Biochemical Characterization of a Flavin Adenine Dinculeotide-Dependent Monooxygenase, Ornithine Hydroxylase from *Pseudomonas aeruginosa*, Suggests a Novel Reaction Mechanism†

Kathleen M. Meneely and Audrey L. Lamb*

Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045

Received May 15, 2007; Revised Manuscript Received August 10, 2007

ABSTRACT: Pyoverdin is the hydroxamate siderophore produced by the opportunistic pathogen *Pseudomonas aeruginosa* under the iron-limiting conditions of the human host. This siderophore includes derivatives of ornithine in the peptide backbone that serve as iron chelators. PvdA is the ornithine hydroxylase, which performs the first enzymatic step in preparation of these derivatives. PvdA requires both flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) for activity; it was found to be a soluble monomer most active at pH 8.0. The enzyme demonstrated Michaelis−Menten kinetics in an NADPH oxidation assay, but a hydroxylation assay indicated substrate inhibition at high ornithine concentration. PvdA is highly specific for both substrate and coenzyme, and lysine was shown to be a nonsubstrate effector and mixed inhibitor of the enzyme with respect to ornithine. Chloride is a mixed inhibitor of PvdA with respect to ornithine but a competitive inhibitor with respect to NADPH, and a bulky mercurial compound (*p*-chloromercuribenzoate) is a mixed inhibitor with respect to ornithine. Steady-state experiments indicate that PvdA/FAD forms a ternary complex with NADPH and ornithine for catalysis. PvdA in the absence of ornithine shows slow substrate-independent flavin reduction by NADPH. Biochemical comparison of PvdA to *p*-hydroxybenzoate hydroxylase (PHBH, from *Pseudomonas fluorescens*) and flavin-containing monooxygenases (FMOs, from *Schizosaccharomyces pombe* and hog liver microsomes) leads to the hypothesis that PvdA catalysis proceeds by a novel reaction mechanism.

Pseudomonas aeruginosa is an opportunistic human pathogen that under iron-limiting conditions produces two siderophores, pyochelin and pyoverdin, that contribute to virulence (*1*, *2*). Pyoverdin, a hydroxamate siderophore, chelates iron with high affinity and has the ability to remove iron from host proteins such as transferrin and lactoferrin (*3*). Derivatives of ornithine, both hydroxyornithine and formylhydroxyornithine, are incorporated into the peptide backbone of pyoverdin and directly coordinate the iron (*4*).

Ornithine hydroxylase (PvdA[1] or L-ornithine N^5-oxygenase) is the first enzyme involved in the derivatization of ornithine, hydroxylating the primary amine of the side chain (Figure 1A). PvdA is part of the pyoverdin locus (*5*) and PvdA deletion mutants are pyoverdin-deficient (*6*, *7*). PvdA is functionally related to the lysine hydroxylase (IucD) of *Escherichia coli*, which is required for production of the aerobactin siderophore (Figure 1B); *p*-hydroxybenzoate hydroxylase (PHBH) of the soil bacterium *Pseudomonas fluorescens*, which is important in the biodegradation of lignin from wood (Figure 1C); and flavin-containing monooxygenases (FMOs) from a variety of organisms from bacteria to mammals, involved in xenobiotic detoxification (Figure 1D). IucD, PHBH, and FMO require two coenzymes: FAD and NADPH (*8*−*10*). FAD is reduced by NADPH and can then donate electrons to molecular oxygen (Figure 1). The end result is the production of H_2O, and a hydroxyl group is added to the side chain amine of lysine (IucD), the activated aromatic ring of the hydroxybenzoate (PHBH), or to a variety of nucleophilic substrates (FMO) (*9*, *11*, *12*). The mechanism that PHBH and FMO use for hydroxylation is considerably different, and the mechanism for IucD is unknown. PHBH requires substrate to be bound to the enzyme for FAD reduction by NADPH, followed by the dissociation of $NADP^+$ from the enzyme and the binding of molecular oxygen. The reduced flavin reoxidizes, passing through two transient intermediate states before finally forming the oxidized flavin, the product (3,4-dihydroxybenzoate), and water (Figure 1c) (*9*, *13*−*15*). FMO does not require substrate for FAD reduction or for oxygen binding. Instead, the flavin is reduced by NADPH and then binds molecular oxygen to form a stable flavin hydroperoxide intermediate that reacts with an oxidizable substrate to form the hydroxylated product and water (Figure 1d) (*9*, *16*−*18*).

† This publication was made possible by the National Center for Research Resources of the National Institute of Health (NIH Grant P20 RR-17708).

* Corresponding author: phone (785) 864-5075; fax (785) 864-5294; e-mail lamb@ku.edu.

[1] Abbreviations: PvdA, ornithine hydroxylase from *Pseudomonas aeruginosa*; IucD, lysine hydroxylase from *Escherichia coli*; PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*; FMO, flavin-containing monooxygenase; mFMO, mammalian or microsomal (hog liver) flavin-containing monooxygenase; yFMO, yeast (*Schizosaccharomyces pombe*) flavin-containing monooxygenase; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide; NADH, nicotinamide adenine dinucleotide; DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoate; V_0, initial velocity; V_{max}, velocity at maximal substrate concentration; K_m, Michaelis constant; k_{cat}, turnover number; K_I, inhibition constant.

10.1021/bi700932q CCC: $37.00
Published on Web 09/28/2007

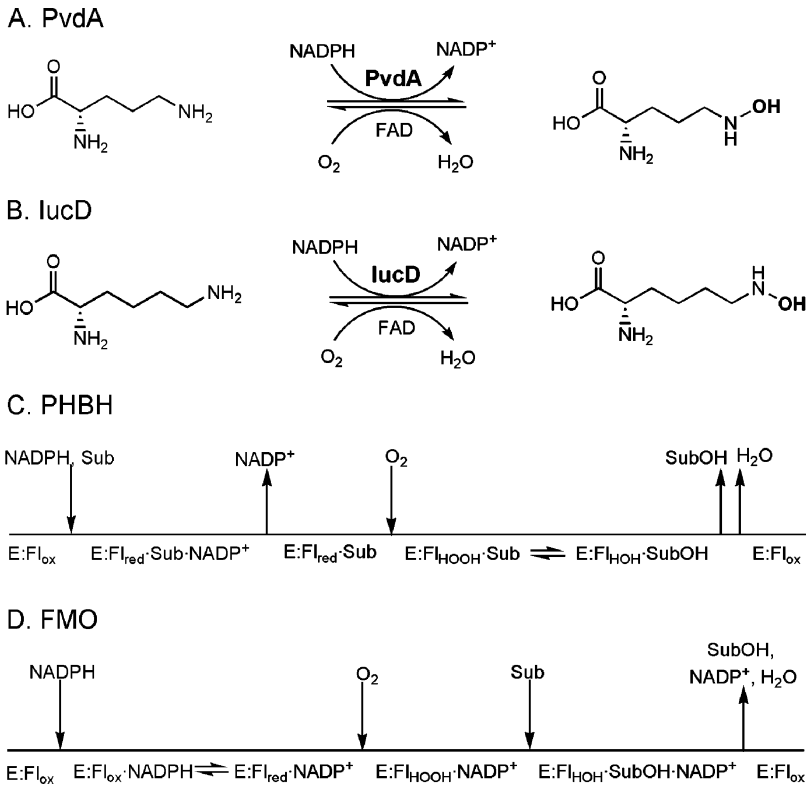

FIGURE 1: Reaction schemes for (A) ornithine hydroxylase (PvdA) from *P. aeruginosa*, (B) lysine hydroxylase (IucD) from *E. coli*, (C) *p*-hydroxybenzoate hydroxylase (PHBH) from *P. fluorescens*, and (D) flavin-containing monooxygenase (FMO) from hog liver microsomes. The substrate for PHBH (*p*-hydroxybenzoate) and FMO (nucleophilic and some electrophilic compounds) is labeled as Sub, and the flavin states are labeled as oxidized (Fl_{ox}), reduced (Fl_{red}), hydroperoxyflavin intermediate (Fl_{HOOH}), and hydroxyflavin intermediate (Fl_{HOH}).

Recently, PvdA has also been shown to be NADPH- and FAD-dependent, but the reaction mechanism has not been elucidated (*19*).

Here we report a comprehensive biochemical characterization of PvdA, including the kinetics of product formation and NADPH oxidation, demonstration of substrate inhibition, inhibition by lysine, chloride, and mercurial compounds, the oligomerization state in solution, and steady-state kinetics. We put these new data into the framework of what is known for IucD, PHBH, and FMO and begin to determine the mechanism of the reaction, and its relation to the mechanisms of the functional homologues. This work is a first step toward using PvdA as an antimicrobial drug target to reduce the virulence of *P. aeruginosa* (and possibly related bacterial species that require an amine hydroxylase for siderophore production), which infect immunocompromised patients and patients with cystic fibrosis.

EXPERIMENTAL PROCEDURES

Cloning of PvdA. The *pvdA* gene was amplified from PAO1 *P. aeruginosa* genomic DNA by polymerase chain reaction by use of Herculase (Stratagene) with 10% dimethyl sulfoxide as an adjuvant. The forward primer (5′-GAA TTC CAT ATG ACT CAG GCA ACT GCA ACC-3′) includes an *Nde*I site (underlined), whereas the reverse primer (5′-CCC AAG CTT TCA GCT GGC CAG GGC GTG-3′) contains a *Hin*dIII site (underlined). The amplified 1329 base-pair fragment was digested with *Nde*I and *Hin*dIII and ligated into the pET28b plasmid (Novagen) digested with the same enzymes. The resultant plasmid encodes the *pvdA* gene with an N-terminal histidine tag. This plasmid was transformed into the BL21(DE3) *E. coli* strain (Stratagene) for overproduction of the PvdA protein.

PvdA Protein Overexpression and Purification. BL21(DE3) *E. coli* containing the PvdA expression plasmid was grown in LB medium containing 50 μg/mL kanamycin at 37 °C with shaking (225 rpm) until an OD_{600} of ~0.8 was reached. Protein expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside, and the cells were harvested by centrifugation (6000*g*, 10 min, 4 °C) after 3−4 h. The cell pellet was resuspended in 15 mL of 20 mM potassium phosphate pH 8.0, 500 mM NaCl, 50 mM sodium citrate, and 5 mM imidazole (buffer A) per liter of culture. Cells were disrupted by use of a French pressure cell (35 000 psi), and cellular debris was removed by centrifugation (12000*g*, 30 min, 4 °C). The supernatant was applied to a chelating Sepharose fast-flow column (Amersham Biosciences) charged with nickel chloride and pre-equilibrated in buffer A. PvdA protein eluted at approximately 300 mM imidazole in a linear gradient of 5−500 mM imidazole in buffer A. The pooled fractions were applied to a Superdex 200 size-exclusion column (Amersham Biosciences) equilibrated with 100 mM potassium phosphate, pH 8.0, and 100 mM sodium citrate. The fractions containing PvdA were pooled and concentrated by use of an Amicon stirred cell with a YM-30 membrane to 9−13 mg/mL as determined by the Bradford assay and stored at −80 °C for use in the activity assays.

Oligomerization Studies. To determine the hydrodynamic radii of the PvdA species in solution, a PD2000DLS^{Plus} dynamic light scattering detector (Precision Detectors) was used with a PvdA concentration of 4.7 mg/mL. The

hydrodynamic radii were also determined in the elution buffer in the presence of 10-fold excess FAD or in the presence of 10-fold excess FAD and ornithine.

NADPH Oxidation Assay. The standard assay buffer contained 100 mM potassium phosphate, pH 8.0, 0.03 mM FAD, and 0.15 mM NADPH. PvdA (0.25 mg, 5 μM) was incubated in 1 mL of assay buffer for 2 min at 24 °C before the reaction was initiated by the addition of 5 mM L-ornithine. NADPH oxidation was monitored at 366 nm (ϵ = 2850 M^{-1} cm^{-1}) on a BioMate 3 spectrometer (Thermo Spectronics) at 24 °C for 40 s with 5 s time points according to the protocol described for IucD (*10*).

Hydroxylation Assay. The amount of hydroxylated product formed by PvdA was assayed by a variation of the Csaky iodine oxidation reaction (*20*−*23*). The standard assay buffer is the same as described for the NADPH oxidation assay. The reaction was initiated by the addition of 5 mM L-ornithine to 0.25 mg of enzyme (5 μM) in 1 mL of assay buffer at 24 °C. At 10-s intervals up to 40 s, aliquots (83 μL) of the assay mixture were withdrawn and added to 42 μL of 0.2 N perchloric acid to terminate the reaction. For each time point, 50 μL of the terminated reaction mixture was transferred into a glass 96-well plate and the reaction mixture was neutralized by the addition of 50 μL of 5% (w/v) sodium acetate solution. To each well were added 50 μL of 1% (w/v) sulfanilic acid in 25% (v/v) acetic acid and 20 μL of 1.3% (w/v) potassium iodide in glacial acetic acid, and the reaction was allowed to incubate at room temperature for 5−7 min. Excess iodine was removed with 20 μL of 0.1 N sodium thiosulfate, and the color was developed by adding 20 μL of 0.6% (w/v) α-naphthylamine in 30% (v/v) acetic acid. The absorbance at 562 nm was measured after 15 min on an Elx800 plate reader (Bio-Tek). A standard curve of hydroxylamine hydrochloride was used to calculate the amount of hydroxylated product produced.

Determination of Enzyme Specificity and Kinetic Parameters. A variety of permutations of the standard assay conditions were tested. By use of the NADPH oxidation assay, pH optimization was conducted with various buffers (100 mM potassium phosphate at pH 6.0−8.0; 100 mM Tris-HCl at pH 7.0−9.0; 100 mM glycine at pH 9.0−10.0; or 33 mM potassium phosphate, 33 mM Tris-SO_4, and 33 mM glycine at pH 6.0−10.0) in steps of 0.5 pH unit in the presence and absence of substrate. An ionic strength of 100 mM promoted protein solubility and stability and was used for all experiments. Both the NADPH oxidation and hydroxylation assays were used to determine enzyme specificity. For the coenzyme substitution reactions, FAD was replaced by FMN or NADPH by NADH at comparable concentrations. For the substrate substitution reactions, 5 mM L-ornithine was substituted with 5 mM DL-2,3-diaminopropionic acid, DL-2,4-diaminobutyric acid, L-lysine, 5-aminopentanoic acid, 1,4-diaminobutane, D-ornithine, or L-norleucine, all purchased from Sigma. The Michaelis−Menten kinetics of PvdA were assayed similarly, by varying the L-ornithine concentrations from 0 to 15 mM.

Determination of FAD Dissociation Constant. PvdA was diluted into 100 mM potassium phosphate, pH 8.0, to a final concentration of 2.5 μM. The FAD concentration was varied from 0 to 60 uM. Protein fluorescence was excited at 280 nm and the emission was detected at 330 nm.

Steady-State Kinetics. By use of the NADPH oxidation assay and 1 mL of standard assay buffer with 5 μM PvdA and 0.03 mM FAD, NADPH concentration (0.05−0.125 mM) was varied relative to ornithine concentration (0.5−10 mM) and the initial velocities were measured.

Inhibition Assays. Inhibition of PvdA by L-lysine was investigated by the hydroxylation assay. The standard assay conditions were as described above with a few modifications. To increase the signal-to-noise ratio, the substrate, coenzymes, and enzyme were increased in concentration to 25 μM PvdA, 0.15 mM FAD, and 0.75 mM NADPH, and the L-ornithine concentrations ranged from 0.15 to 2 mM in 1 mL of assay buffer. L-Lysine was added in concentrations ranging from 0 to 10 mM. Inhibition by chloride was characterized by the NADPH oxidation assay under standard assay conditions. Sodium chloride concentrations ranged from 0 to 250 mM over an L-ornithine concentration range of 0.5−10 mM. Inhibition by *p*-chloromercuribenzoate (PCMB) was characterized by the NADPH oxidation assay and standard assay conditions. The PCMB concentration ranged from 0 to 10 μM over an L-ornithine concentration range of 0.075−5 mM.

PvdA Flavin Reoxidation. To determine the rate constants for flavin reduction and reoxidation, 40 μM PvdA was incubated in 100 mM potassium phosphate, pH 8.0, with 20 μM FAD. Upon the addition of 40 μM NADPH, the absorbance from 300 to 800 nm was measured at 20-s time points on a Cary 50 Bio spectrophotometer (Varian) at 24 °C. The change in absorbance at 451 nm was used to determine the rate constants for reduction and reoxidation.

Data anaylsis. All reactions were carried out in triplicate with standard deviations as indicated. Data analysis was done with SigmaPlot 8.0. All curves were fit to the data points by use of least-squares linear regression analysis and standard algorithms, except the substrate inhibition, which was fit with the equation $V_0 = V_{max}[\text{substrate}]/\{K_m + [\text{substrate}] + ([\text{substrate}]^2/K_I)\}$ (*23*).

RESULTS

PvdA Protein Production and Purification. The *pvdA* gene was cloned from PAO1 *P. aeruginosa* genomic DNA, and an overexpression plasmid was generated such that expression in BL21(DE3) *E. coli* yielded the PvdA protein of 51.6 kDa including an N-terminal His_6 tag (a construct with a C-terminal His_6 tag was insoluble and was produced in inclusion bodies; J. Martin Bollinger, personal communication). Protein purification was carried out by nickel affinity and size-exclusion chromatography, yielding 20−45 mg of PvdA at ∼95% purity per liter of culture. The purified protein did not contain FAD as determined by the lack of an absorbance peak at 450 nm.

Oligomerization Studies. The average hydrodynamic radius for apo-PvdA in the activity assay buffer was 4.77 nm, with a small percentage as larger species suggesting aggregation, as determined by dynamic light scattering (Figure 2). The addition of excess FAD, or excess FAD plus excess ornithine, yielded a hydrodynamic radius comparable to that of the apoprotein.

pH Optimum for Catalytic Activity. The initial velocities for NADPH oxidation by PvdA were measured in a pH range of 6.0−10.0 (Figure 3). The pH for maximal turnover is 8.0−

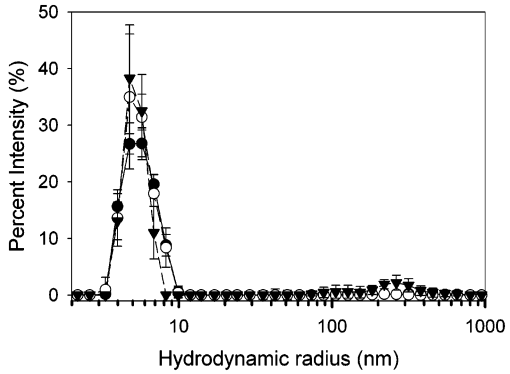

FIGURE 2: Hydrodynamic radii as determined by dynamic light scattering for PvdA in elution buffer. The fraction of species with a particular hydrodynamic radius is plotted for apo-PvdA (●), PvdA with excess FAD (○), and PvdA with excess FAD and ornithine (▼).

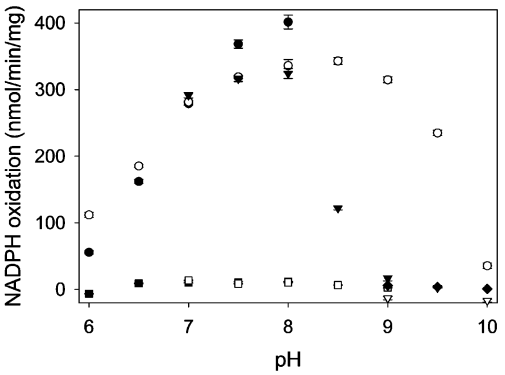

FIGURE 3: Effect of pH on PvdA activity. The rate of NADPH oxidation was measured in the presence of ornithine as a decrease in absorbance at 366 nm in the following buffers: (●) 100 mM potassium phosphate at pH 6.0−8.0; (○) 33 mM potassium phosphate, 33 mM Tris-SO_4, and 33 mM glycine at pH 6.0−10.0; (▼) 100 mM Tris-HCl at pH 7.0−9.0; and (▽) 100 mM glycine at pH 9.0−10.0. The rate of NADPH oxidation was measured in the absence of ornithine in the following buffers: (■) 100 mM potassium phosphate at pH 6.0−8.0; (□) 100 mM Tris-HCl at pH 7.0−9.0; and (◆) 100 mM glycine at pH 9.0−10.0.

8.5. Therefore, all further assays were performed with potassium phosphate, pH 8.0, as the buffer system. Substrate-independent NADPH oxidation was monitored in a pH range of 6.0−10.0 and no change was observed over the entire pH range, indicating that the uncoupling of the enzyme is not pH-dependent.

Coenzyme Specificity. With the NADPH oxidation and hydroxylation assays, the coenzymes required for PvdA activity were determined (Table 1). PvdA is dependent on FAD for activity, and FMN is not an effective coenzyme. NADPH is the electron donor for PvdA catalysis, and no coenzyme oxidation was detected upon substitution with NADH; however, hydroxylated product was detected at 14% of full activity, suggesting the possibility of nonspecific product formation in the presence of NADH. Substrate-independent NADPH oxidation was detected at 2% of full activity.

Substrate Specificity. Shortening of the side-chain length by one or two methylene groups (DL-2,4-diaminobutyric acid or DL-2,3-diaminopropionic acid, respectively) resulted in no significant activity in either assay (Table 1). By extending the side-chain by one methylene group (L-lysine), NADPH oxidation occurred at 80% of the rate of L-ornithine; however, no hydroxylated product was formed. Changing the chirality of the substrate to D-ornithine resulted in no enzymatic activity in either assay (Table 1 and ref *19*). The α-amino and α-carboxyl groups are required for activity and may be involved in substrate binding, since no NADPH oxidation or hydroxylated product was detected with 5-aminopentanoic acid and very little NADPH oxidation (4%) and no hydroxylated product was detected for 1,4-diaminobutane (Table 1). Exchanging the side-chain amine for a methyl group (L-norleucine) resulted in no NADPH oxidation or product formation above background, which was anticipated as this substitution eliminates the amine to be hydroxylated.

Table 1: Summary of PvdA Coenzyme and Substrate Specificities

test conditions	hydroxylated product formation (nmol min^{-1} mg^{-1})	NADPH oxidation (nmol min^{-1} mg^{-1})
Omission Test		
no omission	321 ± 3	534 ± 14
− FAD	22 ± 12	*a*
− NADPH	*a*	*a*
− L-ornithine	*a*	11 ± 1
Specificity		
− FAD		
+ FMN	*a*	*a*
− NADPH		
+ NADH	46 ± 1	*a*
− L-ornithine		
+ DL-2,3-diaminopropionic acid	*a*	8 ± 4[b]
+ DL-2,4-diaminobutyric acid	*a*	*a*
+ L-lysine	*a*	428 ± 4
+ 5-aminopentanoic acid	*a*	*a*
+ 1,4-diaminobutane	*a*	21 ± 2
+ D-ornithine	*a*	5 ± 3[b]
+ L-norleucine	*a*	*a*

[a] Below the limits of detection (5 nmol for the hydroxylation assay; <1 nmol for the NADPH oxidation assay). [b] Less than substrate-independent NADPH oxidation.

Determination of Kinetic Constants. Both the NADPH oxidation assay and the hydroxylation assay were used to determine the kinetic parameters of PvdA at L-ornithine concentrations ranging from 0 to 15 mM (Figure 4). Increasing the ratio of FAD to protein did not affect the kinetic parameters (for example, increase V_{max} or k_{cat}), nor did it increase the uncoupling of the reaction. Substrate inhibition is observed for the hydroxylation assay at ornithine concentrations over 5 mM but not for the NADPH oxidation assay. Above 5 mM ornithine, the initial velocity for the hydroxylation assay continued to decrease with increasing ornithine concentration at all concentrations tested up to 50 mM ornithine (data not shown). V_{max}, K_m, and k_{cat} values are consistent for the two assays (Table 2).

FAD Dissociation Constant. The dissociation constant for FAD was determined to be 26 ± 5 μM. The 2.5-fold difference from the previously reported K_d (*19*) may be due to elimination of chloride (an inhibitor) from our final protein storage buffer, and thus from the buffer system for determination of the dissociation constant.

Ternary Complex Formation. By use of steady-state kinetics and the NADPH oxidation assay, initial velocities were measured for PvdA with excess FAD by varying both NADPH and ornithine concentrations. The resulting velocities, plotted as double-reciprocal plots, indicate the formation

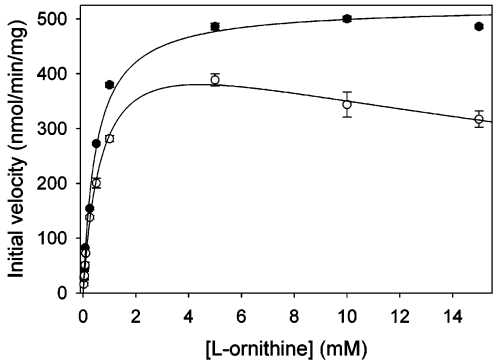

FIGURE 4: Kinetic analysis of PvdA as determined by NADPH oxidation and hydroxyornithine production. Initial velocities were measured as a function of NADPH oxidation versus L-ornithine concentration (●) and as the amount of hydroxyornithine production versus L-ornithine concentration (○). The curve for the NADPH oxidation assay was fit to the standard equation for Michaelis−Menten reactions, and the curve for the hydroxylation assay was fit to $V_0 = V_{max}[\text{substrate}]/\{K_m + [\text{substrate}] + ([\text{substrate}]^2/K_I)\}$ for substrate inhibition due to the decrease in activity at substrate concentrations above 5 mM.

Table 2: Summary of PvdA Kinetic Parameters

	hydroxylated product formation assay	NADPH oxidation assay
V_{max}, nmol min^{-1} mg^{-1}	479 ± 54	528 ± 8
K_m, μM	600 ± 70	593 ± 12
k_{cat}, min^{-1}	24 ± 3	26.4 ± 0.4
k_{cat}/K_m, M^{-1} s^{-1}	670	742
K_I,[a] mM	31 ± 5	na[b]

[a] Inhibition constant determined for substrate inhibition. [b] na, not applicable.

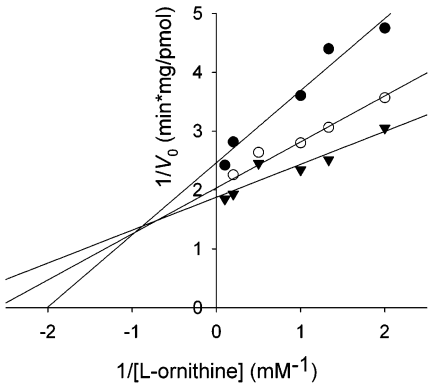

FIGURE 5: Ternary complex formation of FAD-loaded PvdA with NADPH and ornithine: double-reciprocal plot of NADPH oxidation as a function of L-ornithine concentration. NADPH concentrations used were (●) 0.05 mM, (○) 0.075 mM, and (▼) 0.125 mM.

of a ternary complex since lines are not parallel but intersect in the upper left quadrant (Figure 5) (*24*).

Inhibition Assays. In the hydroxylation assay, addition of L-lysine resulted in mixed inhibition (Table 3). Addition of sodium chloride into the reaction mixture resulted in inhibition of NADPH oxidation. Mixed inhibition was observed with respect to L-ornithine, whereas the inhibition is competitive with respect to NADPH. In the NADPH oxidation assay, mixed inhibition by *p*-chloromercuribenzoate (PCMB), a mercury compound that binds cysteines not involved in disulfide bonding, was observed.

Flavin Reoxidation. By use of substoichiometric amounts of FAD and stoichiometric amounts of NADPH, aerobic reduction of flavin by NADPH in PvdA in the absence of ornithine was monitored as a decrease in absorbance at 451 nm. The rate constant was determined as 0.036 ± 0.007 s^{-1}, and no semiquinone intermediates were detected (no absorbance peaks between 600 and 800 nm). Aerobic oxidation of the reduced flavin was observed over time as an increase in absorbance with a rate constant determined as 0.019 ± 0.002 s^{-1} (Figure 6).

DISCUSSION

PvdA is most closely related to a lysine hydroxylase from *E. coli* (IucD). Both enzymes hydroxylate the primary amines of amino acid side chains (Figure 1) and share 46% sequence similarity. The structure of IucD has not been determined, and there has been debate about the solubility and cellular localization (membrane or cytoplasm) of this enzyme (*25*−*28*). Initial attempts to overexpress IucD led to insoluble protein, and hydrophobic stretches of the enzyme were identified that could serve as transmembrane helices (*27*). These sequences were later suggested to be involved in binding of coenzymes, FAD at the N-terminus and NADPH in the center of the protein (*25*). In support of this latter hypothesis, IucD can be produced in an active, soluble form by the addition of an N-terminal His tag (*28*). This purified protein is tetrameric in solution (*10*, *29*). PvdA is also soluble when produced with an N-terminal histidine tag and is monomeric in solution (Figure 2). Neither IucD nor PvdA copurifies with the required flavin coenzyme, and both bind FAD in the micromolar range (*19*, *30*).

PvdA is also functionally related to two previously well-characterized enzymes, flavin-containing monooxygenase (FMO) and *p*-hydroxybenzoate hydroxylase (PHBH). FMOs are individually substrate-promiscuous and hydroxylate a wide variety of nucleophilic substrates including primary, secondary, and tertiary amines as well as sulfur-, phosphorus-, selenium-, and iodine-containing groups (*9*). FMOs in mammalian systems [in particular, the hog liver microsomal FMO (mFMO), which has been biochemically characterized] are membrane-bound tetramers/octamers, but in bacterial and unicellular eukaryotes FMOs are cytoplasmic dimers (*9*, *31*, *32*). The structure of FMO from *Schizosaccharomyces pombe* (yFMO) is composed of two domains, a large domain containing the FAD binding sequence and the FMO identifying sequence (residues 1−175 and 292−457), and a smaller insertion domain (residues 176−291) involved in stabilizing the NADPH (*33*). Sequence similarity between PvdA and yFMO is 37% (determined with the ALIGN algorithm, Genestream Search, CRBM Montepellier, France). PHBH and PvdA are relatively as similar in terms of primary structure (34%). Sequence similarity between PvdA and either yFMO or PHBH is not concentrated in areas of ligand binding but is found throughout the proteins. PHBH hydroxylates the activated aromatic ring of *p*-hydroxybenzoate and is very substrate-specific (*8*). PHBH is a dimer in solution and the X-ray crystal structure shows three domains: an N-terminal Rossmann fold for FAD binding (residues 1−174), a middle domain composed primarily of β-structure for substrate binding (residues 175−295), and a helical C-terminal domain involved in dimerization (residues

Table 3: Summary of PvdA Inhibition

inhibitor	varied component	type of inhibition	K_I,[a] μM	K_I',[b] μM
L-lysine	L-ornithine	mixed	5400 ± 1400	4300 ± 1500
chloride	L-ornithine	mixed	134 ± 31	213 ± 36
chloride	NADPH	competitive	67 ± 6	na[c]
PCMB	L-ornithine	mixed	4.1 ± 0.6	2.9 ± 0.4

[a] Inhibition constant for competitive inhibition: $K_I = [E][I]/[EI]$. [b] Inhibition constant for uncompetitive inhibition: $K_I' = [ES][I]/[ESI]$. [c] na, not applicable.

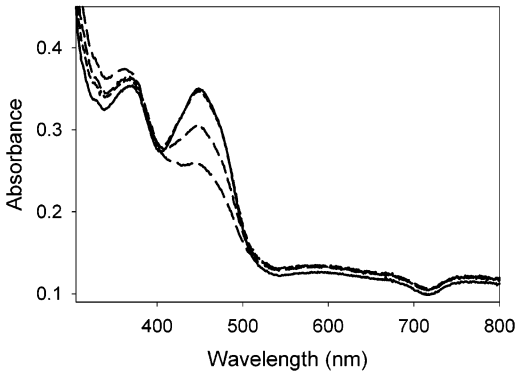

FIGURE 6: Flavin reoxidation as a function of time. Absorbance spectra of PvdA/FAD complex were measured at 300−800 nm. Representative absorbance spectra are shown: (—) oxidized flavin, (− − −) reduced flavin, (- - -) at 60 s after reduction, and (•••) at 120 s after reduction. The rate constants for flavin reduction and reoxidation were determined by the change in absorbance at 451 nm to be 0.036 ± 0.007 s^{-1} and 0.019 ± 0.002 s^{-1}, respectively. PvdA concentration used was 40 μM, and the FAD concentration was 20 μM.

296−394) (*34*). PHBH binds FAD stably in the nanomolar range, and FAD is copurified with both mFMO and yFMO (*33*, *35*).

PvdA is FAD- and NADPH-dependent (Table 1 and ref *19*). For PHBH and mFMO, NADPH reduces FAD for the hydroxylation of substrate, with the hydroxyl group derived from molecular oxygen (Figure 1) (*15*, *35*). Similar to other flavin-containing enzymes (*9*), the oxidized flavin in PvdA shows two peaks at 380 and 451 nm (Figure 6), whereas fully reduced flavin shows only one peak at 380 nm (data not shown). Flavin reduction in PHBH in the presence of substrate occurs at a rate of 256 s^{-1}, but in the absence of substrate, reduction occurs slowly at 0.41 min^{-1} (*13*). Reoxidation of the reduced flavin in the absence of substrate has a millisecond half-life (*36*). In mFMO, flavin reduction is independent of substrate and the reduced enzyme reacts with molecular oxygen to form a stable flavin−peroxide intermediate (half-life of 2 h at pH 7.2) until substrate binds (*9*). In this initial characterization of flavin reduction and oxidation, PvdA does not require substrate for flavin reduction (Figure 6), similar to mFMO. However, reoxidation of the flavin in PvdA is quicker than in mFMO by 2 orders of magnitude but slower than in PHBH by 4 orders of magnitude.

PHBH has been reported to have a k_{cat} of 1370 min^{-1} at 25 °C (*13*, *16*, *17*). In contrast, kinetic analysis of PvdA yields a slow turnover number, 26.4 min^{-1} by the NADPH oxidation assay and 24 min^{-1} by the hydroxylation assay at 25 °C (Table 2), which is comparable to the value for IucD (*23*). Kinetic parameters have not been reported for yFMO, and those reported for mFMO are an order of magnitude slower than those for PvdA, albeit at 4 °C (*16*, *17*). PHBH and IucD exhibit substrate inhibition at high substrate concentrations (*8*, *30*). Similarly, the hydroxylation assay plot shows inhibition of PvdA activity at high L-ornithine concentrations with a K_I of 31 ± 5 mM (Figure 4, Table 2). However, this substrate inhibition is not seen in the NADPH oxidation assay (Figure 4 and ref *19*). Therefore, the excess substrate does not inhibit the oxidation of NADPH but only the formation of hydroxylated product, promoting uncoupling of the reaction.

The PvdA active site is very specific for substrate. The peptide groups and the side-chain amine are required for catalysis and presumably are important for substrate positioning and binding (Table 1). Shortening of the side-chain length resulted in no significant activity. Extending the side-chain by one methylene group (L-lysine) resulted in significant NADPH oxidation without formation of the hydroxylated product, indicating that the reaction was uncoupled. As with PHBH (*37*), uncoupling of the NADPH oxidation from product formation in PvdA leads to the production of hydrogen peroxide (*19*). Therefore, L-lysine acts as a nonsubstrate effector for PvdA. We hypothesize that L-lysine leads to closing of the active site by an induced-fit mechanism, allowing for electron transfer from NADPH to FAD. However, L-lysine is sufficiently different in size that it cannot be hydroxylated efficiently, resulting in uncoupling of the enzyme similar to what is seen for nonsubstrate effectors of PHBH and IucD (*13*, *30*, *37*, *38*). Lysine is a mixed inhibitor (Table 3), also indicating that lysine is sufficiently similar to ornithine to compete for binding at the active site. Ornithine and lysine bind both in the active site (as substrate and competitive inhibitor) and at a second site leading to substrate inhibition (ornithine) or uncompetitive inhibition (lysine). Most probably, this effect is due to two molecules of substrate (substrate inhibition) or one substrate molecule and one inhibitor molecule (mixed inhibition) binding to the active site simultaneously, as described canonically for invertase (*39*). In contrast, competitive inhibition was observed for nonsubstrate effectors of PHBH (*37*) and for 5-aminopentanoic acid, L-2,4-diaminobutyric acid, and L-homoserine for PvdA (*19*).

IucD has been reported to have increased activity with increased chloride concentration (*29*). In contrast, inhibition by halides, including chloride, has been described for PHBH (*40*). The crystal structure of PHBH demonstrates three chloride binding sites, one of which interferes with flavin intermediate formation and substrate hydroxylation (*9*, *41*). Whereas chloride could merely disrupt ionic interactions for decreased enzyme efficiency, competitive chloride inhibition in PvdA with respect to NADPH suggests specific binding of the monovalent anion in the coenzyme-binding site. Both IucD and PHBH are inhibited by bulky mercurial compounds (~60%), and the activity was completely regained by the

addition of reducing agents (*8*, *10*, *42*, *43*). *p*-Chloromercuribenzoate is a mixed inhibitor of PvdA with respect to ornithine (Table 3). While these mercurial compounds are not site-specific, these data suggest that binding of a bulky mercurial compound to a cysteine, possibly in the active or coenzyme binding sites, disrupts activity for all three enzymes.

As demonstrated by steady-state kinetics, FAD-loaded PHBH forms a ternary complex with NADPH and a nonsubstrate effector, 6-hydroxynicotinate (*13*). From this work, it was deduced that PHBH uses a rapid equilibrium random order mechanism (as opposed to a compulsory order mechanism) for catalysis. Yeast FMO/FAD forms a ternary complex with NADPH and substrate; however, the reaction was determined as a compulsory order mechanism (*9*, *17*). PvdA/FAD also forms a ternary complex with substrate and NADPH (Figure 5); however, it cannot be determined from this primary plot if the mechanism will be random order or compulsory order (*24*).

CONCLUSIONS

PvdA shares many biochemical similarities with PHBH: substrate specificity, substrate inhibition, nonsubstrate effectors, and inhibition by halides and bulky mercurials. However, some of the characteristics seen in PvdA are shared with mFMO: primary amine hydroxylation and the reduction of flavin in the absence of substrate. Some characteristics of PvdA are different from either PHBH or the FMOs, including the oligomeric state in solution (monomer) and the rate of reoxidation of the flavin in the absence of substrate. The sequence similarity between PvdA and PHBH (18% identity, 34% similarity) and between PvdA and yFMO (19% identity, 37% similarity) are comparable to that between PHBH and yFMO (18% identity, 38% similarity). However, the structural similarity between PHBH and yFMO is predominantly in the FAD binding domains, with a DALI *Z*-score of 10.5 for less than half the protein (~180 amino acids), and a root-mean-squared deviation of 4.0 Å (*44*). Taken together, our data suggest that catalysis by PvdA will likely proceed by a novel reaction mechanism, and PvdA will have a three-dimensional structure distinct from those previously determined for PHBH and the FMOs.

ACKNOWLEDGMENT

We thank T. C. Gamblin for the use of the Bio-Tek Elx800 plate reader and the Varian Cary 50 Bio spectrophotometer. We thank B. Palfey for helpful discussions.

REFERENCES

1. Cox, C. D. (1982) Effect of pyochelin on the virulence of *Pseudomonas aeruginosa*, *Infect. Immun. 36*, 17−23.
2. Meyer, J. M., Neely, A., Stintzi, A., Georges, C., and Holder, I. A. (1996) Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*, *Infect. Immun. 64*, 518−523.
3. Crosa, J. H. (1989) Genetics and molecular biology of siderophore-mediated iron transport in bacteria, *Microbiol. Rev 53*, 517−530.
4. Meyer, J. M. (2000) Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species, *Arch. Microbiol. 174*, 135−142.
5. Lamont, I. L., and Martin, L. W. (2003) Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*, *Microbiology 149*, 833−842.
6. Visca, P., Serino, L., and Orsi, N. (1992) Isolation and characterization of *Pseudomonas aeruginosa* mutants blocked in the synthesis of pyoverdin, *J. Bacteriol. 174*, 5727−5731.
7. Visca, P., Ciervo, A., and Orsi, N. (1994) Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*, *J. Bacteriol. 176*, 1128−1140.
8. Hosokawa, K., and Stanier, R. Y. (1966) Crystallization and properties of *p*-hydroxybenzoate hydroxylase from *Pseudomonas putida*, *J. Biol. Chem. 241*, 2453−2460.
9. Palfey, B. A., and Massey, V. (1998) Flavin-Dependent Enzymes, in *Comprehensive Biological Catalysis* (Sinnott, M., Ed.) pp 83−154, Academic Press, San Diego, CA.
10. Plattner, H. J., Pfefferle, P., Romaguera, A., Waschutza, S., and Diekmann, H. (1989) Isolation and some properties of lysine N6-hydroxylase from *Escherichia coli* strain EN222, *Biol. Met. 2*, 1−5.
11. Ballou, D. P., Entsch, B., and Cole, L. J. (2005) Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases, *Biochem. Biophys. Res. Commun. 338*, 590−598.
12. Cashman, J. R. (2000) Human flavin-containing monooxygenase: substrate specificity and role in drug metabolism, *Curr. Drug Metab. 1*, 181−191.
13. Howell, L. G., Spector, T., and Massey, V. (1972) Purification and Properties of *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*, *J. Biol. Chem. 247*, 4340−4350.
14. Spector, T., and Massey, V. (1972) *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*, Evidence for an Oxygenated Flavin Intermediate, *J. Biol. Chem. 247*, 5632−5636.
15. Husain, M., and Massey, V. (1979) Kinetic studies on the reaction of *p*-hydroxybenzoate hydroxylase. Agreement of steady state and rapid reaction data, *J. Biol. Chem. 254*, 6657−6666.
16. Beaty, N. B., and Ballou, D. P. (1981) The reductive half-reaction of liver microsomal FAD-containing monooxygenase, *J. Biol. Chem. 256*, 4611−4618.
17. Beaty, N. B., and Ballou, D. P. (1981) The oxidative half-reaction of liver microsomal FAD-containing monooxygenase, *J. Biol. Chem. 256*, 4619−4625.
18. Jones, K. C., and Ballou, D. P. (1986) Reactions of the 4a-hydroperoxide of liver microsomal flavin-containing monooxygenase with nucleophilic and electrophilic substrates, *J. Biol. Chem. 261*, 2553−2559.
19. Ge, L., and Seah, S. Y. (2006) Heterologous expression, purification, and characterization of an L-ornithine N(5)-hydroxylase involved in pyoverdine siderophore biosynthesis in *Pseudomonas aeruginosa*, *J. Bacteriol. 188*, 7205−7210.
20. Csaky, T. Z. (1948) On the Estimation of Bound Hydroxylamine in Biological Materials, *Acta Chem. Scand. 2*, 450−454.
21. Tomlinson, G., Cruickshank, W. H., and Viswanatha, T. (1971) Sensitivity of substituted hydroxylamines to determination by iodine oxidation, *Anal. Biochem. 44*, 670−679.
22. Gillam, A. H., Lewis, A. G., and Andersen, R. J. (1981) Quantitative Determination of Hydroxamic Acids, *Anal. Chem. 53*, 841−844.
23. Stehr, M., Smau, L., Singh, M., Seth, O., Macheroux, P., Ghisla, S., and Diekmann, H. (1999) Studies with lysine N6-hydroxylase. Effect of a mutation in the assumed FAD binding site on coenzyme affinities and on lysine hydroxylating activity, *Biol. Chem. 380*, 47−54.
24. Cornish-Bowden, A. (1995) *Fundamentals of Enzyme Kinetics*, Portland Press Ltd., London.
25. Stehr, M., Diekmann, H., Smau, L., Seth, O., Ghisla, S., Singh, M., and Macheroux, P. (1998) A hydrophobic sequence motif common to N-hydroxylating enzymes, *Trends Biochem. Sci. 23*, 56−57.
26. Krone, W. J., Luirink, J., Koningstein, G., Oudega, B., and de Graaf, F. K. (1983) Subcloning of the cloacin DF13/aerobactin receptor protein and identification of a pColV-K30-determined polypeptide involved in ferric-aerobactin uptake, *J. Bacteriol. 156*, 945−948.
27. Herrero, M., de Lorenzo, V., and Neilands, J. B. (1988) Nucleotide sequence of the *iucD* gene of the pColV-K30 aerobactin operon and topology of its product studied with *phoA* and *lacZ* gene fusions, *J. Bacteriol. 170*, 56−64.
28. Seth, O., Smau, L., Welte, W., Ghisla, S., Stehr, M., Diekmann, H., and Macheroux, P. (1998) A reply to Dick et al., *Trends Biochem. Sci. 23*, 414−415.

29. Thariath, A., Socha, D., Valvano, M. A., and Viswanatha, T. (1993) Construction and biochemical characterization of recombinant cytoplasmic forms of the IucD protein (lysine:N6-hydroxylase) encoded by the pColV-K30 aerobactin gene cluster, *J. Bacteriol. 175*, 589−596.
30. Macheroux, P., Plattner, H. J., Romaguera, A., and Diekmann, H. (1993) FAD and substrate analogs as probes for lysine N6-hydroxylase from *Escherichia coli* EN 222, *Eur. J. Biochem. 213*, 995−1002.
31. Schlenk, D. (1998) Occurrence of flavin-containing monooxygenases in non-mammalian eukaryotic organisms, *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol., Endocrinol. 121*, 185−195.
32. Choi, H. S., Kim, J. K., Cho, E. H., Kim, Y. C., Kim, J. I., and Kim, S. W. (2003) A novel flavin-containing monooxygenase from *Methylophaga* sp, strain SK1 and its indigo synthesis in *Escherichia coli*, *Biochem. Biophys. Res. Commun. 306*, 930−936.
33. Eswaramoorthy, S., Bonanno, J. B., Burley, S. K., and Swaminathan, S. (2006) Mechanism of action of a flavin-containing monooxygenase, *Proc. Natl. Acad. Sci. U.S.A. 103*, 9832−9837.
34. Entsch, B., and Van Berkel, W. J. H. (1995) Structure and mechanism of *para*-hydroxybenzoate hydroxylase, *FASEB J. 9*, 476−483.
35. Poulsen, L. L., and Ziegler, D. M. (1979) The liver microsomal FAD-containing monooxygenase. Spectral characterization and kinetic studies, *J. Biol. Chem. 254*, 6449−6455.
36. Spector, T., and Massey, V. (1973) *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*, Reactivity with Oxygen, *J. Biol. Chem. 247*, 7123−7127.
37. Spector, T., and Massey, V. (1972) Studies of the Effector Specificity of *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*, *J. Biol. Chem. 247*, 4679−4687.
38. Howell, L. G., and Massey, V. (1970) A Non-substrate Effector of *p*-Hydroxybenzoate Hydroxylase, *Biochem. Biophys. Res. Commun. 40*, 887−893.
39. Combes, D., and Monsan, P. (1983) Sucrose Hydrolysis by Invertase. Characterization of Products and Substrate Inhibition, *Carbohydr. Res. 117*, 215−228.
40. Steennis, P. J., Cordes, M. M., Hilkens, J. H., and Muller, F. (1973) On the interaction of *para*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* with halogen ions, *FEBS Lett. 36*, 177−180.
41. Gatti, D. L., Palfey, B. A., Lah, M. S., Entsch, B., Massey, V., Ballou, D. P., and Ludwig, M. L. (1994) The Mobile Flavin of 4-OH Benzoate Hydroxylase, *Science 266*, 110−114.
42. Dick, S., Marrone, L., Duewel, H., Beecroft, M., McCourt, J., and Viswanatha, T. (1999) Lysine: N6-hydroxylase: stability and interaction with ligands, *J. Protein Chem. 18*, 893−903.
43. Marrone, L., and Viswanatha, T. (1997) Effect of selective cysteine → alanine replacements on the catalytic functions of lysine:N6-hydroxylase, *Biochim. Biophys. Acta 1343*, 263−277.
44. Holm, L., and Sander, C. (1993) Protein structure comparison by alignment of distance matrices, *J. Mol. Biol. 233*, 123−138.

BI700932Q