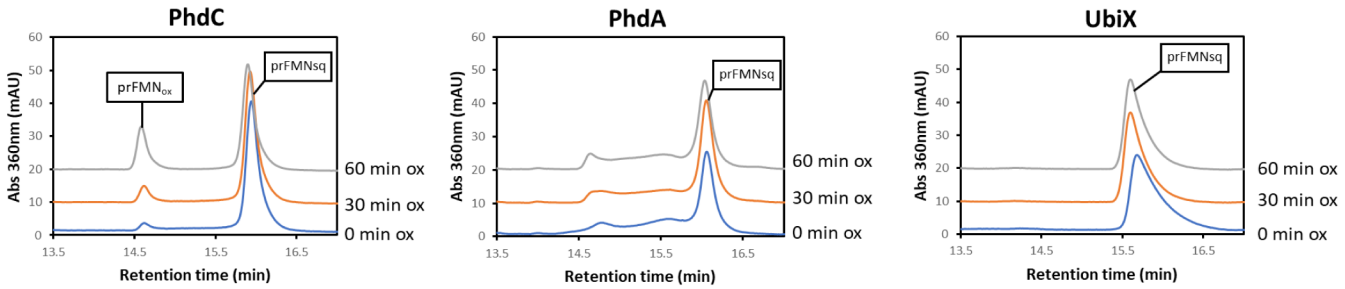

Figure 3. PhdC-catalyzed oxidative maturation of prFMN$_{red}$. PhdC was reconstituted with prFMN$_{red}$ and air admitted slowly to the reaction vial as described in the text; prFMN$_{sq}$ forms immediately. Conversion of prFMN$_{sq}$ to the correctly oxidized form was followed by HPLC. Analogous control experiments were performed with PhdA and UbiX; these enzymes do not efficiently catalyze prFMN oxidation.

PhdA, the recovered PhdC contained predominantly prFMN$_{ox}$ with some prFMN$_{sq}$ also present (Figure 4). However, in the presence of PhdA, PhdA was now found to contain prFMN$_{ox}$, whereas no cofactor was associated with PhdC. The specific activity of the reconstituted PhdA was found to be very similar to that of the enzyme isolated from the *E. coli* strain coexpressing PhdB and PhdC (Table 1). These experiments demonstrate that PhdC catalyzes the conversion prFMN$_{red}$ to the catalytically active, oxidized form, prFMN$_{ox}$, which is then released and bound by PhdA.

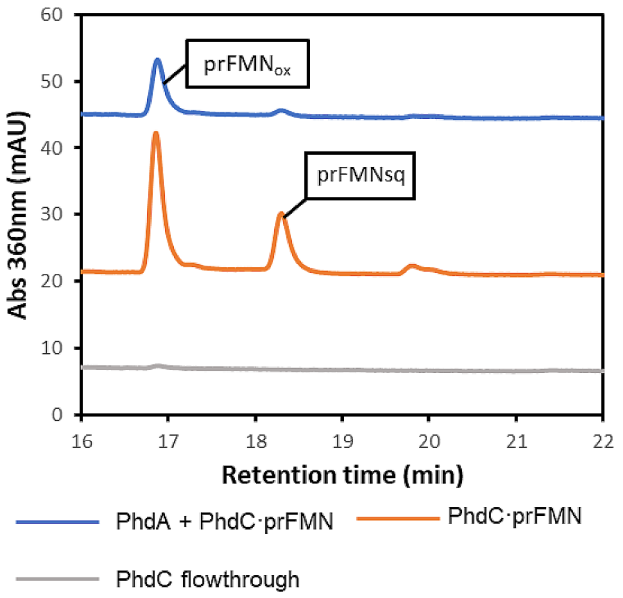

Figure 4. *In vitro* reconstitution of holo-PhdA from prFMN$_{red}$ and PhdC under aerobic conditions. Top trace: cofactor analysis of PhdA after incubation with the PhdC:prFMN complex under aerobic conditions. The trace indicates PhdA now contains prFMN$_{ox}$. Middle trace: cofactor analysis of the PhdC:prFMN complex incubated under aerobic conditions shows a mixture of prFMN$_{ox}$ and prFMN$_{sq}$ bound to PhdC. Bottom trace: no cofactor remains bound to PhdC after incubation of the PhdC:prFMN$_{ox}$ complex with PhdA under aerobic conditions.

Reconstitution of 2,4-Furan Dicarboxylic Acid Decarboxylase Activity by PhdC. Having demonstrated that PhdC effectively reconstitutes PhdA, we were curious whether the PhdC could reconstitute other UbiD-like enzymes that have proved hard to express as holo-enzymes. To answer this question, we selected 2,4-furan dicarboxylic acid decarboxylase, HmfF. Much like PhdA, HmfF has been reported to show very low activity, even when coexpressed in *E. coli* with UbiX.[25] We therefore coexpressed HmfF in *E. coli* with either PhdB alone or both PhdB and PhdC and compared the activities of HmfF purified from each strain. HmfF purified from the PhdC-expressing strain was about 12-fold more active (turnover number ~ 15 min^{-1}) than the strain lacking PhdC (Table 1). PhdC was also used to reconstitute purified apo-HmfF *in vitro*, using a similar procedure to that described above for PhdA. In this case, a substantially higher turnover number of ~40 min^{-1} was measured for HmfF, which is similar to that previously reported for the *in vitro* reconstituted enzyme.[25] These results suggest that PhdC is a general prFMN maturase, rather than it specifically acting on PhdA.

Conversion of PrFMN Semiquinone to Fully Oxidized PrFMN by PhdC. We further investigated the ability of PhdC to convert the one-electron oxidized prFMN$_{sq}$ species to the mature cofactor form. Samples of prFMN$_{red}$ were enzymatically synthesized using UbiX, and the protein was removed by spin filtration under anaerobic conditions. Next, the prFMN$_{red}$ solution was exposed to air whereupon it immediately oxidized to give the purple semiquinone radical species. PhdC was added to a final concentration of 100 μM, and the solution incubated aerobically at room temperature for 5 min. Any unbound flavin species were removed by desalting, and the prFMN products bound by PhdC were analyzed by HPLC. We found that the mixture of incorrectly oxidized prFMN species produced by air oxidation, comprising mainly prFMN$_{sq}$, was largely converted to prFMN$_{ox}$ by incubation with PhdC (Figure 5A). Repeating this experiment with PhdA yielded only prFMN$_{sq}$ bound to the protein, which showed no decarboxylation activity.

To clarify the role of molecular oxygen in the PhdC-catalyzed conversion of prFMN$_{sq}$ to prFMN$_{ox}$, the experiment was repeated, but after exposing prFMN$_{red}$ to air, the solution was then made anaerobic before adding PhdC. After incubating the solution for 5 min, any unbound flavins were removed by desalting through a spin filtration column under anaerobic conditions. HPLC analysis of the products bound to PhdC showed only prFMN$_{sq}$. However, exposure of the protein to air for 5 min led to the complete conversion of the radical species to active prFMN$_{ox}$ (Figure 5B). This observation shows that PhdC requires oxygen to convert prFMN$_{sq}$ to prFMN$_{ox}$. When a similar experiment was repeated with PhdA, only prFMN$_{sq}$ was observed without any formation of prFMN$_{ox}$ after the initial one-electron oxidation (Figure 5C).

Insights from N5-Alkyl FMN Model Compounds. The pathway by which prFMN$_{red}$ is oxidized to the active cofactor is not well understood, in large part because of the very limited

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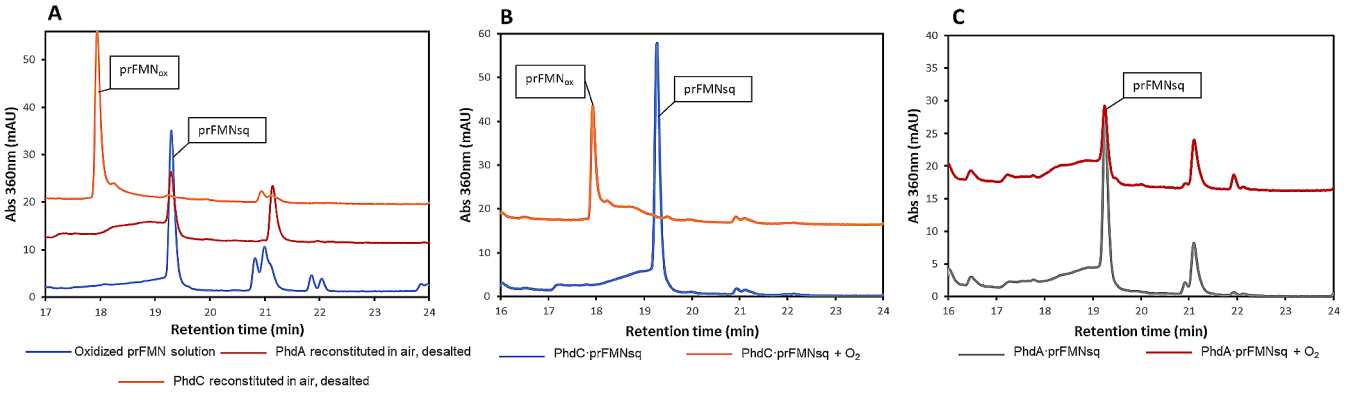

Figure 5. (A) HPLC traces of PhdA and PhdC after aerobic reconstitution. (B) Conversion of prFMNsq to prFMN$_{ox}$ by PhdC. (C) PhdA is unable to oxidize prFMNsq to prFMN$_{ox}$.

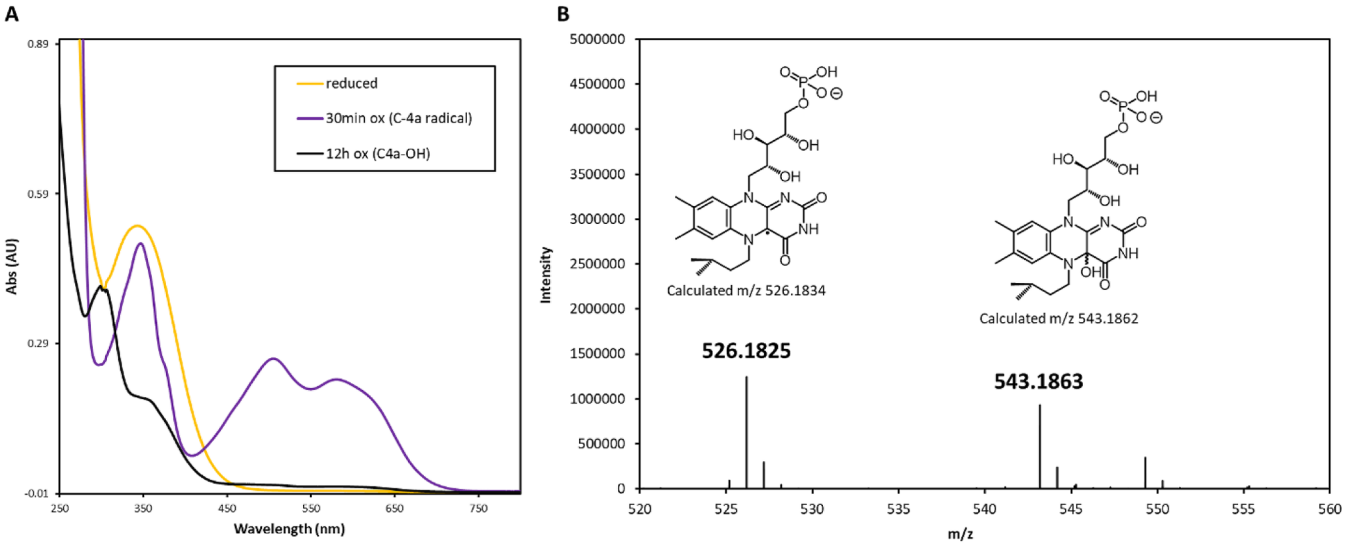

Figure 6. Oxidation of N5-isopentyl-FMN does not produce a prFMN-like ylide. (A) UV−visible spectra of fully reduced N5-isopentyl-FMN; the purple radical formed after 30 min oxidation and the colorless C4a-hydroxy-FMN derivative formed after 12 h. (B) MS spectrum confirming the formation of the radical- and hydroxy-N5-isopentyl-FMN after air oxidation for 12 h.

quantities of the coenzyme that can be enzymatically synthesized. To better understand the oxidation process, we synthesized the N5-isopentyl-FMN analog, shown in Figure 6, using a previously described method.[17] This compound is uncyclized but otherwise chemically similar to reduced prFMN. Exposing this compound to air resulted in the rapid formation of the one-electron oxidized purple radical form. Prolonged air oxidation resulted in the purple color fading to produce a colorless compound. MS analysis of this compound showed it to have a mass consistent with the formation of the C4a hydroxide (m/z = 543.19). There was no evidence for the formation of the prFMN-like ylide form, pointing to the general unfavorability of eliminating water from N5-alkyl flavins.

Kinetics of PrFMN Oxidation by PhdC. We examined the kinetics of the first step in the oxidation of prFMN$_{red}$ by stopped-flow UV−visible spectrometry. Solutions of either the 100 μM PhdC−prFMN$_{red}$ complex or 100 μM prFMN$_{red}$ in the absence of PhdC were introduced into one syringe of a stopped-flow spectrophotometer. The solutions were mixed with buffer containing O_2 at various concentrations, and the increase in absorbance at 510 nm was monitored to detect formation of the prFMN$_{sq}$ radical. Under either condition, the reaction was second order with the observed first order rate constant for the formation of prFMN$_{sq}$ exhibiting a linear dependence on [O_2] (Figure 7A). k_{2app}, = 6100 ± 300 M^{-1} s^{-1} calculated for the oxidation of the PhdC−prFMN$_{red}$ complex did not significantly differ from the nonenzymatic oxidation of prFMN$_{red}$, k_{2app}, = 6500 ± 300 M^{-1} s^{-1}. These observations indicate that the first oxidation occurs spontaneously rather than being catalyzed by PhdC.

The second phase of the reaction, conversion of prFMN$_{sq}$ to prFMN$_{ox}$, occurs much more slowly and was monitored in a spectrophotometer with the cuvette open to the air. Over the course of ∼20 min, the absorbance at 510 nm due to the radical species decreases and correspondingly the band at 385 nm, characteristic of prFMN$_{ox}$, increases (Figure 7B,C). Unlike the oxidation of prFMN$_{red}$, the oxidation of prFMN$_{sq}$ to prFMN$_{ox}$ does not occur nonenzymatically.

Interestingly, the products of the reaction appear to differ, depending upon whether PhdA is also present during oxidation. These differences show up in the long wavelength absorbance bands of the cofactor, which are compared in Figure 7C,D. When a stoichiometric amount of PhdA was included in the reaction, the kinetics appear biphasic (Figure 7B) and can be fit by two first-order processes with apparent

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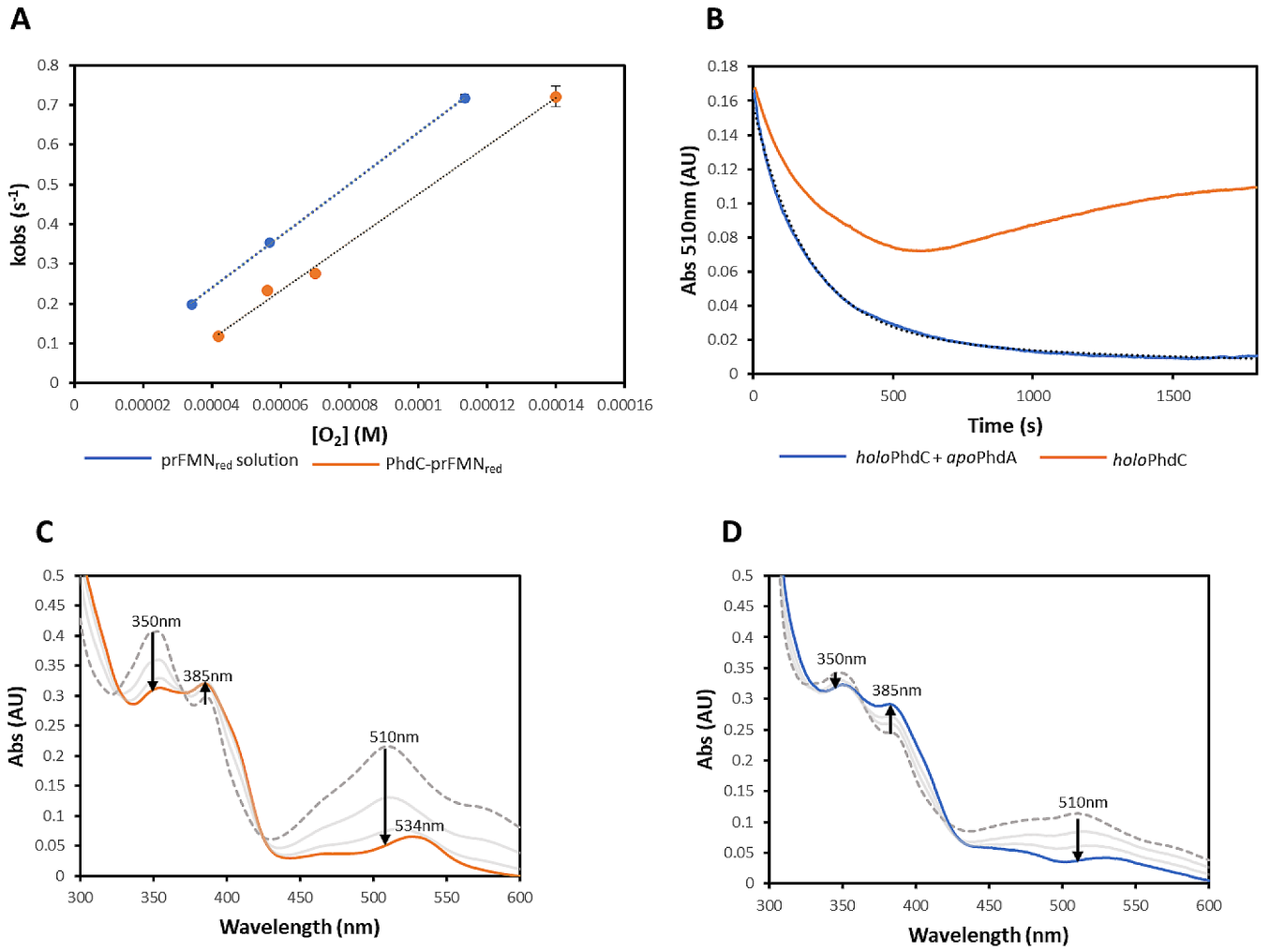

Figure 7. Kinetics of PhdC-catalyzed oxidation of prFMN. (A) Plots of observed first order rate constant vs $[O_2]$ for PhdC−prFMN$_{red}$ complex (orange dots) and free prFMN$_{red}$ (blue dots) reacting with oxygen to form prFMN$_{sq}$. (B) Aerobic reaction of the PhdC−prFMN$_{red}$ complex monitored at 510 nm over 30 min: top trace (orange), in the absence of PhdA; bottom trace (blue), in the presence of stoichiometric PhdA; and dotted line, biphasic exponential line of fit. (C) Spectral changes observed over 30 min in the aerobic reaction of the PhdC−prFMN$_{red}$ complex, representative spectra shown. (D) Spectral changes observed over 30 min in the aerobic reaction of the PhdC−prFMN$_{red}$ complex in the presence of stoichiometric PhdA, representative spectra shown.

rate constants $k_1 = 0.32 \pm 0.004$ min^{-1} and $k_2 = 0.074 \pm 0.008$ min^{-1}. In contrast, in the absence of PhdA, a further very slow reaction occurred that resulted in an increase in absorption at 510 nm and the appearance of a new absorption band with a maximum at 535 nm. The kinetics of this last reaction were not well described by a first order process, suggesting that it may result from the reaction of free prFMN$_{ox}$ with other molecules in the buffer solution.

DISCUSSION

The UbiD pfam of putative prFMN-dependent (de)-carboxylases now contains over 35 000 sequences, pointing to the widespread occurrence of this class of enzymes in microbes. There has been a significant expansion of our knowledge surrounding the mechanisms,[18−23] structures,[24−29] and substrate range[12,27,30,31] of these enzymes since the first prFMN-dependent enzyme, FDC, was described in 2015.[10] However, the biosynthesis of the prFMN cofactor has received much less attention. It is well established that the additional ring is derived from dimethylallyl phosphate[6] or dimethylallyl pyrophosphate,[8] depending upon the organism, and is installed by specialized prenyl-transferases represented by UbiX-like proteins,[32] but this reaction requires reduced FMN that subsequently must be reoxidized to form active prFMN. This oxidation process has remained poorly understood − in some cases, e.g., FDC, active holo-enzyme can be produced simply by coexpressing either the cognate or a homologous UbiX enzyme. However, in many cases, including PhdA, coexpression of the cognate UbiX does not result in the production of active holoenzyme.

Previous *in vitro* studies with FDC revealed that reduced prFMN must be bound by the enzyme prior to oxidation for the coenzyme to mature into the active form, implying that the enzyme plays an active role in the maturation process.[9,33] However, FDC appears to be exceptional in this regard, as most UbiD enzymes studied do not efficiently install and oxidize reduced prFMN to generate active holoenzyme. A conserved Arg residue in the active site of FDC was postulated to be important in the maturation process because mutating this residue to Ala resulted in impairment of maturation activity of an enzyme,[33] but this Arg residue is conserved in other UbiD enzymes, including PhdA, that do not efficiently

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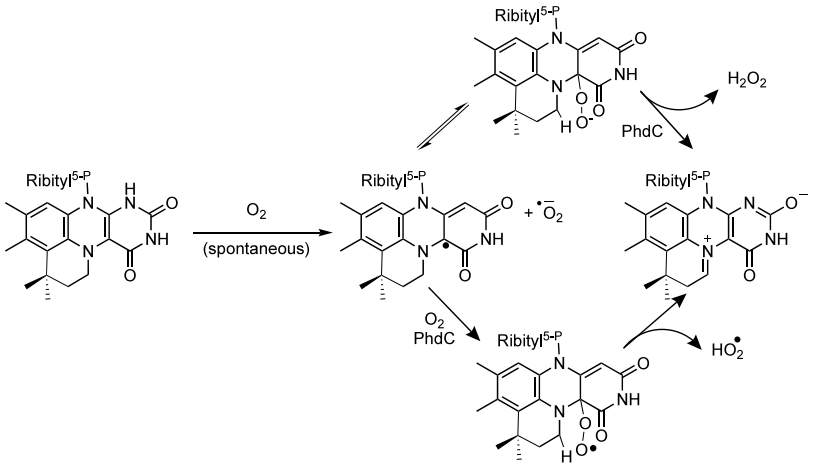

Figure 8. Alternate pathways for the PhdC-catalyzed oxidation of reduced prFMN.

install prFMN, suggesting that other features of the active site must be important. Our studies on reduced N5-isopentyl-FMN illustrate the problem inherent in forming the $prFMN_{ox}$ ylide: although oxidation to the C4a semiquinone radical (or peroxide) is facile, the protons at C1' are not acidic and therefore oxidation at C1' is kinetically disfavored. In the absence of an enzyme, oxidation of prFMN is thus stalled at the C4a semiquinone radical stage and, in the model compound, N5-isopentyl-FMN, this species ultimately oxidizes to the C4a hydroxide.

The mechanistic details for the PhdC-catalyzed oxidation of prFMN remain to be established. The initial one-electron oxidation of $prFMN_{red}$ to give $prFMN_{sq}$ occurs spontaneously in air and thus does not require catalysis by PhdC. Indeed, we found that PhdC can scavenge the free prFMN semiquinone radical formed by nonenzymatic oxidation and in the presence of oxygen convert this species to the fully oxidized form. Therefore, it seems that the enzyme's primary role is to catalyze the second one-electron oxidation and mediate proton abstraction from the C1' carbon. We envisage two mechanisms by which this oxidation could occur.

In the first mechanism, the superoxide radical anion generated by the initial oxidation step recombines with the $prFMN_{sq}$ radical to give the C4a peroxide, a well-established species in the reactions of flavins with oxygen. The C4a peroxide then acts as a general base to remove the C1' proton and undergoes elimination to form $prFMN_{ox}$ and hydrogen peroxide. This mechanism represents the consensus view, but an objection to it is that it requires a relatively weak base, peroxide to remove a nonacidic proton at C1'.

As an alternative mechanism, we propose reaction of $prFMN_{sq}$ with a second molecule of oxygen, thereby generating a peroxy-radical. This radical is then poised to oxidize C1' by either a hydrogen atom transfer (HAT) or proton coupled electron transfer (PCET) mechanism (Figure 8).[34] Elimination of the hydrogen superoxide radical then results in the formation of oxidized prFMN. This mechanism is supported by the observation that when PhdC is reconstituted with $prFMN_{sq}$ under anaerobic conditions, no reaction occurs until exposure to oxygen, which results in conversion of the radical to fully oxidized prFMN.

We note that very recently, a protein with similar activity to PhdC was reported.[35] This protein, LpdD from *Lactobacillus plantarum*, appears to facilitate the maturation of the UbiD-like gallic acid (3,4,5-trihydroxybenzoic acid) decarboxylase, LpdC. The crystal structure of LpdD (determined without prFMN bound) shows it to be a dimeric enzyme, with the dimer interface formed by a β-strand sandwich. However, LpdD has only 18% sequence identity to PhdC and its structure shows little similarity to the structures of PhdC predicted by SWISS-MODEL (Figure 1C) or AlphaFold.

To examine whether PhdC-like enzymes may be more widely distributed, we used sequence similarity network[36,37] (SSN) analysis to identify other potential enzymes that might act as prFMN maturases and group them by sequence similarity. This analysis identified PhdC-like proteins encoded in the operons of a number of prFMN-dependent decarboxylases, although none have so far been characterized (Figure 9). Interestingly, LpdD and its homologues do not co-occur in the SSN generated for PhdC. This points to the possibility that PhdC and LpdD may have convergently evolved similar functions. The lack of a well conserved family of prFMN maturases that occur in proximity to *ubiX/ubiD* operons is likely one reason why they have not been identified as additional components of the UbiX/UbiD system until now.

There is considerable interest in using prFMN-dependent (de)carboxylases for biocatalysis applications,[38−41] as these enzymes have the potential to selectively functionalize otherwise unreactive alkenes and aromatic compounds under mild conditions. However, such applications require that active holoenzymes can be reliably produced when recombinantly expressed in a suitable microbial host because *in vitro* reconstitution is impractical on an industrial scale. The identification of a prFMN maturase, PhdC, that can be coexpressed with UbiX to produce UbiD-like enzymes such as PhdA and HmfF as highly active catalysts removes an important roadblock to using this class of (de)carboxylases in industrial applications.

CONCLUSIONS

We have identified an enzyme associated the oxidative maturation of prFMN, which we term PhdC due to its genomic location in the phenazine-1-carboxylate decarboxylase operon, *phd*. This enzyme facilitates the one-electron oxidation of the extremely stable $prFMN_{sq}$ radical to the biologically active $prFMN_{ox}$ using molecular oxygen as the oxidant. Significantly, coexpression of PhdA in *E. coli* with PhdC and the prenyl-FMN synthase, PhdB, resulted in highly active holo-

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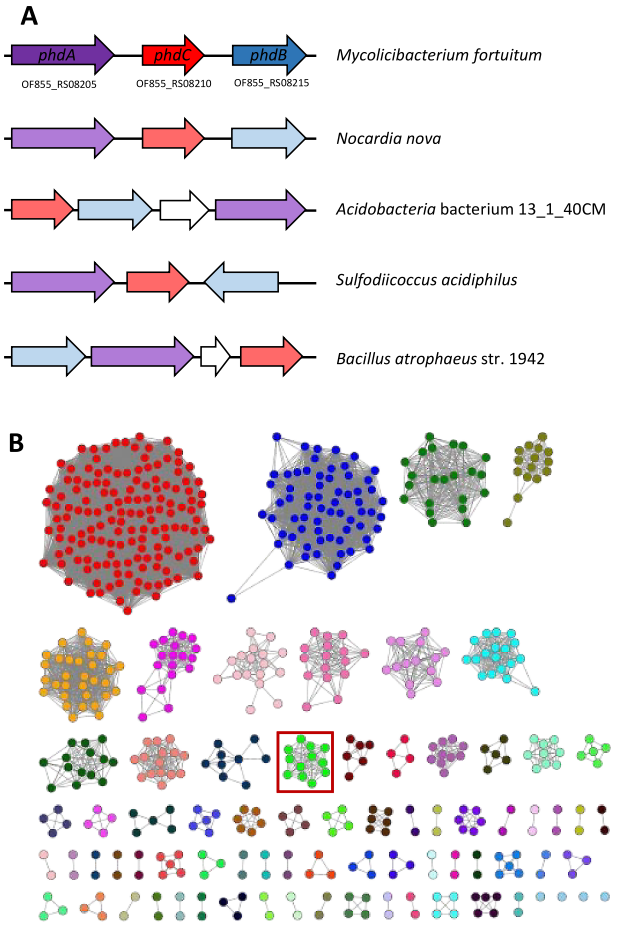

Figure 9. Identification of other PhdC-like proteins in other microorganisms. (A) Location of *phdC*-like genes (red) relative to *ubiD* (purple) and *ubiX* (blue) genes in representative bacterial genomes. (B) Sequence similarity network of PhdC-like proteins. Red box denotes the cluster containing *phdC*.

PhdA. In contrast, coexpression of only PhdB with PhdA resulted in mainly apo-PhdA being produced. The production of active holo-HmfF, another UbiD-like enzyme that is hard to obtain in holo-form, points to the general utility of PhdC, and similar prFMN maturases, for producing holo-UbiD enzymes.

MATERIALS AND METHODS

Strains and Plasmids. A codon-optimized gene encoding *phdC* was commercially synthesized and subcloned in the dual expression vector pCDFduet-1 (GenScript Biotech Co.). For coexpression of *phdC* and *phdA* genes, *phdA* was amplified from the pET20b(+) vector using PCR and cloned into the pCDFduet-1 vector between the NdeI and XhoI sites using a Gibson Assembly kit (New England Biolabs).

E. coli Rosetta strains with the pET20b(+) vector individually containing either *phdA* or *phdB* were kindly provided by Prof. Dianne Newman (Caltech). A codon-optimized gene encoding *ubiX* from *Pseudomonas aeruginosa* was commercially synthesized and subcloned in the expression vector pET28b(+) (GenScript Biotech Co.). For coexpression of *phdA* and *phdB* genes, *phdB* was amplified from the pET20b(+) vector using PCR and cloned into the pET28b(+) vector between the NcoI and *Bam*HI sites using a Gibson Assembly kit (New England Biolabs). For protein expression, all plasmids were transformed into *E. coli* BL21 DE3 (New England Biolabs).

Protein Expression and Purification. All *E. coli* BL21 DE3 strains were grown at 37 °C with shaking at 180 rpm in LB broth supplemented with 50 μg/mL spectinomycin and/or 50 μg/mL kanamycin. After reaching an OD_{600} of 0.6−0.8, protein expression was induced by adding IPTG, final concentration 0.25 mM. For cells expressing UbiX or PhdB, the medium was also supplemented with 1 mL per liter of prenol. The cultures were incubated overnight at 18 °C with shaking at 180 rpm. Cells were harvested by centrifugation (4 °C, 6200 *g*, 15 min) and stored at −80 °C.

To purify proteins, cells were resuspended in buffer A (20 mM Bis-tris/Cl (pH 7.2), 500 mM KCl, 1 mM $MnCl_2$, 10 mM imidazole, 5% glycerol) supplemented with complete EDTA-free protease inhibitor cocktail (Roche) and sonicated using 5 s pulses separated by 10 s for a total time of 18−20 min. The lysate was clarified by centrifugation at 4 °C and 16 000 *g* for 45 min. Proteins were purified from the supernatant by Ni-NTA affinity chromatography using a HisTrap (GE Healthcare) column. Protein purification was performed on a Bio-Rad NGC chromatography system at a flow rate of 2 mL/min. Initially, the column was equilibrated with buffer A. Proteins were eluted using a linear gradient of 0.1 to 1.0 M imidazole in buffer A over a volume of 40−45 mL. Fractions were analyzed by SDS-PAGE on a 4−20% gel (Bio-Rad). Protein containing fractions were concentrated by centrifugation filters (4 °C, 5000 rcf, 15 min) using Amicon Ultra-15 30k Centrifugal Filters (Millipore Sigma). Protein purity was assessed by SDS-PAGE (Figure S1). All purified proteins were stored at −80 °C without desalting.

Enzymatic Synthesis of PrFMN. prFMN was synthesized under anaerobic conditions following previously described methods.[12] Typically, 500 μM FMN in 20 mM Bis-tris/Cl, pH 7.2, and 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 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.

***In Vitro* Reconstitution of PhdA and PhdC with Reduced PrFMN.** 50−200 μM PhdC or PhdA was incubated with ∼500 μM reduced prFMN under anaerobic conditions in 20 mM Bis-tris/Cl, pH 7.2, 1 mM $MnCl_2$, and 500 mM KCl. After 10−20 min, unbound flavins were removed by spin-filtering proteins into 20 mM Bis-tris/Cl, pH 7.2, 1 mM $MnCl_2$, and 500 mM KCl using 7K Zeba spin desalting columns (Thermo Fischer Co.). Prior to use, desalting columns were scrubbed of residual O_2 by first passing through 20 μL of 0.1 M sodium dithionite solution, after which they were equilibrated with buffer until no dithionite was detected in the flowthrough. Aerobic reconstitution of PhdC and PhdA was performed similarly, except that dithionite was omitted in equilibration of the desalting columns. The prFMN species

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bound by the proteins were analyzed by HPLC as described below.

Reconstitution of holo-PhdA Using PhdC. 50−200 μM PhdC containing oxidized prFMN prepared as described above, was mixed with 50 μM apo-PhdA and incubated for 5 min at 4 °C either anaerobically or aerobically. To separate the resulting holo-PhdA from PhdC, the mixture was subjected to spin filtration (9000 *g*, 20 min) using Amicon Ultra-0.5 100k centrifugal filters; PhdA (M_r = 310 kDa, hexamer) was retained, whereas the much smaller PhdC (M_r = 32 kDa, dimer) passed through the filter. Retentate and flowthrough were analyzed by SDS-PAGE on a 4−20% gel to confirm the presence of each protein. The activity of the reconstituted PhdA was determined by measuring the rate of phenazine-1-carboxylic acid decarboxylation as described previously.[12]

Stopped-flow Kinetic Analysis. Reduced prFMN was reconstituted with a 2-fold excess of PhdC, as described above, to ensure all the cofactor was bound, and the PhdC−prFMN$_{red}$ solution was stored in an anaerobic tonometer prior to use. The reduced PhdC−prFMN$_{red}$ (50 μM after mixing) was mixed with 20 mM Bis-tris/Cl buffer, pH 7.2, containing 500 mM KCl and 1 mM $MnCl_2$ at 4 °C, and the reaction monitored at 510 nm. To obtain buffer with different oxygen concentrations, the buffer was sparged with mixtures of N_2 and O_2 ranging from 30% to 100% O_2.

UV−visible Spectroscopy. All UV−visible measurements were performed at room temperature with a UV-2500i UV−vis Spectrometer (Shimadzu). Full spectrum measurements were performed with 1 nm intervals and 2 nm slit width. To follow prFMN oxidation, 100 μM (final concentration) PhdC reconstituted with reduced prFMN under anaerobic conditions was mixed with oxygenated buffer (20 mM Bis-tris/Cl, pH 7.2, 500 mM KCl, 1 mM $MnCl_2$) and immediately introduced into the cuvette. The decarboxylase activities of FDC and HmfF were determined spectrophotometrically at 20 °C using assay conditions previously described.[7,25]

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 10 mM trifluoroacetic acid in water (buffer A) and 10 mM trifluoroacetic acid in acetonitrile (buffer B). The column was equilibrated in 5% buffer B prior to 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. To assay PhdA activity, samples containing PCA and phenazine were separated using the following gradient: 5% B for 1 min, 5−55% B over 1 min, held at 55% B for 3 min, 55−60% B over 10 min, and 60−95% B over 1 min.

Mass Spectrometry. To analyze prFMN 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 × 4.6 mm) was used with the same prFMN HPLC method as 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 to 1200 *m*/*z*.

Sequence Similarity Networks. Sequence similarity networks were produced using the Enzyme Similarity Network (ESN) tool developed by the Enzyme Function Initiative (Woese Institute for Genomic Biology, U. Illinois).[36,37] An initial group of candidate sequences was generated using BLAST with the sequence of PhdC as the query sequence (UniProt BLAST query e-value = 1, SSN Edge threshold, *E* = 1). These sequences were subjected to Gene Neighborhood Analysis (neighborhood size = 5, co-occurrence cutoff = 20%) to determine which clusters co-occurred with predicted UbiDs. From the initially generated group of sequences, two PhdC-like genes immediately downstream of predicted UbiDs were selected and used to identify further candidate sequences by the same process as described above. The nonredundant sequences from all three groups were pooled and used to create the final SSN (SSN edge calculation e-value = 5, Edge threshold, *E* = 70).

Pyridoxamine Phosphate Oxidase Activity Assay. To determine whether PhdC displayed PNP_{ox} activity, 100 μM PhdC was incubated with 1 mM FMN and then desalted to produce FMN-reconstituted PhdC. PNP_{ox} reactions were performed by incubating 50uM PhdC-FMN overnight with 1 mM pyridoxine 5′-phosphate (PNP) in 20 mM Bis-tris, pH 7, at rt. Oxidation of PNP to PLP was monitored by LC-MS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.4c02747.

SDS-PAGE of PhdC; UV−vis of PhdC bound to prFMN forms; UV−vis of PhdC bound to FMN; cross-linking; native-PAGE; SEC of PhdC, PhdB, and PhdA (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PCA phenazine-1-carboxylic acid
PhdA phenazine-1-carboxylic acid decarboxylase
FDC ferulic acid decarboxylase
prFMN prenylated-flavin mononucleotide

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