Enzyme Catalysis

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Mechanistic Studies on the Conversion of Arylamines into Arylnitro Compounds by Aminopyrrolnitrin Oxygenase: Identification of Intermediates and Kinetic Studies**

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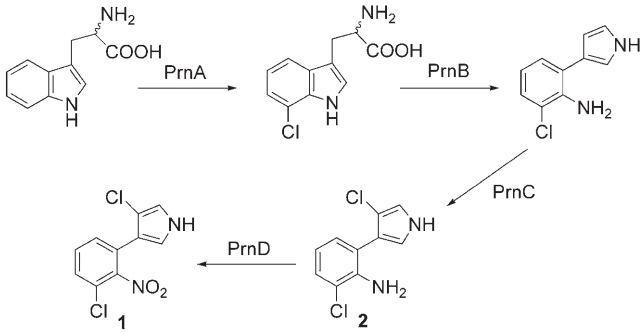
Rieske oxygenases are widespread in nature and catalyze a diverse set of oxidation reactions including *cis*-dihydroxylation, monohydroxylation, desaturation, sulfoxidation, and *O*- and *N*-dealkylation.[1,2] Recently, we reported the characterization of a Rieske *N*-oxygenase, aminopyrrolnitrin oxygenase (PrnD), which catalyzes unusual arylamine oxidation reactions.[3] Although arylamine oxidases seem to be widely distributed and used in a variety of metabolic reactions,[4–9] PrnD represents one of only two known examples of arylamine oxidases or *N*-oxygenases involved in the formation of nitro groups, the other being AurF involved in aureothin biosynthesis.[9] PrnD is involved in the biosynthesis of antibiotic pyrrolnitrin[3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole] (**1**), which is produced by many Pseudomonads and displays broad-spectrum antifungal activity.[10] In the proposed biosynthetic pathway of **1** (Scheme 1),[11] PrnD catalyzes the oxidation of the amino group of aminopyrrolnitrin (**2**) to a nitro group to form **1**. Although PrnD has been characterized, no experimental evidence has been available for the mechanism of conversion of the arylamine. Therefore,



Scheme 1. Proposed biosynthetic pathway for pyrrolnitrin **1**.[11]

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we decided to investigate the intermediates formed during the conversion of arylamine into arylnitro compounds, as these might provide valuable mechanistic information.

Rieske oxygenases are typically comprised of two protein components: a terminal oxygenase and a flavin reductase.[12] Thus, a reductase was deemed necessary for the function of PrnD. On the basis of the nonspecificity of the flavin reductase,[21] the unknown reductase for PrnD from the *Pseudomonas* strain was substituted by flavin reductase SsuE from *E. coli*, with optimization of the concentration of SsuE for PrnD activity.[3] As compound **2**, the physiological substrate for PrnD, is not commercially available and is difficult to obtain in large amounts either from natural sources or by chemical synthesis, we used an alternative substrate 4-aminobenzylamine (pABA) to investigate the conversion mechanism of the arylamine. To observe the intermediates, PrnD was rapidly mixed with a 1 mM solution of pABA at 15 °C to slow the reaction, and the reaction mixture was analyzed every 30 min. Rieske [2Fe-2S] cluster-reconstituted PrnD[3] in combination with SsuE reductase showed a significant conversion of the substrate pABA to produce several compounds that migrated in HPLC with retention times of 4.9 min (authentic 4-nitrobenzylamine (pNBA)), 1.9 min (product A), and 4.5 min (product B; Figure 1). These products were absent if heat-denaturated

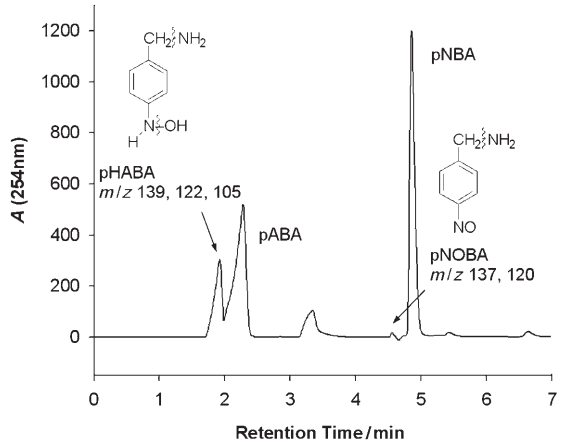


Figure 1. HPLC analysis of the PrnD reaction product. Each product was identified by LC-MS/MS analysis, and the fragmentation patterns are indicated.

PrnD was used or if pABA was omitted from the assay which indicated that the new metabolites were derived enzymatically from pABA. LC-MS/MS analysis of products A and B revealed that these compounds showed the calculated masses ($[M+H]^+$: m/z 139 and 137) of 4-hydroxylaminobenzylamine (pHABA) and 4-nitrosobenzylamine (pNOBA), respectively. Furthermore, it showed the characteristic fragmentation pattern of a hydroxylamine derivative (m/z 122 $[M-16]^+$ and 105 $[M-33]^+$) and a nitroso derivative (m/z 120 $[M-16]^+$), respectively. Thus, pHABA and pNOBA can be considered as intermediates or dead-end products in the dioxygenation of pABA by PrnD. To establish that the hydroxylamine and nitroso compounds are the intermediates in the dioxygenation reaction, it is necessary to show that the partial reaction does occur and that it does so at a rate equal to or greater than the rate of the overall reaction. The intermediacy of the hydroxylamine and nitroso compounds was further assessed through their chemical synthesis and kinetic characterization.

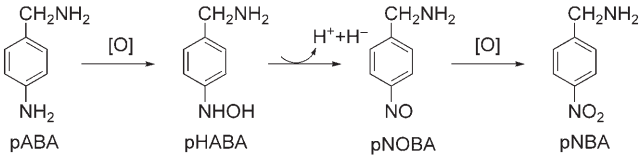
The intermediates pHABA and pNOBA were synthesized from pNBA and pABA, respectively, as described previously[14,15] and were characterized by ^{1}H NMR spectrometry and high-resolution (EI) mass spectrometry.[16] Initial-velocity studies were performed with pHABA and pNOBA as variable substrates in the presence of a fixed concentration of PrnD (≈ 1 μM). The data presented in Table 1 demonstrate

Table 1: Kinetic constants for aminopyrrolnitrin oxygenase.[a]

Substrate	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [μM^{-1} min^{-1}]
2	6.8 ± 0.59	191 ± 20	0.036 ± 0.004
pABA	6.5 ± 0.62	379 ± 34	0.017 ± 0.002
pHABA	7.9 ± 0.98	404 ± 56	0.019 ± 0.003
pNOBA	84.5 ± 9.72	580 ± 73	0.146 ± 0.021

[a] K_m = Michaelis constant.

that PrnD catalyzes the oxygenation of pHABA and pNOBA to the common product pNBA, indicating that the rate of oxygenation of the intermediates was high enough for them to be intermediates in the dioxygenation of the arylamine catalyzed by PrnD. As could be predicted from the fact that only small amounts of nitroso intermediate can be detected in the reaction mixture catalyzed by PrnD, the turnover rate of the nitroso compound is approximately 13-fold greater than the overall rate of dioxygenation. Thus, PrnD catalyzes at least three consecutive reactions (Scheme 2), in which the



Scheme 2. Proposed mechanism for bioconversion of arylamines into arylnitro compounds by PrnD.

conversion of arylamine into arylnitro compounds proceeds in two monooxygenation steps and one dehydrogenation step, via hydroxylamine and nitroso compounds as intermediates. The involvement of radical species in the reaction catalyzed by PrnD was excluded in the previous studies.[3]

To investigate the kinetic competence of intermediate pHABA, several PrnD mutants were prepared. The position of site-directed mutagenesis was decided based on homology modeling followed by analysis of the substrate-binding pocket. A homology model of PrnD was built based on the structure of the α-subunit of the naphthalene dioxygenase of *Pseudomonas* sp. strain NCIB 9816-4—the Rieske oxygenase for which a crystal structure was determined.[17] The α-subunit contains a Rieske [2Fe-2S] center and mononuclear non-heme-iron site, which is believed to be the site of activation of molecular oxygen and substrate oxygenation.[18] In consider-

ation of the hydrophobicity of the residues and the distance between the substrate and the residues in the substrate-binding pocket, three residues (D269, F312, W209) were mutated. The catalytic activities of PrnD mutants of D269A, F312V, and W209A toward pHABA were determined and compared with those of pABA. Wild-type (WT) PrnD exhibited similar activity toward pHABA and pABA, consistent with the notion that formation of intermediate pHABA is a rapid step in WT PrnD. F312V and W209A mutants showed no conversion of either pHABA or pABA. However, D269A mutant PrnD showed much higher activities toward the intermediate pHABA ($k_{cat} = 8.4\ min^{-1}$, $k_{cat}/K_m = 0.020\ \mu M^{-1} s^{-1}$) than pABA ($k_{cat} = 0.3\ min^{-1}$, $k_{cat}/K_m = 0.004\ \mu M^{-1} s^{-1}$). These results indicate that the hydroxylamine intermediate is kinetically competent. In addition, the accumulation of the hydroxylamine intermediate in WT PrnD indicates that its formation is fast relative to its decay. The lack of significant accumulation of the nitroso intermediate suggests that it decays faster than it is formed. Thus, the rate-limiting step is the dehydrogenation of pHABA to give pNOBA. Of note, in the absence of NADPH flavin reductase system, the hydroxylamine intermediate was not oxidized by O_2 to the nitroso intermediate and H_2O_2 was not formed. Artificial electron acceptors such as methylene blue and ferricyanide were less than 10% as effective as O_2 under anoxic conditions. In addition, the D269A mutation significantly slows down the formation of hydroxylamine, suggesting that Asp269 is involved in the early steps of the reaction and likely responsible for the abstraction of a proton from the substrate. A homology model shows that Asp269 is properly positioned in the substrate-binding pocket to perform the proposed function.

To confirm whether one or both oxygen atoms of O_2 were incorporated into the product, we carried out isotope-labeling experiments with $^{18}O_2$. Incorporation of the label was analyzed by ESI/LC-MS. In the first experiment, PrnD was incubated with pABA in an $^{18}O_2$ atmosphere. Unlabeled pNBA showed the molecular ion, $[M+H]^+$, at m/z 153. In contrast, most of the pNBA produced under the $^{18}O_2$ atmosphere revealed the molecular ion at m/z 157, indicating the incorporation of two ^{18}O atoms. In addition, incorporation of one ^{18}O atom into pHABA was demonstrated by the signal for the molecular ion at m/z 141, in comparison to m/z 139 for the unlabeled pHABA. For the second labeling experiment, unlabeled pHABA and pNOBA were incubated in an $^{18}O_2$ atmosphere with PrnD. The incorporation of one ^{18}O atom into pNBA was demonstrated by the molecular ion at m/z 155. In addition, no ^{18}O was incorporated into pNOBA in the reaction of pHABA with PrnD, ruling out the possibility of the involvement of the dihydroxylamine intermediate. Thus two ^{18}O atoms are sequentially incorporated into the product, confirming that the dioxygenation by PrnD proceeds in a consecutive monooxygenase-type reaction. Note that some iron(II)-dependent oxygenases have been reported to be bifunctional or even trifunctional, catalyzing several consecutive oxidative transformations within a single biosynthetic pathway.[19,20]

In this study, we have obtained direct evidence for the involvement of hydroxylamine and nitroso intermediates in the PrnD-catalyzed oxygenation reaction of arylamines, substantiating for the first time the catalytic mechanism for the conversion of arylamines into arylnitro compounds. The intermediates are at least partially released from the active site of the enzyme during catalysis, providing a rare example of three consecutive chemical reactions catalyzed by one active site. This may be the primary mechanism by which arylamines are oxygenated to give arylnitro compounds in biological processes. The characterization of PrnD adds a new and interesting member to the family of Rieske nonheme-iron oxygenases and demonstrates the pathway for the formation of arylnitro compounds in nature.

Experimental Section

Site-directed mutagenesis of pTKXb-PrnD was carried out by using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The pTKXb-prnD plasmid was used as the DNA template.[3] Trp209 and Asp269 residues were mutated to Ala individually. Residue Phe312 was mutated to Val. The plasmids containing the correct mutant genes were cloned into pMAL-c2x and then used to transform *E. coli* BL21(DE3), and colonies selected by ampicillin resistance were used for protein expression. The PrnD mutants were expressed, purified, and reconstituted with Fe^{2+} and S^{2-} according to the same procedure for the wild-type enzyme, as described elsewhere.[3]

Enzyme activity was routinely assayed by HPLC. The assay mixture (0.5 mL final volume) contained 500 μM NADPH, 3 μM FMN, 500 μM substrate, and SsuE and PrnD (SsuE/PrnD molar ratio of 4.0) in 20 mM TrisHCl at pH 7.8 and was stirred at 30 °C. Reactions were initiated by addition of PrnD to the reaction mixture and analyzed by HPLC. One unit of activity was defined as the amount of enzyme forming 1 μmol of product per minute at 30 °C under standard assay conditions, calculated from the rate of substrate depletion. Kinetic parameters determined in atmospheric oxygen were obtained by fitting the data to the Michaelis–Menten equation.

For labeling studies with $^{18}O_2$, two vials, one containing substrate and one containing holo-PrnD reaction mixture, were degassed by application of a vacuum and flushed with argon three times. The anaerobic holo-PrnD solution was transferred to the vial containing the substrate. The argon was removed by application of a vacuum, and finally $^{18}O_2$ was allowed to enter into the vial. After incubation for 1 h at 30 °C, the reaction was analyzed by HPLC coupled to an electrospray ionization (EI) mass spectrometer (TSQ Quantum, ThermoFinnigan, San Jose, CA) in positive-ion mode. A linear gradient of MeOH (0–75%) in aqueous acetic acid (0.1%) was used.

Products of enzyme reactions were analyzed by an Agilent 1100 Series HPLC System. The sample was eluted on a ZORBAX SB-C8 Column (4.6 × 150 mm^2, Agilent). HPLC parameters were as follows: 25 °C; solvent A: 1% acetic acid in water; solvent B: methanol; gradient: 5% B for 2 min; then to 100% B in 18 min, and finally maintain at 100% B for 2 min; flow rate: 1.0 mL min^{-1}; detection by UV absorbance at 254 nm. Hydrogen peroxide was determined by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in the presence of horseradish peroxidase at 725 nm, as described previously.[21]

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