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Research Article

# Decarboxylation of Aromatic Carboxylic Acids by the Prenylated-FMN-dependent Enzyme Phenazine-1-carboxylic Acid Decarboxylase

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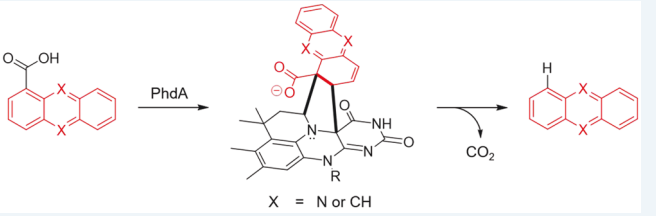
**ABSTRACT:** Phenazine-1-carboxylic acid decarboxylase (PhdA) is a member of the expanding class of prenylated-FMN-dependent (prFMN) decarboxylase enzymes. These enzymes have attracted interest for their ability to catalyze (de)carboxylation reactions on aromatic rings and conjugated double bonds. Here we describe a method to reconstitute PhdA with prFMN that produces an active and stable form of the holo-enzyme that does not require prereduction with dithionite for activity. We establish that oxidized phenazine-1-carboxylate (PCA) is the substrate for decarboxylation, with $k_{cat}$ = 2.6 $s^{-1}$ and $K_M$ = 53 $\mu$M. PhdA also catalyzes the much slower exchange of solvent deuterium into the product, phenazine, with an apparent turnover number of 0.8 $min^{-1}$. The enzyme was found to catalyze the decarboxylation of a broad range of polyaromatic carboxylic acids, including anthracene-1-carboxylic acid. Previously described prFMN-dependent aromatic (de)carboxylases have utilized electron-rich phenolic or heterocyclic molecules as substrates. PhdA extends the substrate range of prFMN-dependent (de)carboxylases to electron-poor and unfunctionalized aromatic systems, suggesting that it may prove a useful catalyst for the regioselective (de)carboxylation of otherwise unreactive aromatic molecules.




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

## INTRODUCTION

Decarboxylation and carboxylation reactions are essential to metabolism, but they typically incur a highly unfavorable transition state because of the buildup of negative charge on the $\alpha$-carbon. Nature has therefore evolved a remarkably wide range of cofactors to catalyze (de)carboxylation reactions that can act as electron sinks to stabilize the negatively charged transition state.[1] The most recently discovered decarboxylation cofactor is prenylated flavin mononucleotide (prFMN),[2,3] in which the conventional isoalloxazine of the flavin is modified by the addition of a fourth ring derived from an isoprene unit. This unusual modification introduces a nitrogen ylide functionality into the ring system that converts this quintessential redox cofactor into one that supports (de)-carboxylation reactions at $sp^2$-hybridized carbon atoms.[4−7]

The mechanism by which prFMN-dependent enzymes catalyze decarboxylations is equally unusual, and differs depending upon the substrate undergoing decarboxylation. Ferulic acid decarboxylase (FDC) is the first-discovered and best characterized prFMN-dependent enzyme.[3,8−13] For this enzyme, there is good evidence that the reaction is initiated through a 1, 3-dipolar cycloaddition between the nitrogen ylide of the cofactor with the double bond adjacent to the carboxyl-group of the substrate.[9,11] This sets up the flavin nucleus to act as an electron sink in the decarboxylation step (Figure 1).[3] However, for enzymes that catalyze decarboxylation of electron-rich aromatic carboxylic acids, such as AroY,[14] a different mechanism seems more likely. In this case, electrophilic addition to prFMN is proposed to facilitate decarboxylation of the aromatic ring (Figure 1).[14]

Since the discovery of prFMN, it has become clear that prFMN-dependent (de)carboxylases are widespread in microbes.[14,15] This class of enzymes are commonly referred to as "UbiD-like" enzymes, after a widely distributed prFMN-dependent decarboxylase involved in bacterial ubiquinone biosynthesis.[16] Although the reactions catalyzed by the majority of putative prFMN-dependent enzymes remain to be determined, it appears that many are involved in the bacterial metabolism of aromatic hydrocarbons. This observation has sparked intense interest in UbiD-like enzymes for their potential to function as selective and environmentally benign catalysts for (de)carboxylation reactions in industrial processes, with recent studies highlighting their potential for C−C bond-forming reactions.[7,17,18]

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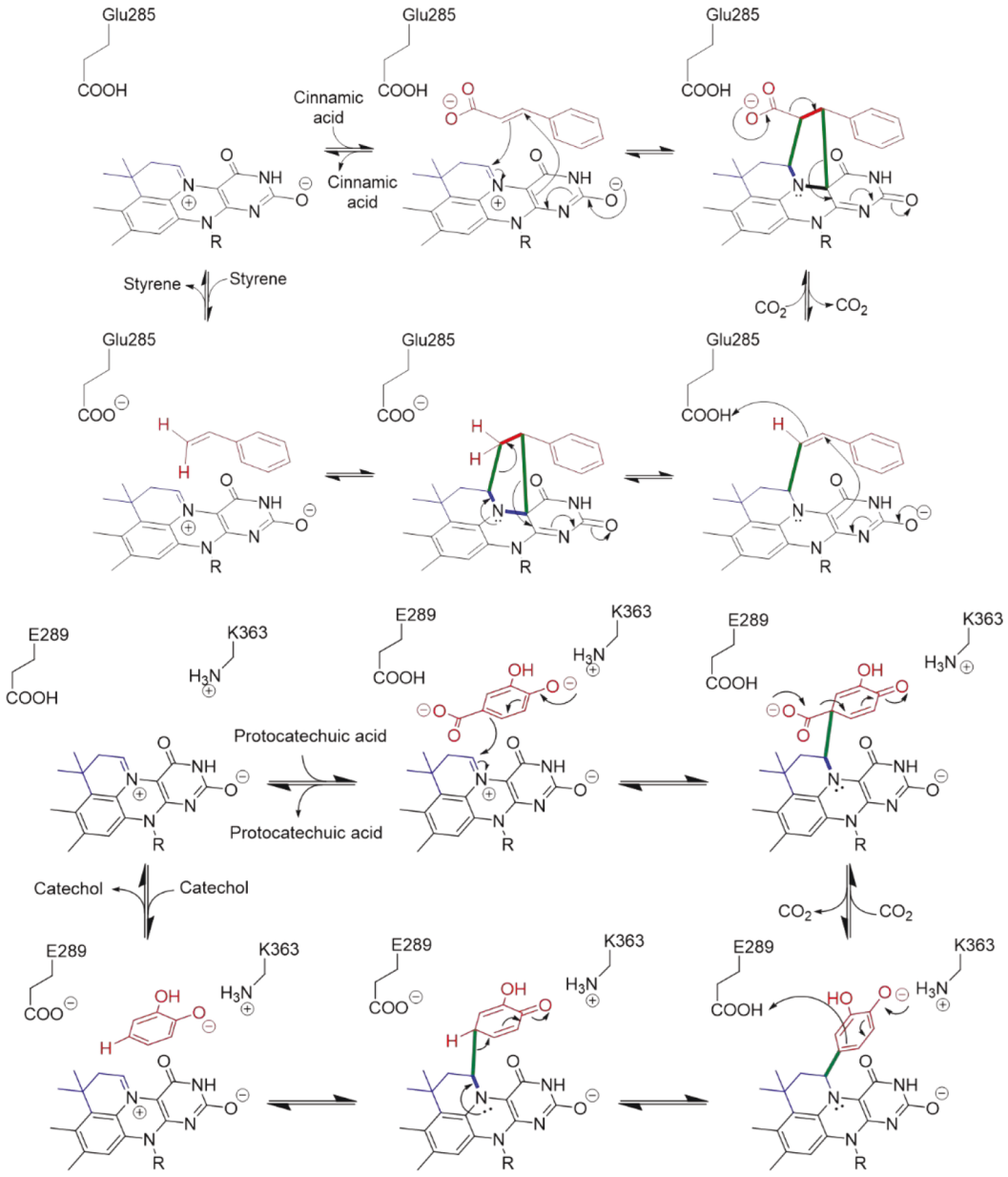


**Figure 1.** Divergent reaction mechanisms of prFMN-dependent decarboxylases: (Top) 1,3−dipolar cycloaddition mechanism proposed for the decarboxylation of cinnamic acid by FDC. (Bottom) Electrophilic addition mechanism proposed for the decarboxylation of protocatechuic acid by AroY.

PhdA is a recently identified prFMN-dependent enzyme that catalyzes the decarboxylation of phenazine-1-carboxylic acid (PCA) (Figure 2A).[19] Phenazines are redox-active metabolites that are secreted by a wide variety of bacteria,[20] for which PCA serves as the precursor. For organisms such as *Pseudomonas* spp, phenazine secretion confers a competitive advantage by facilitating anoxic survival and biofilm formation, thereby inhibiting other microbes. Phenazine-producing microbes are found in clinical, environmental, and agricultural contexts. Clinically, infection with *P. aeruginosa* poses a serious health risk, in part because the biofilms it forms, render the bacterium resistant to antibiotic treatment.[21] In agriculture, phenazines secreted by *Pseudomonas* spp. are important in biocontrol, where they protect cereal crops from a variety of fungal and parasitic diseases.[22] Degradation of phenazines by competing bacteria renders the plants more susceptible to infection.

The reaction catalyzed by PhdA is intriguing because, *a priori*, the decarboxylation reaction could occur by either the cycloaddition mechanism used by FDC or by the electrophilic addition mechanism used by AroY, depending on whether PCA is in the reduced or oxidized state. A recent report[19] described the initial characterization of PhdA, from *Mycobacterium fortuitum*. Among the interesting observations, PhdA activity was found to be dependent on the presence of substoichiometric amounts (w.r.t. substrate) of the one-electron reducing agent sodium dithionite, and the activity was enhanced by the presence of paraquat radical, a common electron mediator. Furthermore, it appeared that neither the fully reduced nor fully oxidized forms of PCA were active as substrates for PhdA, suggesting a possible radical mechanism for decarboxylation.[19]


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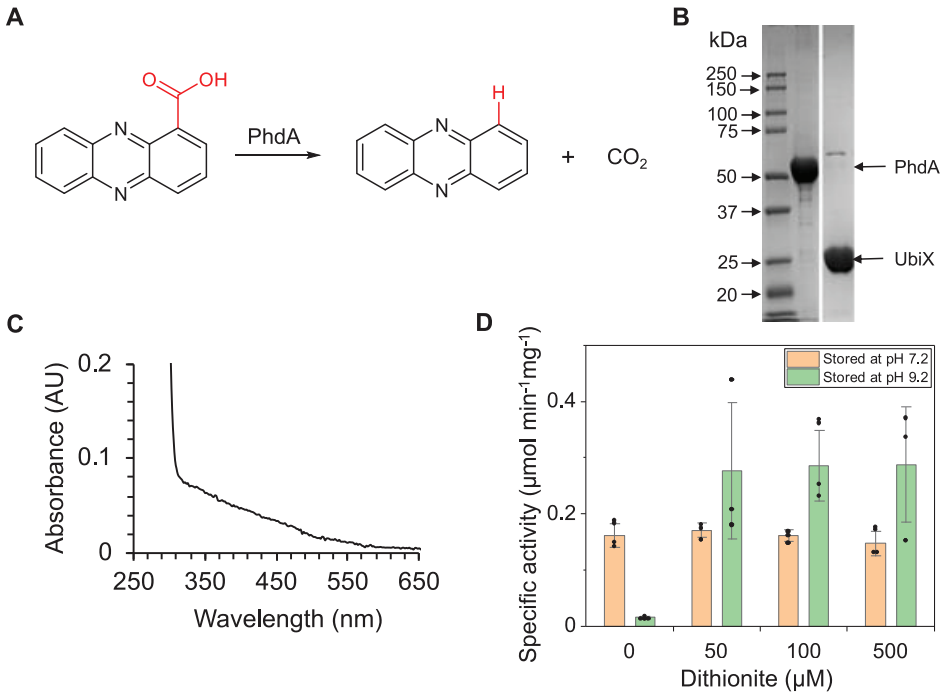


**Figure 2.** Purification and activity of PhdA. (A) Decarboxylation of PCA to phenazine catalyzed by PhdA. (B) SDS-PAGE of purified PhdA and UbiX, gel stained with Coomassie blue. (C) UV−visible spectrum of purified PhdA. (D) Comparison of the specific activities of PhdA stored at pH 7.2 or pH 9.2 and reduced with increasing concentrations of Na-dithionite prior to assay.

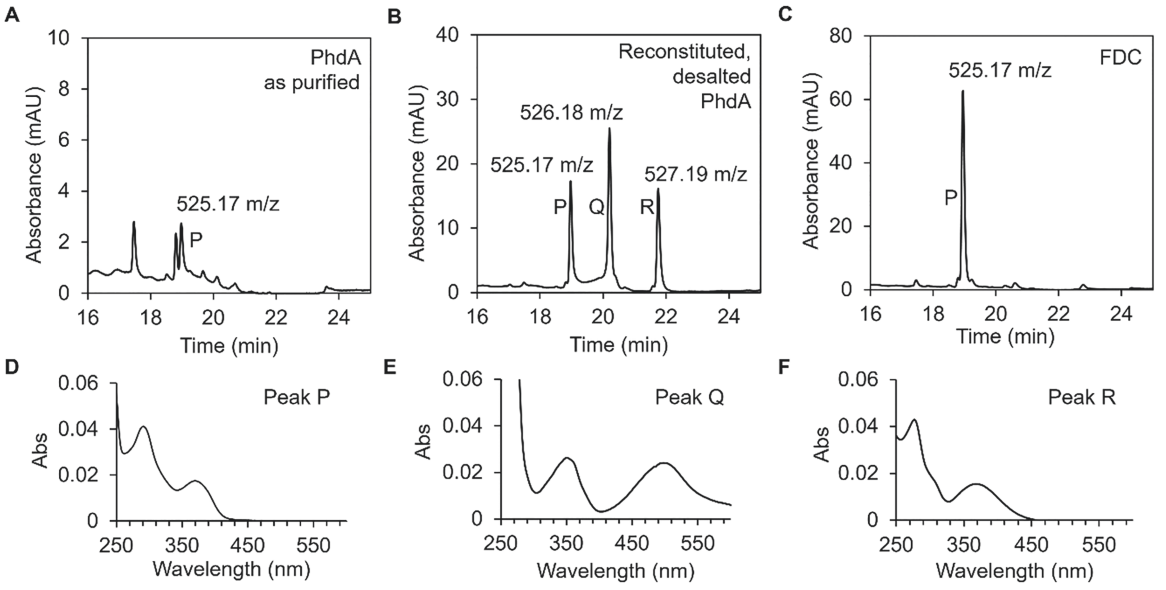


**Figure 3.** prFMN content and *in vitro* reconstitution of PhdA. (Top) HPLC chromatographs comparing the prFMN content of PhdA and FDC. (A) PhdA as purified from *E. coli*. (B) PhdA reconstituted *in vitro* with prFMN. (C) Holo-FDC as purified from *E. coli*. (D−F) UV−visible spectra of prFMN species bound to PhdA; peak P corresponds to the oxidized iminium form of prFMN; peak Q represents the stable prFMN radical; the structure of the prFMN species represented by peak R is unclear.

Here we report a detailed characterization of PhdA, in which we have established conditions for reconstituting the enzyme in a highly active and stable form, which does not require reducing agents, and have investigated the substrate range for the enzyme. We show that PhdA will decarboxylate a range of polyaromatic compounds, including unfunctionalized anthracene carboxylic acids.

## RESULTS

**Purification and Reconstitution of PhdA.** In initial experiments, PhdA was overexpressed in *E. coli* in the absence of a coexpressed prFMN synthase (UbiX). PhdA was expressed well and could be easily purified using NTA-nickel affinity chromatography, however the protein was obtained as the inactive apoenzyme. Although *E. coli* synthesizes prFMN, the endogenous levels of prFMN appear to be insufficient to


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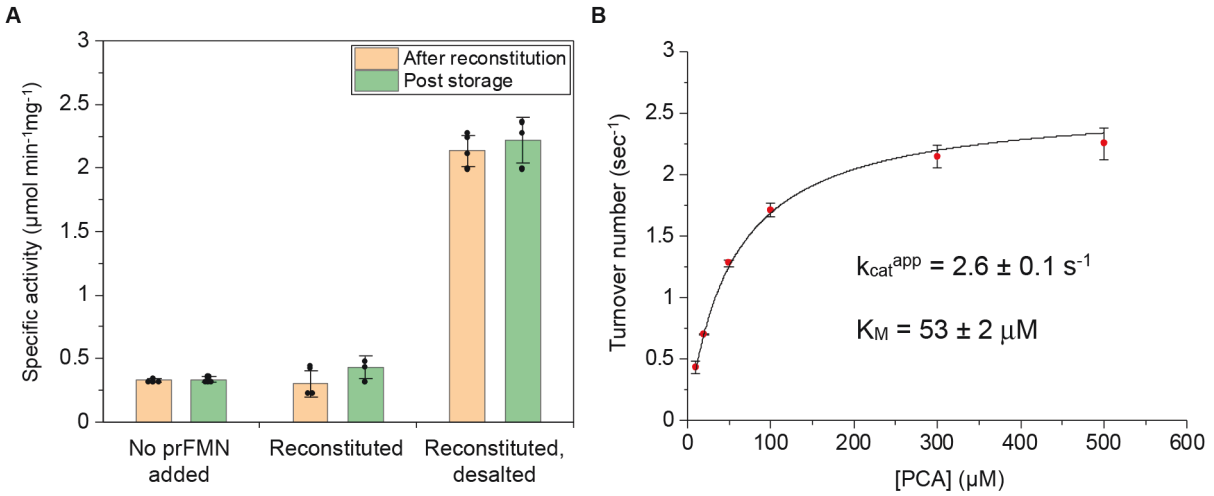


**Figure 4.** Reconstitution and kinetic parameters for PhdA. (A) Comparison of the specific activity of PhdA before reconstitution; reconstituted but not desalted; reconstituted and desalted. (B) Steady-state kinetic parameters for decarboxylation of PCA by PhdA.

obtain detectable amounts of the enzyme in active form. To obtain active enzyme, it was found necessary to coexpress PhdA with the prFMN synthase, UbiX, from *P. aeruginosa*.[23] Under these conditions, the enzyme as isolated was pale yellow and exhibited a broad, featureless absorption shoulder extending from ~300−550 nm (Figure 2B,C).

Initially, purification of PhdA was conducted at pH 9.2, following the protocol described by Costa and co-workers.[19] Under these conditions, the enzyme required incubation with sodium dithionite (>50 $\mu$M) at pH 6.5 for activity, as previously reported (Figure 2D). However, in our hands, it proved hard to obtain consistent values for PhdA activity, and large variations in enzyme activity between individual assays were apparent (Figure 2D). We also investigated whether, as previously reported, the electron mediator, paraquat, was necessary for activity. However, we found only a marginal improvement in activity in the presence of the paraquat radical (Figure S1).

Because prFMN is sensitive to hydrolysis and is known to isomerize between the active iminium and inactive enamine and ketimine forms,[3,10,24] we suspected that the relatively high pH at which the enzyme was purified and stored might result in some degradation of the cofactor. Therefore, we repeated the purification of PhdA at pH 7.2. At this pH, the activity of PhdA was not affected by the presence of dithionite or paraquat radical in the assay, and was similar to that exhibited by the dithionite-treated enzyme purified at pH 9.2. Therefore, for further experiments, the enzyme was purified and stored at pH 7.2.

Regardless of whether PhdA was purified at pH 7.2 or 9.2, the enzyme showed quite low levels of activity, suggesting that not all the active sites contained active cofactor. Maturation and installation of the prFMN cofactor is still poorly understood and has been reported to be problematic for some other UbiD-like enzymes such as AroY.[14,24] Therefore, we undertook further analysis of the flavin content of PhdA by HPLC. As a reference, we also analyzed the flavin content of FDC, for which prFMN is known to be efficiently installed when coexpressed with a prFMN synthase.[13] This analysis confirmed that, compared with FDC, only a small fraction of PhdA active sites appeared to contain prFMN (Figure 3A) and that other chromophores, possibly degraded forms of prFMN, were present.

**Reconstitution of holo-PhdA.** We attempted to improve the fraction of PhdA molecules containing active prFMN by supplementing the growth medium with the prFMN precursor, prenol, which has been shown to increase the amount of prFMN synthesized by *E. coli* strains expressing UbiX.[25] We also examined the effect of coexpressing PhdA with PhdB, which is the UbiX homologue from the parent strain of *Mycobacterium*. However, neither of these approaches resulted in enzyme preparations with significantly higher activities. We therefore turned to reconstituting holo-PhdA *in vitro* using enzymatically synthesized prFMN.

prFMN was synthesized from reduced FMN and dimethylallyl phosphate (DMAP) using recombinantly produced UbiX from *P. aeruginosa*,[23] as described in the Materials and Methods section (Figure S2). After removing UbiX from the reaction mixture, PhdA (50 $\mu$M, final concentration) was added and incubated under anaerobic conditions for 10−20 min at 4 °C. After incubation, PhdA was purified from the reaction mixture by desalting into fresh buffer and the reduced cofactor was allowed to oxidize in air to produce the active form of prFMN bound to PhdA. Interestingly, this final desalting step proved to be essential to produce highly active PhdA; if this step was omitted, the reconstituted enzyme showed no increase in activity. The reconstituted enzyme obtained by this method was ~8-fold more active than the "holo"-PhdA initially purified from *E. coli* (Figure 4A).

Even so, LC-MS analysis showed that, in addition to the active, iminium form of prFMN, two other derivatives of the cofactor were also present in significant amounts (Figure 3B). The mass ($m/z$ = 526.18) and UV−visible spectrum of peak Q (Figure 3E) suggests that it is the stable prFMN radical, which is known to be formed as an off-pathway byproduct of prFMN maturation;[24] the mass ($m/z$ = 527.19) of and UV−visible spectrum of peak R indicates it is a form of prFMN, the structure of which remains unknown.

**Kinetics of PCA Decarboxylation.** Having established the conditions for reconstituting PhdA, we examined the reaction in more detail. Using the reconstituted enzyme, we measured the steady-state kinetic parameters for the decarboxylation of PCA (Figure 4B); we determined the


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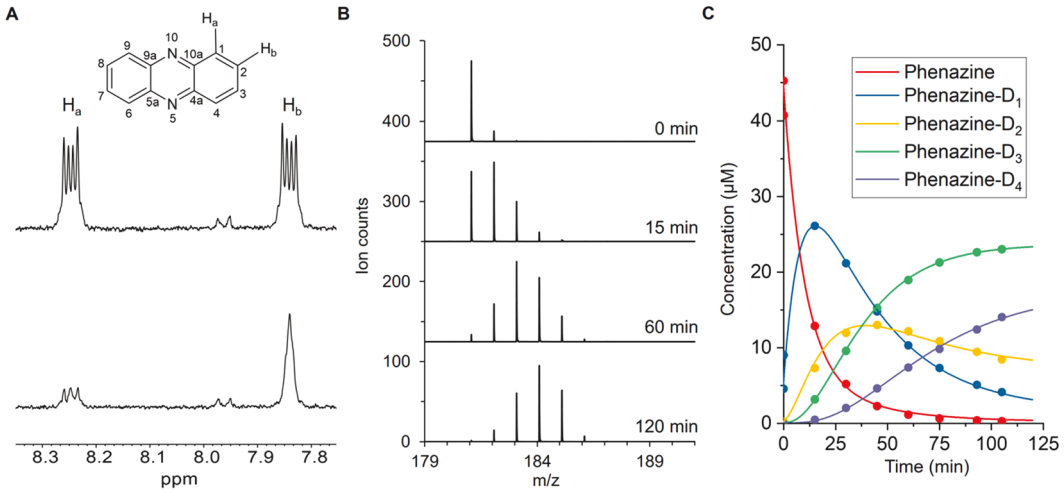
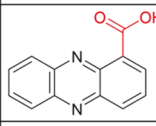
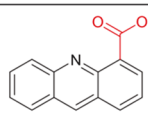
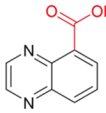
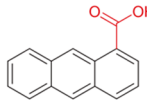
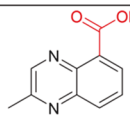
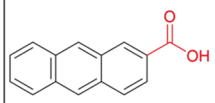
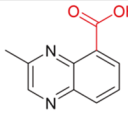
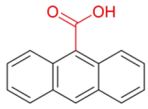
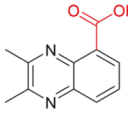



**Figure 5.** Deuterium exchange into phenazine catalyzed by PhdA. (A) (Top) $^1$H NMR spectrum for phenazine, (bottom) spectrum after incubating with PhdA overnight in buffered $D_2O$. (B) Representative mass spectra monitoring the time course for deuterium exchange in phenazine. (C) Time course for deuterium exchange into phenazine determined by peak integration of MS data; the data are shown fitted to a four-step sequential model.

**Table 1. Structures and Percentage Decarboxylation of the Alternative Substrates for PhdA Evaluated in This Study**

| Compound | Substrate | % Conversion | Compound | Substrate | % Conversion |
|---|---|---|---|---|---|
| **PCA** |  | 100 | **5** |  | 55.5 ± 3.9 |
| **1** |  | 0.14 ± 0.01 | **6** |  | 15.0 ± 1.7 |
| **2** |  | 10.2 ± 0.2 | **7** |  | 0 |
| **3** |  | 9.7 ± 0.4 | **8** |  | 0.54 ± 0.06 |
| **4** |  | 76.5 ± 6.6 | | | |

apparent $k_{cat}$ to be 2.6 ± 0.1 $s^{-1}$ and the $K_M$ for PCA to be 53 ± 2 μM. Given that our cofactor analysis showed that prFMN is present in only a fraction of the active sites, the true $k_{cat}$ is likely several fold higher.

We also investigated whether PhdA could catalyze the reverse reaction—carboxylation of phenazine. PhdA was incubated at pH 7.5 overnight at room temperature with phenazine (0.5 mM) and a high concentration of ammonium bicarbonate (2.0 M), in an attempt to shift the chemical equilibrium in favor of carboxylation. The organic compounds were then extracted, and the amount of PCA produced was determined by HPLC. A very small amount of PCA corresponding to <0.1% conversion was formed in the reaction (Figure S3), suggesting that, although reversible, the equilibrium constant for the reaction heavily favors decarboxylation.

**Reduced PCA Is Not a Substrate for Decarboxylation.** The initial report describing the characterization of PhdA left unresolved which oxidation state(s) of PCA are substrates for decarboxylation.[19] As discussed earlier, the reduced form of PCA is an electron-rich system which can, in principle, undergo an electrophilic addition reaction, similar to the mechanism proposed for AroY. To resolve the ambiguity, we prepared the fully reduced form of PCA and evaluated it as a substrate for PhdA under anaerobic conditions, as the compound is readily oxidized in air. Under these conditions, only a trace amount of phenazine was detected after an overnight incubation with PhdA, which we consider was most likely due to residual traces of oxidized PCA. Therefore, we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consider that only the oxidized form of PCA is a substrate for decarboxylation. We note that reduced PCA is expected to adopt a kinked, rather than planar, conformation. It is unclear whether it is the change in electronic structure, or the accompanying change in conformation (that may prevent it binding to PhdA), that prevents the enzyme from decarboxylating reduced PCA.

**PhdA Catalyzes Deuterium Exchange into Phenazine.** The cleavage of aromatic C−H bonds by prFMN-dependent enzymes is central to their ability to catalyze carboxylation reactions. Following the kinetics of deuterium exchange into the product provides a method to study this activation step directly, even if the overall carboxylation reaction is thermodynamically unfavorable. Therefore, to better understand this step in PhdA, we investigated the ability of the enzyme to exchange deuterium from the solvent into phenazine. In deuterated buffer, the enzyme readily exchanged up to 4 deuterium atoms into phenazine, as determined by LC-MS analysis. $^1$H NMR of the deuterated phenazine confirmed that, as expected, the chemically equivalent protons at positions 1, 4, 6, and 9 were exchanged, as is evident from the loss of the doublet of doublets at 8.25 ppm and simplification of the doublet of doublets at 7.85 ppm to a singlet (Figure 5A). In contrast to FDC, where $CO_2$ appears to be required for deuterium exchange into styrene,[12] removing dissolved $CO_2$ from the buffer did not appear to affect deuterium exchange into phenazine by PhdA (Figure S4). The time course for deuterium exchange was monitored using LC-MS, which allowed the formation of mono-, di-, tri-, and tetra-deuterated phenazine to be followed (Figure 5B). These data were well fitted by a 4-step sequential kinetic model (Figure 5C; Table S1). After correcting for the mole fraction of $D_2O$ and the ratio of phenazine to enzyme in the experiment, an apparent rate constant for exchange of the first deuterium into phenazine was calculated as 0.83 ± 0.06 min$^{-1}$. It is evident that the deuterium exchange reaction is much slower than $k_{cat}$ for the decarboxylation for PCA (2.6 s$^{-1}$), which suggests that, as might be expected, deprotonation of phenazine (or a step preceding deprotonation) is rate-limiting in the exchange reaction.

**Substrate Scope of PhdA.** We next examined the substrate range of PhdA, with the objective of determining what features of the phenazine ring system were important for substrate recognition and reactivity. The various potential aromatic carboxylic acid substrates listed in Table 1 (500 $\mu$M final concentration) were incubated with 10 $\mu$M PhdA in reaction buffer at room temperature for 17 h. The fraction of substrate decarboxylated was then determined by HPLC analysis (Figures S5 and S6), with the amount of product formed determined by reference to a standard curve for each compound (Figure S7).

Quinoxaline-5-carboxylic acid (**1**), which lacks the distal phenyl ring, proved to be a very poor substrate, with only 0.1% of the compound undergoing decarboxylation under the conditions of the reaction. However, addition of a methyl group at either the 2 or 3 positions, markedly improved reactivity. Both 2-methyl-quinoxaline-5-carboxylic (**2**) and 3-methyl-quinoxaline-5-carboxylic acid (**3**) underwent 10% decarboxylation. 2, 3-Dimethylquinoxaline-5-carboxylic acid (**4**) proved an even better substrate, undergoing 76% decarboxylation to 2, 3-dimethylquinoxaline. These results demonstrate that the extended aromatic system of the phenazine nucleus is not required for reaction; rather, the distal ring likely contributes more to substrate recognition, as its steric bulk can be substituted with methyl groups.

Lastly, we examined the contribution of the heterocyclic nitrogen atoms to the reactivity of the substrate. The isosteric compound, acridine-4-carboxylic acid (**5**) (which contains only one nitrogen atom) proved to be a substrate for PhdA and was decarboxylated to a moderate extent (56%), thereby demonstrating that the quinoxaline functionality is not required for activity. We extended this line of investigation to examine the reactivity of PhdA with unactivated polyaromatic compounds. No reaction was observed with naphthalene-1-carboxylic acid or naphthalene-2-carboxylic acid. However, anthracene-1-carboxylic acid (**6**) proved to be surprisingly reactive and underwent 15% decarboxylation under the conditions of the reaction. We further investigated the regioselectivity of PhdA decarboxylation using anthracene carboxylic acids. No reaction was observed with anthracene-2-carboxylic acid (**7**), which is consistent with the regioselectivity of deuterium exchange observed in phenazine; however, interestingly, a small amount of decarboxylation (0.5%) was observed for anthracene-9-carboxylic acid (**8**), which suggests an alternate mode of substrate binding.

## DISCUSSION

It is now apparent that the UbiD class of (de)carboxylases are widely distributed in microbes and decarboxylate a wide range of aromatic or polyunsaturated molecules. A recent phylogenetic analysis[15] identified over 200 UbiD-like decarboxylases that, by sequence analysis, divided into three subgroups: phenylacrylic acid decarboxylases, typified by FDC;[4] $\alpha,\beta$-unsaturated aliphatic decarboxylases, e.g. tautomycetin-D decarboxylase (TtnD);[15] and a large subgroup of aromatic (de)carboxylases. The reactions catalyzed by the enzymes in these subgroups most likely occur through a dipolar cycloaddition mechanism. The latter subgroup contains the archetypal 4-hydroxy-3-octaprenylbenzoic acid decarboxylase[26] (UbiD), 4-hydroxybenzoate decarboxylase,[27] 3,4-dihydroxybenzoic acid decarboxylase (AroY),[14] furan-dicarboxylate decarboxylase (HmfF)[28] and pyrrole-carboxylate decarboxylase (HudA).[29] Interestingly all the aromatic decarboxylases so far identified operate on electron-rich aromatic or heteroaromatic systems in which prFMN acts as an electrophilic catalyst, cf. the reaction catalyzed by AroY[14] (Figure 1). The one exception may be HudA, for which DFT calculations suggest that formation of a cycloadduct intermediate with pyrrole-carboxylate to be feasible; however, an electrophilic mechanism appears to better explain the observed trends in substrate reactivity.[29]

Although the reaction catalyzed by PhdA places it among the aromatic decarboxylase subgroup, the previous ambiguity surrounding the oxidation state of the substrate meant that *a priori* the reaction could plausibly proceed by either an electrophilic mechanism, if reduced PCA was the substrate, or a cycloaddition mechanism if oxidized PCA was the substrate. Our experiments clearly establish that the substrate for PhdA is oxidized PCA, which is electron-poor and thus unlikely to react by an electrophilic mechanism. An electrophilic mechanism is also inconsistent with the decarboxylation of anthracene carboxylic acids. Indeed, if we consider an electrophilic mechanism for the decarboxylation of anthracene carboxylic acids, the reactivity at C-9 should be higher than at C-1,[30] which is the opposite of what is observed.


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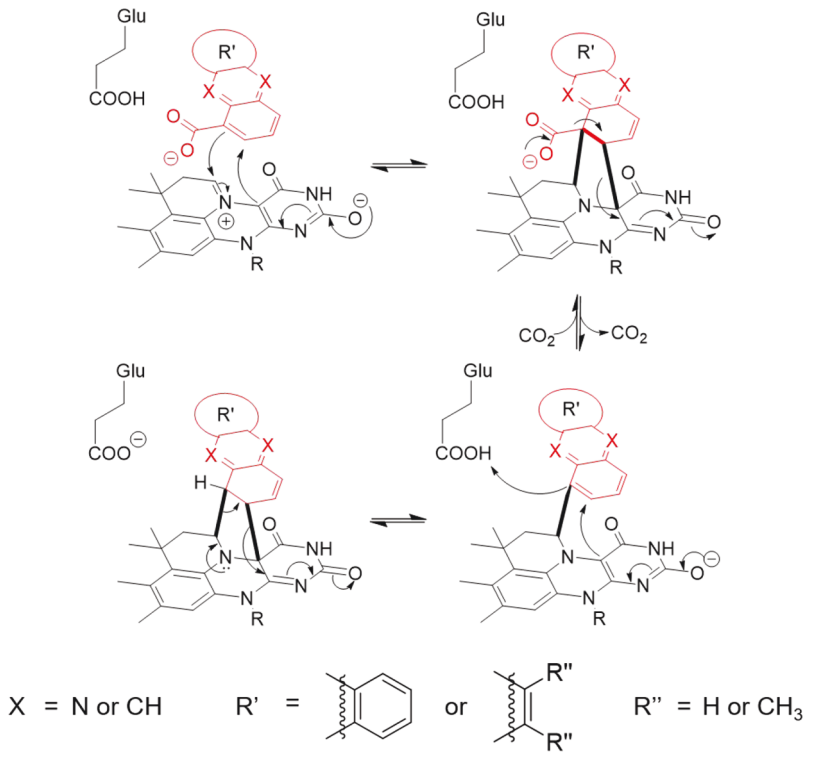


**Figure 6.** Proposed reaction mechanism for PhdA consistent with the observed substrate scope and the regioselectivity of deuterium exchange from solvent.

On the other hand, nonenzymatic reactions of anthracene and acridine with strong dipoles such as nitrile oxides form 1, 3-dipolar cycloadducts[31−33] in a regioselective manner, with the nucleophilic oxygen of nitrile oxides attacking only the C-2 position of anthracene or acridine. Assuming that a similar cycloadduct is formed for PhdA, the implied regioselectivity suggests a bond between C-4a of prFMN and C-2 of phenazine/anthracene (Figure 6). This mode of reaction is consistent with the observed regioselectivity of deuterium exchange in phenazine and the decarboxylation of anthracene carboxylic acids. Therefore, although the 1,3-dipolar cycloaddition mechanism for prFMN reacting with PCA remains to be rigorously established for PhdA, we consider this mechanism is more likely.

One promising application of UbiD (de)carboxylases lies in their potential to activate C−H bonds through carboxylation. Carboxylation reactions are valuable as they provide a facile way to functionalize otherwise unreactive hydrocarbons.[14] Although the reaction equilibrium generally favors decarboxylation,[17] it is in principle possible to couple carboxylation to a thermodynamically favorable second step such as reduction. The equilibrium constant for carboxylation of phenazine appears to be quite unfavorable as we observed only tiny amounts of PCA to be formed when the reaction was run in reverse with high concentrations of bicarbonate. The deuterium exchange experiments provide useful information as they report on the rate at which C−H cleavage occurs in the absence of a complete turnover to form carboxylic acid. In the case of PhdA reacting with phenazine, the rate of C−H cleavage calculated from deuterium exchange is ∼0.8 $min^{-1}$, which is about 200-fold slower than $k_{cat}$ for decarboxylation = ∼ 160 $min^{-1}$. These observations suggest that, even if phenazine carboxylation were coupled to a favorable second step, the reaction would proceed much more slowly in this direction, with deprotonation of the substrate as the likely rate-limiting step.

The oxidative maturation of prFMN, after its synthesis by UbiX, is still not well understood. It appears that maturation requires the UbiD enzyme in question to bind reduced prFMN prior to its oxidation for efficient reconstitution of the holoenzyme. Studies on the maturation of prFMN in FDC, the best understood system, found that incubating the enzyme with oxidized prFMN actually led to loss of activity.[34] Also, an inverse relationship was observed between the amount of oxidized prFMN present in the reconstitution reaction and the final activity of the reconstituted FDC. These observations are in accord with our observation that to efficiently reconstitute PhdA, it was necessary to remove inhibitory prFMN species prior to oxidation. Even then, subsequent on-enzyme oxidation led to 3 different prFMN species bound to PhdA (Figure 3), whereas, in contrast, only the active iminium form of prFMN is found in FDC. This hints at the possibility that other protein components might be needed as chaperones to efficiently reconstitute some UbiD-like enzymes with prFMN.

The trends for the reaction of compounds **1**, **2**, **3**, and **4** with PhdA suggest that the extended $\pi$-system afforded by the distal aromatic ring of PCA is not important for the reactivity of the substrate, but rather contributes to substrate binding. Although compound **1**, lacking the distal aromatic ring, is a very poor substrate, compound **4** in which the phenyl ring is replaced with 2 methyl groups is a quite effective substrate. Similarly, the interesting and somewhat surprising observation that anthracene-1-carboxylic acid is a substrate for decarboxylation demonstrates that the nitrogen atoms of phenazine are not required for activity. Furthermore, the broad substrate scope of PhdA, including its ability to catalyze the decarboxylation of


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unfunctionalized aromatic molecules, point to the potential utility of this enzyme for catalyzing regioselective decarboxylation and carboxylation reactions on otherwise unreactive aromatic molecules under mild conditions.

## MATERIALS AND METHODS

**Reagents and Chemicals.** Aromatic carboxylic acids and the corresponding compounds lacking carboxyl groups were purchased from Apollo Scientific Co., Sigma-Aldrich Co., ChemScene, TCI Co., Thermo Fischer Scientific Co., 1 ClickChemistry Inc., Enamine Ltd., and used without further purification. All other reagents were purchased from Sigma-Aldrich Co. or Thermo Fischer Co. Deuterated solvents were purchased from Cambridge Isotopes Laboratories Inc. or Thermo Fischer Co.

Reduced PCA was synthesized under anaerobic conditions by mixing 9−10 mM PCA with 230 mM sodium dithionite in $H_2O$. The resulting bright orange precipitate was collected by centrifugation and washed with $H_2O$ to remove excess sodium dithionite.[35]

**Strains and Plasmids.** *E. coli* Rosetta strains with pET20b(+) vector individually containing either *phdA* or *phdB* were kindly provided by Prof. Dianne Newman (Caltech). The plasmids were purified using standard methods and transformed into *E. coli* BL21DE3 (Invitrogen) to facilitate expression of the proteins. A codon-optimized gene encoding *ubiX* from *Pseudomonas aeruginosa* (*paubiX*) was commercially synthesized and subcloned in the expression vector pET28b-(+) (GenScript Biotech Co.) For coexpression of PhdA and *Pa*UbiX, pET20b(+) containing *phdA* and pET28b(+) containing *paubiX* were co-transformed in *E. coli* BL21DE3 (Invitrogen).

For co-expression of *phdA* and *phdB* genes, *phdB* was amplified from pET20b(+) vector using PCR and cloned into pET28b(+) vector between the NcoI and *Bam*HI sites using a Gibson Assembly kit (New England Biolabs). The resulting pET28b(+) vector containing *phdB* (without His tag) was co-transformed with pET20b(+) vector containing *phdA* in *E. coli* BL21 DE3.

**Protein Expression and Purification.** All *E. coli* BL21 DE3 strains were cultivated at 37 °C with shaking at 200 rpm in LB broth supplemented with 50 μg/mL Ampicillin and/or 50 μg/mL Kanamycin. After reaching an $OD_{600}$ of 0.6−0.8, protein expression was induced by adding 0.1 mM IPTG. The medium was supplemented with 1 mL of prenol as a precursor to prFMN[25] and 1 mM $MnCl_2$. The cultures were incubated overnight at 20 °C with shaking at 170 rpm. Cells were harvested by centrifugation (4 °C, 5000 rpm, for 15 min) and stored at −80 °C.

To purify proteins, cells were resuspended in buffer A (20 mM Tris/Cl pH 7.2 or pH 9.2, 500 mM KCl, 1 mM $MnCl_2$, 10 mM imidazole, 5% glycerol) supplemented with complete EDTA-free protease inhibitor cocktail (Roche) and sonicated using 3 s pulses separated by 5 s for a total time of 18−20 min. The lysate was clarified by centrifugation at 4 °C and 12 000 rpm for 45 min. PhdA was 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 1 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 10% gel (Bio-Rad). Relevant fractions of PhdA were combined, desalted using a 10-DG column (Bio-Rad) into buffer C (20 mM Tris/Cl pH 7.2 or pH 9.2, 500 mM KCl, 1 mM $MnCl_2$, 5% glycerol) and stored. paUbiX was stored directly without desalting. All purified proteins were stored at −80 °C.

**Enzymatic Synthesis of prFMN.** prFMN was synthesized under anaerobic conditions (Coy chamber with $N_2$ atmosphere containing 2.3% - 2.5% $H_2$ gas), following previously described methods.[23] 300−400 μM FMN was reduced by titrating it with sodium dithionite (5−10 mM final concentration) and mixed with 2 mM DMAP in the presence of 20−30 μM *Pa*UbiX in 20 mM Tris/Cl, pH 7.2, 100 mM KCl, and 5% glycerol. *Pa*UbiX was used directly without desalting. Interestingly, *Pa*UbiX precipitated immediately but still catalyzed almost 100% conversion of FMN to prFMN as determined by HPLC analysis. The reaction was incubated at room temperature overnight following which *Pa*UbiX was separated by centrifugation at 10 000 rpm for 15 min. The resulting supernatant was used to reconstitute PhdA.

***In Vitro* Reconstitution of holo-PhdA.** 50 μM PhdA was reconstituted with 150−200 μM prFMN under anaerobic conditions in 20 mM Tris/Cl, pH 7.2, 10 mM $MnCl_2$, 100 mM KCl, and 5% glycerol. After 10−20 min, reconstituted PhdA was desalted into 20 mM Tris/Cl, pH 7.2, 1 mM $MnCl_2$, 500 mM KCl, and 5% glycerol using Zeba spin desalting columns (Thermo Fischer Co.). The desalted protein with bound prFMN was exposed to air and allowed to oxidize. Reconstituted PhdA was stored at −80 °C. To verify the presence of prFMN, reconstituted PhdA as well as holo-PhdA purified from *E. coli* were directly injected on HPLC. The protein and cofactor separated in line and provided distinct peaks. For comparison, holo-FDC was also analyzed.

**Enzymatic Assays.** Assays to study the effect of dithionite on PhdA activity were performed under anaerobic conditions. 0.1 μM PhdA was incubated with different concentrations of sodium dithionite in 20 mM Bis-Tris/Cl buffer at pH 6.5 for 5−10 min. Reaction was initiated by addition of 100 μM PCA, and quenched at various times by adding 100 mM NaOH (final concentration); centrifuged at 14 000 rpm for 20 min, and the supernatant was analyzed by HPLC.

To study the effect of paraquat radical on PhdA activity, a solution of 1 mM paraquat dichloride was mixed with 0.5 mM sodium dithionite under anaerobic conditions. The presence of paraquat radical was evident by the rapid formation a dark blue solution. 0.2 μM PhdA was incubated with 100 μM freshly prepared paraquat radical in 20 mM Bis-Tris/Cl buffer (pH 6.5) for 10 min after which the reaction was initiated by addition of 100 μM PCA. Reactions were quenched as described above.

Activity assays of reconstituted PhdA were performed at room temperature under aerobic conditions. Typical reactions consisted of 0.2 μM PhdA and 100 μM PCA in 20 mM Bis-Tris/Cl buffer at pH 6.5. Reactions were quenched at various time points by adding 100 mM NaOH (final concentration) and analyzed using HPLC. For steady-state kinetic analyses, different concentrations of PCA were used. To detect activity in the reverse direction, 500 μM phenazine was mixed with 2 M $NH_4HCO_3$ in the presence of 10 μM reconstituted PhdA and 5% DMSO. Appropriate controls were set up in parallel. The reactions were quenched with 100 mM NaOH (final concentration) after 17 h and analyzed using HPLC.

To study the substrate scope of PhdA, 10 μM enzyme was incubated with 500 μM of the aromatic carboxylic acid under


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investigation in 20 mM Bis-Tris/Cl buffer, pH 6.5. Control reactions without PhdA were performed in parallel. Reactions were quenched after 17 h by adding an equal volume of acetonitrile, centrifuged at 14 000 rpm for 20 min, and the supernatant was analyzed using HPLC.

**HPLC Analysis.** All HPLC analysis was performed on a Shimadzu Prominence LC-20AT series chromatography system equipped with a diode array detector. A Phenomenex kinetex C18 column (5 $\mu$m particle size, 250 × 4.6 mm) was used to obtain separation at a flow rate of 0.5 mL/min and a detection wavelength of 360 nm (unless specified otherwise). The mobile phase consisted of 10 mM trifluoroacetic acid in water (buffer A) and 10 mM trifluoroacetic acid in acetonitrile (buffer B). Different methods were employed for different analytes.

For studying the conversion of FMN to prFMN, the system was used at a flow rate of 0.4 mL/min. The gradient consisted of 5% buffer B for 5 min, 5% - 100% B over 25 min and 100% B for 5 min. This was followed by a re-equilibration at 5% B for 5 min.

For separating PCA and phenazine the column was subjected to 5% B over 1 min, 5−55% B over 1 min, held at 55% B for 3 min, a slow gradient from 55−60% B over 10 min, 60−95% B over 1 min, 95% B for 3 min and re-equilibration at 5% B for 6 min.

For separating compound **4** from the product, 2,3-dimethylquinoxaline, a similar gradient was employed with initial equilibration at 5% B for 1 min, 5−45% B over 9 min, held at 45% B for 5 min, 45−95% B over 1 min, held at 95% B over 4 min and re-equilibration at 5% B for 5 min. Detection was carried out at 310 nm. For studying conversion of compounds **5**, **6**, **7**, and **8** to their respective hydrocarbons, the system was equilibrated at 50% B for 5 min followed by a gradient from 50−100% B over 10 min, holding at 100% B for 5 min and re-equilibrating to 50% B for 7 min.

For separating **1**, **2**, and **3** from their respective products, the following modified solvent system was used: Buffer A consisted of 10 mM Tris/Cl pH 7.2 in water and buffer B was 10 mM Tris/Cl pH 7.2 in 60% acetonitrile:40% water mixture. The detection wavelength was 310 nm. Initially, column was equilibrated with 10% B for 5 min followed by a gradient from 10−100% B over 7 min, holding at 100% B for 5 min and re-equilibration to 10% B over 5 min.

For each substrate analyzed, the amount of product formed was determined by reference to a standard curve constructed from known amounts of an authentic standard, see Figure S7.

**H/D Exchange Assays.** Reactions were performed in $D_2O$ with 0.5−10% DMSO-$d_6$, 50−500 $\mu$M phenazine, and 5 $\mu$M reconstituted PhdA. Control reactions without the enzyme were also performed. After overnight incubation, the reactions were quenched and analyzed by either LC-MS or NMR. To study deuterium exchange under low $CO_2$ levels, $CO_2$ was removed by bubbling argon gas for 6−8 h through the buffers, with gaseous $CO_2$ removed by an in-line 5 M KOH trap.

For LC-MS analysis, the reactions were quenched by adding 100 mM NaOD (final concentration), centrifuged at 14 000 rpm for 20 min, and the supernatant was injected onto an Agilent 1290 series LC system equipped with an Agilent 6545 quadrupole-TOF mass spectrometer. An Agilent Zorbax Eclipse Plus C18 column (1.8 $\mu$m particle size, 50 × 2.1 mm) was used at a flow rate of 0.4 mL/min. Analytes were eluted with 0.1% formic acid in water (buffer A) and 0.1% formic acid in 95% acetonitrile, 5% water (buffer B). The method consisted of 5% B for 1 min followed by a gradient from 5% - 95% B over 3 min, 95% B for 1 min and a final re-equilibration to 5% B over 1 min. Mass acquisition was carried out in positive ion mode from 50−1200 $m/z$.

For NMR analysis, the reactions were quenched by adding an equal volume of $CDCl_3$, vortexed to extract the phenazine in $CDCl_3$, and centrifuged at 14 000 rpm for 10 min. The $CDCl_3$ layer was collected separately, dried with $Na_2SO_4$, and analyzed on a Varian MR 400 MHz spectrometer.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c03040.

Apparent rate constants, effect of including paraquat in addition to sodium dithionite on PhdA activity, HPLC analysis of the reaction products obtained by in vitro synthesis of prFMN by UbiX, formation of PCA from phenazine and ammonium bicarbonate after overnight reaction, effect of dissolved $CO_2$ on PhdA-catalyzed solvent deuterium exchange into phenazine, representative HPLC chromatograms monitoring the decarboxylation of compounds (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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