Kinetic mechanism of ornithine hydroxylase (PvdA) from
*Pseudomonas aeruginosa*: substrate triggering of $O_2$ addition but not flavin reduction†

Kathleen M. Meneely‡, Eric W. Barr§, J. Martin Bollinger Jr.§, and Audrey L. Lamb*,‡

‡Department of Molecular Biosciences, University of Kansas, 1200 Sunnyside Avenue, Lawrence, Kansas 66045
§Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802.

Abstract

PvdA catalyzes the hydroxylation of the sidechain primary amine of ornithine in the initial step of the biosynthesis of the *Pseudomonas aeruginosa* siderophore pyoverdin. The reaction requires FAD, NADPH, and $O_2$. PvdA uses the same co-substrates as several flavin-dependent hydroxylases that differ one from another in the kinetic mechanisms of their oxidative and reductive half-reactions. Therefore, the mechanism of PvdA was determined by absorption stopped-flow experiments. By contrast to some flavin-dependent hydroxylases (notably, *p*-hydroxybenzoate hydroxylase), binding of the hydroxylation target is not required to trigger reduction of the flavin by NADPH: the reductive half-reaction is equally facile in the presence and absence of ornithine. Reaction of $O_2$ with FADH$_2$ in the oxidative half-reaction is accelerated by ornithine 80-fold, providing a mechanism by which PvdA can ensure coupling of NADPH and ornithine oxidation. In the presence of ornithine, the expected C(4a)-hydroperoxyflavin intermediate with 390-nm absorption accumulates and decays to the C(4a)-hydroxyflavin in a kinetically competent fashion. The slower oxidative half-reaction that occurs in the absence of ornithine involves accumulation of an oxygenated flavin species and two subsequent states that are tentatively assigned as C(4a)-peroxy- and -hydroperoxyflavin intermediates and the oxidized flavin. The enzyme generates stoichiometric hydrogen peroxide in lieu of hydroxyornithine. The data suggest that PvdA employs a kinetic mechanism that is a hybrid of those previously documented for other flavin-dependent hydroxylases.

The opportunistic human pathogen *Pseudomonas aeruginosa* produces pyoverdin, a hydroxamate siderophore, to scavenge iron in the iron-limiting conditions of the host, a process that has been linked to virulence (1–3). Pyoverdin contains a chromophore and a peptide backbone of 8–12 amino acids, formed by nonribosomal peptide synthetase enzymes (NRPS) (2,4,5). The amino acids of the peptide backbone are both proteinogenic and non-proteinogenic and include two formyl-hydroxyornithines. These two unusual amino acids provide hydroxamate ligands for iron chelation. Two accessory enzymes to the NRPS, the ornithine hydroxylase (PvdA) (6,7) and the hydroxyornithine transformylase (PvdF) (8,9), prepare ornithine for incorporation into the siderophore by the NRPS assembly system.

PvdA was recently characterized by steady-state kinetics and found to require both FAD and NADPH and to have high specificity for ornithine (Figure 1A) (10). The enzyme was tested

†This publication was made possible by NIH Grant Number P20 RR-17708-05 from the National Center for Research Resources of the National Institute of Health. K.M.M. was a recipient of a National Institutes of Health Predoctoral Training Grant Fellowship (GM08545).

*corresponding author; phone: (785)864-5075; fax: (785)864–5294; email: E-mail: lamb@ku.edu.
for activity by two methods: the formation of hydroxylated product, which showed inhibition at high ornithine concentrations, and the oxidation of NADPH, which demonstrated simple saturation kinetics. PvdA was inhibited by chloride ions, and lysine served as a non-substrate effector, activating NADPH oxidation without the formation of hydroxylysine. It was noted that, in several respects, PvdA is functionally homologous to para-hydroxybenzoate hydroxylase (PHBH) and flavin-containing monooxygenase (FMO), two monooxygenases to which PvdA is comparably similar (∼35% at the level of primary structure). In both PHBH and FMO, addition of O₂ to the reduced flavin generates a C(4a)-hydroperoxyflavin intermediate, which decays by substrate hydroxylation to the C(4a)-hydroxyflavin (11–15). This flavin intermediate sequence has been demonstrated by stopped-flow absorption and fluorescence experiments, in which both the hydroperoxy- and hydroxyflavins have absorbance maxima at 375–395 nm, whereas the hydroxyflavin is also highly fluorescent (excitation ∼400 nm and emission > 500 nm) (16–19). Despite the similarity of their chemical mechanisms, the two enzymes (PHBH and FMO) employ different kinetic mechanisms. PHBH requires the presence of substrate for the reduction of the flavin by NADPH (Figure 1B), which is hypothesized to be a regulatory mechanism to prevent uncoupled NADPH oxidation (12, 13, 18). In contrast, the flavin of FMO can be reduced by NADPH regardless of whether the target substrate is present (Figure 1C). The C(4a)-hydroperoxyflavin intermediate produced by subsequent addition of O₂ to the reduced cofactor is remarkably stable (half-life ∼2 hrs) and awaits binding of the substrate. The protection of the intermediate ensures that, once NADPH has been oxidized, substrate oxidation ensues (11, 14, 18, 20). Thus, PHBH and FMO embody two distinct mechanistic strategies to ensure coupling of NADPH and substrate oxidation.

The previous steady-state kinetic experiments on PvdA suggested that FAD reduction does not require ornithine. However, the upper limit that could be set for the half-life of the presumptive hydroxylating intermediate was much shorter than would seem to be required for PvdA to use the FMO coupling strategy (10). This work thus raised the question of whether PvdA ensures coupling of NADPH oxidation and ornithine hydroxylation, and how the enzyme might accomplish that coupling. Here, we have used stopped-flow absorption measurements to elucidate the PvdA mechanism. The results show that, whereas the enzyme follows the canonical chemical mechanism involving the key C(4a)-hydroperoxyflavin intermediate, PvdA coupling is regulated by a mechanism that is distinct from those employed by PHBH and FMO. This novel mechanism involves substrate triggering of O₂ addition to the reduced flavin.

Materials and Methods

PvdA Purification

PvdA protein was over-expressed and purified as described previously (10). The purified protein in 100 mM potassium phosphate, pH 8.0 with 100 mM sodium citrate was concentrated to 260 – 290 µM using an Amicon stirred-cell.

Stopped-Flow Absorption

Stopped-flow experiments were performed at 22 °C using an Applied Photophysics SX.18MV stopped-flow apparatus (Surrey, U.K.) equipped with a photomultiplier detector housed in an anoxic chamber (MBraun). Absorbance traces were recorded with an optical path length of 1 cm. For the experiments determining the dependence of the flavin intermediate formation on [O₂], the O₂-free PvdA-flavin-NADPH complex was mixed in the stopped-flow apparatus with buffer containing varying [O₂], which were prepared by mixing appropriate volumes of O₂-saturated and O₂-free buffer. The O₂-saturated buffer was equilibrated at 22 °C, resulting in a solution with an oxygen concentration of 1.3 mM (21). These experiments were conducted
with and without ornithine. The stopped-flow mixing ratio was 1:1. For multi-wavelength detection, a photodiode array detector was used in place of the photomultiplier. Experimental details are given in the figure legends. Curve-fitting analysis was performed with KaleidaGraph 4 (Synergy Software) to determine experimental rate constants from best-fit curves.

Hydrogen peroxide formation in the absence of substrate

The amount of NADPH oxidation and hydrogen peroxide formed were measured as follows. PvdA (5 µM) was incubated with 0.03 mM FAD and 0.15 mM NADPH in 100 mM potassium phosphate, pH 8.0 in a 1 ml final volume. NADPH oxidation was measured continuously for 4 minutes by monitoring the decrease in absorbance at 366 nm (ε = 2,850 M⁻¹ cm⁻¹) with a BioMate 3 spectrometer (Thermo Spectronics) at 24 °C (10). The amount of hydrogen peroxide produced was determined independently with an Amplex Red (Invitrogen) assay measuring the increase in absorbance at 572 nm (ε = 72,000 M⁻¹ cm⁻¹) at 24 °C. At 4 minutes, 250 µl of the assay was diluted into 750 µl of 100 mM potassium phosphate, pH 8.0 containing 0.02 mg/ml horseradish peroxidase and 0.05 mM Amplex Red. The change in absorbance was measured in triplicate with the BioMate 3 spectrometer.

Results

Rapid reaction kinetics

As with all flavin-dependent monooxygenases, the flavin chemistry by which PvdA effects hydroxylation of ornithine (Orn) can be studied as two half-reactions. First, FAD bound to the enzyme is reduced by NADPH in the reductive half-reaction. This is followed by the oxidative half-reaction, in which O₂ adds to the enzyme-bound FADH₂ and hydroxyornithine (OrnOH) is produced. FADH₂ oxidation is expected also to occur in the absence of substrate. In this case, production of hydrogen peroxide accounts for the two oxidizing equivalents that are not transferred to ornithine. Using stopped-flow experiments monitoring absorbance, the flavin reaction intermediates were detected and rate constants determined for each catalytic step.

Conditions for reduction of PvdA-bound FAD

The previously determined dissociation constant for PvdA•FAD (26 µM (10); 10 µM (22)) is indicative of relatively weak binding. To limit the complications of free FAD in solution, all experiments were conducted such that FAD was predominantly bound to enzyme, and the PvdA•FAD complex was saturated with NADPH. Concentrations amenable to spectroscopic observation were (post-mixing): PvdA (145 µM), FAD (30 µM) and NADPH (180 µM).

Reduction of flavin is ornithine independent

Several well-studied flavin-dependent monooxygenases require that the substrate be bound for NADH or NADPH to reduce the flavin (FAD or FMN), whereas others have no such requirement. To determine if reduction is dependent on ornithine for PvdA, the kinetics of the reduction of FAD by NADPH were examined (Figure 2). Enzyme that had been pre-mixed with no, one or two substrates was mixed in the stopped-flow apparatus with the other substrate(s). The effect of ornithine on the reduction kinetics was examined by separately including or omitting the substrate in the stopped-flow solutions. Pre-incubation of enzyme with ornithine, with or without flavin, and mixing with the remaining co-substrate(s) was also examined. A family of bi-phasic curves, presumably representing FAD reduction followed by NADP⁺ release, was generated. Flavin reduction occurs in the absence of ornithine, and the presence of the hydroxylation target accelerates the reaction by at most 2-fold. By comparison, p-hydroxybenzoate accelerates flavin reduction in PHBH by 10⁴–10⁵-fold (12,13). The data in Figure 2 do not resolve the order of addition of all components, and further experiments monitoring the rate of reduction as a function of NADPH and ornithine concentrations are
warranted to establish binding order. The important point is that FAD reduction by NADPH is essentially equally facile in the presence and absence of ornithine, inconsistent with the possibility that PvdA ensures coupling by the same mechanism employed by PHBH.

**Formation of the flavin intermediates in the presence of ornithine**

The oxidative half-reaction was investigated by mixing a solution containing PvdA (290 µM), FAD (60 µM), NADPH (360 µM) and ornithine (10 mM) with O₂-containing buffer (1.3 mM) (Figure 3A). The hydroperoxyflavin intermediate accumulates rapidly under these reaction conditions, as evidenced by the characteristic absorption feature at 380 nm, which is similar to those from C(4a)-hydroperoxyflavin intermediates in related monooxygenases (23, 24). Measuring at 390 nm, the observed first-order rate constant for formation of the hydroperoxyflavin intermediate is linearly dependent on the oxygen concentration (Figure 3B). From this plot, the second-order rate constant for oxygen addition can be estimated as $150 \pm 30 \text{ mM}^{-1}\text{s}^{-1}$. In Figure 3C, the increase in absorbance at 390 nm without an increase in 450 nm is also evident, correlating to this formation of hydroperoxyflavin. The oxygen is transferred from the hydroperoxyflavin to the substrate ornithine to produce the C(4a)-hydroxyflavin and the hydroxylated product, detected as an increase in absorbance at both 390 and 450 nm. The rate constant determined from the absorbance trace at 450 nm is $3.5 \pm 0.7 \text{ s}^{-1}$. The final species formed is the oxidized flavin, as a result of the dehydration of the hydroxyflavin. This species is characterized by an increase in absorbance at 450 nm and decrease in absorbance at 390 nm. The rate constant determined from the absorbance trace at 450 nm is $0.6 \pm 0.1 \text{ s}^{-1}$. The rates reported here are consistent with the steady-state $k_{cat}$ previously reported to be $0.4 \text{ s}^{-1}$, which was determined with enzyme pre-equilibrated with excess FAD and NADPH (10).

**Formation of the flavin intermediates in the absence of ornithine**

The oxidative half-reaction was also investigated in the absence of ornithine by mixing a solution containing PvdA (290 µM), FAD (60 µM), and NADPH (360 µM) with O₂-containing buffer (1.3 mM). Close inspection of Figure 4A indicates that, in the first reliable spectrum (0.003 s), the absorbance maximum is at 361 nm, which shifts to 376 nm at later timepoints. In the reactions of both cyclohexanone monooxygenase (CHMO) (25) and phenol hydroxylase (PHHY) (26–28), C(4a)-peroxyflavin intermediates have been detected. For CHMO, the intermediate is characterized by a wavelength of maximum absorbance ($\lambda_{max}$) of 366 nm, blue-shifted from the 383-nm $\lambda_{max}$ for the hydroperoxyflavin intermediate. Therefore, we have assigned the first flavin intermediate as a peroxyflavin ($k_{obs} = 1.8 \pm 0.4 \text{ mM}^{-1}\text{s}^{-1}$). Interestingly, this species shows an increase in absorbance at 450 nm, which may also be attributed to oxidized flavin in these turnover experiments (Figure 4B). After the formation of the peroxyflavin, the next step is protonation to form the hydroperoxyflavin. This second step is characterized by the spectral shift documented above and a decrease in absorbance at 450 nm, whereas the absorbance at 390 remains unchanged, characteristic of the hydroperoxyflavin detected in the presence of ornithine (Figure 4B). Finally, oxidized flavin begins to form by 100 s, as the increasing absorbance at 450 nm and decreasing absorbance at 390 nm indicate. This phase presumably represents the slow decay of the hydroperoxyflavin to the oxidized flavin with production of hydrogen peroxide rather than hydroxylated product.

**Hydrogen peroxide detection when ornithine is absent**

To confirm the formation of hydrogen peroxide in the reaction without ornithine, and to correlate H₂O₂ production to NADPH oxidation, the amount of NADPH oxidation and hydrogen peroxide formed in the absence of substrate were measured. At a four-minute timepoint, $7 \pm 2$ nmol of NADPH were oxidized while $6.6 \pm 0.2$ nmol of hydrogen peroxide
were formed. Therefore, in the absence of the substrate ornithine, the hydroperoxyflavin decays to form oxidized flavin, and hydrogen peroxide is released.

**Discussion**

Flavin monooxygenases use a variety of methods to prevent wasteful NADPH consumption. In the case of PHBH (12,13) and PHHY (29), the flavin reduction is greatly accelerated by the addition of substrate. Other flavin-containing enzymes are reduced quickly regardless of the presence or absence of substrate. In the case of FMO (11,14,20), addition of oxygen then forms a long-lived hyroperoxyflavin as the enzyme awaits substrate, whereas CHMO (25) forms a peroxyflavin. Excess substrate causes uncoupling of NADPH oxidation and ornithine hydroxylation in PvdA (10). At physiological ornithine concentrations, we hypothesize that PvdA uses a hybrid of the methods for reaction coupling already demonstrated for flavin monooxygenases. Reduction of the flavin by NADPH is independent of the presence of ornithine, and addition of the oxygen to the flavin is accelerated by the presence of ornithine.

In Figure 5 we have redrawn the $A_{390}$ traces from Figure 3B and Figure 4B to emphasize the delay in oxygen addition to the flavin due to the absence of ornithine.

We propose that PvdA produces a peroxyflavin intermediate, also seen previously in cyclohexanone monooxygenase (25) and phenol hydroxylase (26–29). For CHMO, the peroxyflavin ($\lambda_{\text{max}} = 366$ nm) is the reactive intermediate (the hydroperoxyflavin, $\lambda_{\text{max}} = 383$ nm, is not reactive) to perform a Baeyer-Villiger rearrangement in which the cyclic ketone is converted to e-caprolactone (25). In addition to their absorption signatures, the crucial evidence for the assignment of the two intermediates as protonated and unprotonated peroxide complexes is that they can be inter-converted by varying pH. In PvdA, the spectral shift is clearly evident without ornithine (Figure 4A) and is also evident with ornithine at very early timepoints (Figure 3A), suggesting that an unprotonated peroxide may also form early in the PvdA reaction and be rapidly converted to the C(4a)-hydroperoxyflavin by protonation. In the absence of ornithine, the formation of the peroxyflavin is 80-fold slower than in the presence of ornithine. The peroxy-and hydroperoxyflavin intermediates of CHMO are in equilibrium, and the reported p$K_a$ for this proton is 8.4 (25). In PvdA, it is tempting to hypothesize that the protonation of the peroxyflavin to make the hydroperoxyflavin is linked to deprotonation of the ornithine ($pK_a = 8.7$, (30,31)) making the ornithine reactive, ready to accept a hydroxyl group (Scheme 1). The similarity of the ornithine and hydroperoxyflavin p$K_a$s bolsters our hypothesis that the ornithine deprotonation and peroxyflavin protonation may be linked.

PvdA was previously hypothesized to be mechanistically similar to either PHBH or FMO (10). PvdA is comparably similar to both enzymes (~35% sequence similarity) despite the lack of structural homology between PHBH and FMO. Based on this new mechanistic information, the sequence of PvdA and PHHY were aligned. With the removal of the C-terminal domain of unknown function of PHHY (32) and alignment of the proposed co-enzyme and co-substrate binding regions, the similarity reaches 50% (20% identical). We therefore hypothesize that PvdA may be most similar in structure to the two N-terminal domains of PHHY.

**Conclusions**

PvdA, the ornithine monooxygenase from *Pseudomonas aeruginosa*, demonstrates a unique kinetic mechanism. In the reductive half-reaction, substrate is not required for NADPH-reduction of FAD. The oxidative half-reaction includes three flavin intermediates: peroxy-, hydroperoxy-, and hydroxyflavins; of which the hydroperoxyflavin is the reactive intermediate. Therefore, ornithine accelerates oxygen addition to the flavin, suggesting a novel mechanism for the conservation of NADPH in the absence of substrate.
Abbreviations and Full Textual Notes

CHMO, cyclohexanone monooxygenase from Acinetobacter sp.
F_{HOO}^-, peroxyflavin
F_{HOOH}, hydroperoxyflavin
F_{HOH}, hydroxyflavin
F_{ox}, oxidized FAD
F_{red}, reduced FAD
FMO, flavin-containing monooxygenase
NRPS, nonribosomal peptide synthetase
Orn, ornithine
OrnOH, N^5-hydroxyornithine
PvdA, ornithine hydroxylase or L-ornithine N^5-oxygenase from Pseudomonas aeruginosa
PvdF, hydroxyornithine transformylase from Pseudomonas aeruginosa
PHBH, para-hydroxybenzoate hydroxylase from Pseudomonas fluorescens
PHHY, phenol hydroxylase from Trichosporon cutaneum.

Acknowledgements

We thank T. C. Gamblin for the use of the Cary WinUV Microplate Spectrophotometer. We are indebted to B. Palfey for continued discussions about flavin intermediates.

References


Figure 1.
Reaction catalyzed by (A) ornithine hydroxylase (PvdA) from *P. aeruginosa*, (B) p-hydroxybenzoate hydroxylase (PHBH) from *P. fluorescens*, and (C) flavin-containing monooxygenase (FMO) from hog liver microsomes. The substrate for PHBH (p-hydroxybenzoate (33)) and FMO (variety of hydrophobic nitrogen and sulfur containing compounds (34–36)) is labeled as S, SOH represents the hydroxylated substrate (product), and the flavin states are oxidized (Fl\textsubscript{ox}), reduced (Fl\textsubscript{red}), C4a-hydroperoxyflavin intermediate (Fl\textsubscript{HOOH}) and hydroxyflavin intermediate (Fl\textsubscript{HOH}). (D) Flavin oxidation states.
Reductive half-reaction kinetics of PvdA. Absorbance-versus-time traces under different mixing conditions. Eight different mixing conditions were tested in oxygen-free 100 mM potassium phosphate pH 8.0 buffer containing PvdA (290 μM) at 22 °C. The coenzyme and cosubstrate concentrations were: FAD (60 µM) and NADPH (360 µM) in the same buffer and the spectra were recorded using a stopped-flow spectrometer equipped with a photomultiplier tube. Ornithine (10 mM) was included in some of the reaction mixtures. The curves were fit to a double exponential equation presumably for FAD reduction and NADP+ release (KaleidaGraph 4). $E \rightarrow FN (\bullet)$ where $E$ = enzyme, $F$ = FAD, $N$ = NADPH, and $v$ = versus (mixed with), $k_{obs} = 0.63$ and 0.11 s$^{-1}$; $ES \rightarrow FN (\bullet)$ where $S$ = ornithine, $k_{obs} = 0.50$ and 0.08 s$^{-1}$; $E \rightarrow FNS (\bullet)$, $k_{obs} = 0.47$ and 0.07 s$^{-1}$; $EN \rightarrow FS (+)$, $k_{obs} = 0.55$ and 0.21 s$^{-1}$; $EN \rightarrow F (\times)$, $k_{obs} = 0.45$ and 0.04 s$^{-1}$; $EF \rightarrow N (\circ)$, $k_{obs} = 0.39$ and 0.08 s$^{-1}$; $EF \rightarrow NS (\square)$, $k_{obs} = 0.33$ and 0.11 s$^{-1}$; $EFS \rightarrow N (\bigdiamond)$, $k_{obs} = 0.24$ and 0.04 s$^{-1}$. The data points for each mixture were measured in duplicate and averaged. Every 20th data point is displayed for each fit curve.

Figure 2.

Biochemistry. Author manuscript; available in PMC 2010 May 26.
PvdA flavin oxidation in the presence of ornithine. A. An oxygen-free solution containing 290 µM PvdA, 60 µM FAD, 360 µM NADPH, 10 mM ornithine in 100 mM potassium phosphate pH 8.0 was mixed with an equal volume of the same buffer containing 1.3 mM oxygen at 22 °C. The spectra were recorded with a photodiode array. B. Representative absorbance-versus-time traces recorded at 390 nm using a photomultiplier tube. An oxygen-free solution containing 290 µM PvdA, 60 µM FAD, 360 µM NADPH, 10 mM ornithine in 100 mM potassium phosphate pH 8.0 was mixed with an equal volume of the same buffer containing oxygen concentrations such that the final O$_2$ concentrations were 220 µM (■), 430 µM (▲), and 650 µM (▼). Each oxygen concentration was measured in duplicate and fit independently.
(KaleidaGraph 4) to produce the points for the inset plot. Inset: $k_{\text{obs}}$ versus oxygen concentration plot, indicating a second order rate constant of $150 \pm 30 \text{ mM}^{-1} \text{ s}^{-1}$. Residual oxygen was detected in the system as evidenced by the non-zero rates at $0 \mu\text{M O}_2$, and therefore the line has a positive y-intercept. C. Representative absorbance-versus-time traces for absorbance at 390 nm (○) and 450 nm (●) were recorded using a photomultiplier tube. Enzyme, NADPH, FAD, ornithine and $\text{O}_2$ mixing scheme as described in part A.
[Figure 4.](#)
PvdA flavin oxidation in the absence of ornithine. A. An oxygen-free solution containing 290 µM PvdA, 60 µM FAD, 360 µM NADPH in 100 mM potassium phosphate, pH 8.0 was mixed with an equal volume of the same buffer containing 1.3 mM oxygen at 22 °C. The spectra were recorded with a photodiode array. B. Representative absorbance-versus-time traces for absorbance at 390 nm (○) and 450 nm (●) were recorded using a photomultiplier tube. Enzyme, FAD, NADPH, and O₂ mixing scheme as described in part A.
Figure 5.
Representative absorbance-versus-time traces for absorbance at 390 nm in the presence (○) and absence (●) of ornithine replotted from Figure 3B and Figure 4B for direct comparison.
Scheme 1.