Identification and Characterization of the Flavin:NADH Reductase (PrnF) Involved in a Novel Two-Component Arylamine Oxygenase

Jung-Kul Lee\textsuperscript{1,3} and Huimin Zhao\textsuperscript{1,2,*}

University of Illinois at Urbana-Champaign, Department of Chemical and Biomolecular Engineering\textsuperscript{1} and Departments of Chemistry and Bioengineering, Institute for Genomic Biology, and Center for Biophysics and Computational Biology,\textsuperscript{2} 600 South Mathews Avenue, Urbana, Illinois 61801, and Konkuk University, Institute of Biomedical Science and Technology, 1 Hwayang-Dong, Gwangjin-Gu, Seoul, Korea 143-701\textsuperscript{3}

Received 3 July 2007/Accepted 25 September 2007

Two-component oxygenases catalyze a wide variety of important oxidation reactions. Recently we characterized a novel arylamine N-oxygenase (PrnD), a new member of the two-component oxygenase family (J. Lee et al., J. Biol. Chem. 280:36719–36728, 2005). Although arylamine N-oxygenases are widespread in nature, aminopyrrolnitrin N-oxygenase (PrnD) represents the only biochemically and mechanistically characterized arylamine N-oxygenase to date. Here we report the use of bioinformatic and biochemical tools to identify and characterize the reductase component (PrnF) involved in the PrnD-catalyzed unusual arylamine oxidation. The prnF gene was identified via sequence analysis of the whole genome of Pseudomonas fluorescens Pf-5 and subsequently cloned and overexpressed in Escherichia coli. The purified PrnF protein catalyzes reduction of flavin adenine dinucleotide (FAD) by NADH with a $k_{cat}$ of $65 \text{ s}^{-1}$ ($K_m = 3.2 \mu M$ for FAD and $43.1 \mu M$ for NADH) and supplies reduced FAD to the PrnD oxygenase component. Unlike other known reductases in two-component oxygenase systems, PrnF strictly requires NADH as an electron donor to reduce FAD and requires unusual protein-protein interaction with the PrnD component for the efficient transfer of reduced FAD. This PrnF enzyme represents the first cloned and characterized flavin reductase component in a novel two-component arylamine oxygenase system.

For oxygenases that require NAD(P)H, the catalyzed reaction can be separated into two steps, i.e., the oxidation of NAD(P)H to generate two reducing equivalents and the oxygenation of substrates. Most of the monooxygenases catalyzing the oxygenation are flavoprotein enzymes that carry out the two reactions on a single polypeptide chain (31). However, two-component monooxygenases where NAD(P)H oxidation and the oxygenation reaction are catalyzed by separate polypeptides linked by an electron transport chain also have been described (12, 17, 29). Bacterial two-component monooxygenase systems that utilize reduced flavin as a substrate are also continually being identified (3, 9, 13, 39, 47). Although these enzyme systems catalyze distinct reactions, a central theme in the two-component oxygenase family is the presence of a flavin-dependent reductase involved in flavin reduction followed by the transfer of reduced flavin to the oxygenase component.

Recently we reported the characterization of a Rieske N-oxygenase, aminopyrrolnitrin oxygenase (PrnD), that catalyzes unusual arylamine oxidation (23). Although arylamine N-oxygenases seem to be widely distributed and used in a variety of metabolic reactions, PrnD represents the only characterized example of arylamine N-oxygenases involved in aryl nitro group formation. PrnD is involved in the biosynthesis of the antibiotic pyrrolnitrin (compound 1 in Fig. 1), which is produced by many pseudomonads and has broad-spectrum antifungal activity (5, 22, 36). In the proposed biosynthetic pathway of pyrrolnitrin (Fig. 1), PrnD catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group, forming pyrrolnitrin (41). In addition, we have obtained direct evidence for the involvement of hydroxylamine and nitroso intermediates in the PrnD-catalyzed arylamine oxygenation reaction, substantiating a two-component monooxygenase-type catalytic mechanism for the conversion of arylamine to aryl nitro compounds (24). This may be the primary mechanism by which arylamines are oxygenated to give aryl nitro compounds in biochemical processes. The other arylamine-oxygenating enzyme, AurF, was recently identified as a monooxygenase based on whole-cell experiments, not on in vitro experiments with the purified enzyme (37, 42).

Although PrnD, a Rieske N-oxygenase, has been characterized and the chemical mechanism regarding arylamine oxygenation has been suggested, little information is available about the coupled flavin reductase, the enzyme responsible for the supply of the reduced flavin. Flavin reductase catalyzes the reduction of flavins, such as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin by NAD(P)H to form reduced flavins that are required to activate oxygen by the terminal oxygenases. It has been reported that flavin reductases from Vibrio harveyi and Photobacterium fischeri stimulated the enzyme activities of various monooxygenases from different microorganisms (43, 44). Luciferase (40), styrene monooxygenase (30), 4-hydroxyphenylacetate 5-monooxygenase (12), pyrrole-2-carboxylate monooxygenase (2), nitritolactate monooxygenase (21, 45), and EDTA monooxygenase (34) have been characterized as two-component monooxygenases. Other members of the two-component mono-

\textsuperscript{*} Corresponding author. Mailing address: University of Illinois at Urbana-Champaign, Department of Chemical and Biomolecular Engineering, 600 South Mathews Avenue, Urbana, IL 61801. Phone: (217) 333-2233. Fax: (217) 333-0750. E-mail: zhao5@uiuc.edu.

\textsuperscript{†} Supplemental material for this article may be found at http://jb.asm.org/.

\textsuperscript{‡} Published ahead of print on 5 October 2007.
oxygenase class have also been described, including the monoxygenases involved in biosynthesis of antibiotics such as pristinamycin IIA and valanimycin (32, 39).

The elucidation of the *Pseudomonas fluorescens* Pf-5 genome sequence in 2005 opened the door for genome-scale research with this important microbial strain (33). Therefore, we decided to identify the flavin reductase to complete the two-component arylamine oxygenase system for *P. fluorescens* Pf-5. Because the genes involved in the same antibiotic biosynthetic pathway are usually clustered on the chromosome (15, 28), we started our study by carrying out sequence analysis around a previously identified *prnD* gene and chose a gene (*prnF*) as a candidate encoding the flavin reductase based on the bioinformatics studies. Here we describe the cloning of *prnF* from *P. fluorescens* Pf-5 and the characterization of the recombinant protein. We also provide experimental evidence that PrnF is the flavin:NADH reductase component of the two-component arylamine oxygenase system in *P. fluorescens* Pf-5. The characterization of the PrnD/PrnF arylamine oxygenase system adds a new and interesting member to the family of two-component monoxygenases and gives a better understanding of the formation of aryl nitro compounds in nature.

**MATERIALS AND METHODS**

**Materials.** The pMal-c2x expression vector, maldE primer, factor Xa, amylase resin, Taq DNA polymerase, T4 DNA ligase, DNase I, and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Plasmid pQE30Xa was obtained from Qiagen (Valencia, CA). The bichromonichinic acid protein assay kit, AMP, NADH, NADPH, NAD+, FAD, FMN, riboflavin, lumiflavin, 4-aminobenzyl amine (pABA), and 4-nitrobenzyl amine (pNBA) were from Sigma-Aldrich (St. Louis, MO).

**Bacterial strains and growth conditions.** *Escherichia coli* BL21(DE3) and DH5α were obtained from Novagen (Madison, WI) and the University of Illinois Biochemistry Department’s Cell and Media Facility (Urbana, IL), respectively. *Pseudomonas fluorescens* Pf-5 (ATCC no. BAA-447) was purchased from the American Type Culture Collection (Manassas, VA). *E. coli* strains DH5α and BL21(DE3) were grown aerobically at 37°C or 30°C in Luria-Bertani (LB) medium with constant shaking (220 rpm). When necessary, kanamycin was added at 50 μg/ml, ampicillin at 100 μg/ml, and chloramphenicol at 25 μg/ml. Solid media were prepared by addition of 1.5% (wt/vol) agar.

**Construction of pQE30Xa-prnF expression plasmid.** The *prnF* gene was amplified by PCR from *P. fluorescens* Pf-5 genomic DNA using two oligonucleotide primers, 5'-GGGGATCCATGAATGCTGCCACCGAAAC-3' (BamHI restriction site is underlined) and 5'-GGAGAACCTTCTTATCTGTCGGAGACGC-3' (HindIII restriction site is underlined). The PCR amplification was conducted using *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) to minimize potential point mutations introduced by PCRs under standard conditions. The PCR program was as follows: 2 min at 96°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and a final elongation of 10 min at 72°C. The PCR products were cleaved by BamHI and HindIII and purified using the Qiagen QiAquick gel purification kit (Qiagen). The purified product was cloned into the BamHI- and HindIII-digested expression vector pQE30Xa. The resulting pQE30Xa-prnF is, under the control of the T5 promoter and expresses PrnF as a protein fused to the N terminus of a His6 tag. The cloned *prnF* gene was confirmed to be free of point mutations by DNA sequencing at the Biotechnology Center of the University of Illinois using the BigDye Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA).

**Expression and purification of PrnF and PrnD proteins.** *E. coli* BL21(DE3) cells (Invitrogen) overexpressing PrnF were grown in LB medium supplemented with ampicillin (100 μg/ml). Cells were grown at 30°C until the absorbance at 600 nm reached ∼0.6. Then, protein expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) and grown for an additional 6 h at 20°C. Cells were harvested by centrifugation at 4°C for 20 min at 6,000 × g, rinsed with phosphate-buffered saline, and frozen and stored at −20°C. The yield was approximately 3 g bacterial wet weight/liter of culture. For the purification of the His6-tagged PrnF protein, 3 g of cells were resuspended in 10 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5) supplemented with 25 μg/ml DNase I. A bacterial lysate was prepared by thawing and resuspending this suspension; this suspension was treated with 10 mg of lysozyme for 30 min. After clarification by centrifugation at 30 min at 20,000 × g and 4°C, the volume of the crude extract was adjusted to 10 ml with binding buffer. The crude extract was then loaded at a flow rate of 25 ml/h onto a 2.5-ml HisBind resin column (Novagen), and the PrnF protein was purified according to the manufacturer’s protocol. An additional wash step with a buffer containing 100 mM imidazole was performed for 6 column volumes prior to PrnF elution with 250 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 20% glycerol, pH 7.5. For cleavage with factor Xa, the purified His6–PrnF fusion protein was incubated with factor Xa (1 μg/200 μg of fusion protein) for 16 h at 4°C. The His6 tag was removed after cleavage by a second adsorption to the nickel-nitritotriacetic acid (Ni-NTA) column. Fractions from the Ni-NTA column were then concentrated with CentriToc-10 ultrafiltration units (Amicon) and adjusted to 100 mM NaCl, 20 mM Tris-HCl, and 1 mM diithiothreitol, pH 7.5. The PrnD enzyme was expressed, purified, and reconstituted as described elsewhere (23).

**Construction of a recombinant *E. coli* strain coexpressing prnD and prnF genes.** The PCR product obtained by using the same oligonucleotide primers with BamHI and HindIII restriction sites was cleaved by BamHI and HindIII and purified using the Qiagen II gel purification kit (Qiagen). The purified product was ligated into the plasmid pACYC-Duet (Novagen), which had been treated with the T5 promoter, and expressed PrnF as a protein fused to the N terminus of a His6 tag. The cloned *prnF* gene was confirmed to be free of point mutations by DNA sequencing at the Biotechnology Center of the University of Illinois using the BigDye Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems, Foster City, CA).

**Construction of a recombinant *E. coli* strain coexpressing prnD and prnF genes.** The PCR product obtained by using the same oligonucleotide primers with BamHI and HindIII restriction sites was cleaved by BamHI and HindIII and purified using the Qiagen II gel purification kit (Qiagen). The purified product was ligated into the plasmid pACYC-Duet (Novagen), which had been treated with the same restriction endonucleases. The resulting plasmid, pACYC-prnF, was transformed into *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA). The plasmid was reisolated and transformed into *E. coli* strain BL21(DE3) harboring the
plasmid pTKXb-prnD (23). The strain was cultured in LB medium containing chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml). Recombinant E. coli cells carrying pTKXb-prnD and pACYC-prnF or pTKXB-prnD only were cultivated at 30°C until the absorbance at 600 nm reached ~0.6 and then induced with 100 μM IPTG and grown for an additional 12 h at 20°C. The cells were harvested and washed as described above and suspended in 50 mM Tris-HCl (pH 7.0).

Optical spectra were recorded on a Varian Cary 100 Bio UV-Vis spectrophotometer (Varian, Palo Alto, CA) (10, 26). Appropriate amounts of PrnF were incubated at 30°C in a reaction mixture (final volume, 1 ml) containing 200 μM NADH and 30 μM FAD in 50 mM NaCl, 20 mM Tris-HCl, pH 7.0. Reactions were started by addition of enzyme to the reaction mixture. An assay mixture without FAD was used as a blank. One unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH/min at 30°C under aerobic conditions. PrnD oxygenase activity was assayed as described previously (23).

Enzyme assay. Flavin reductase activity was determined by measuring the decrease of the absorbance at 340 nm (ε(24) = 6.22 mM⁻¹ cm⁻¹) due to the oxidation of NADH, using a Varian Cary 100 Bio UV-Vis spectrophotometer (Varian, Palo Alto, CA) (10, 26). Appropriate amounts of PrnF were incubated at 30°C in a reaction mixture (final volume, 1 ml) containing 200 μM NADH and 30 μM FAD in 50 mM NaCl, 20 mM Tris-HCl, pH 7.0. Reactions were started by addition of enzyme to the reaction mixture. An assay mixture without FAD was used as a blank. One unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH/min at 30°C under aerobic conditions. PrnD oxygenase activity was assayed as described previously (23).

Analytical methods. Optical spectra were recorded on a Varian Cary 100 Bio UV-Vis spectrophotometer. Enzyme reaction products were analyzed by addition of enzyme to the reaction mixture. An assay mixture without FAD was used as a blank. One unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH/min at 30°C under aerobic conditions. PrnD oxygenase activity was assayed as described previously (23).

RESULTS

Identification of genes involved in pyrrolnitrin biosynthesis. The sequence analysis of the whole genome of P. fluorescens Pf-5 suggested the presence of a pyrrolnitrin biosynthetic gene cluster (Fig. 2), including four open reading frames (ORFs) (prnA to -D), which have been previously described as a complete operon for pyrrolnitrin biosynthesis (14, 41). All ORFs were in the same orientation from prnA (the first ORF) to the eighth ORF. The ORFs from the fifth to the eighth were annotated as a Na⁺/H⁺ antiporter transmembrane transporter, a putative flavin:NAD(P)H reductase, an RNA polymerase sigma factor, and a sigma factor regulatory protein, respectively, suggesting that the sixth ORF might encode a

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino Acids</th>
<th>Closest Similar Protein (% Identity), Accession Number</th>
<th>Proposed Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1</td>
<td>513</td>
<td>RemN protein (57), ABA53555</td>
<td>drug resistance transporter</td>
</tr>
<tr>
<td>orf2</td>
<td>360</td>
<td>hypothetical protein ml5355 (38), BAB15856</td>
<td>oxidoreductase</td>
</tr>
<tr>
<td>orf3</td>
<td>581</td>
<td>pyruvate dehydrogenase (80), AAM35116</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>prnA</td>
<td>537</td>
<td>tryptophan halogenase (93), EOA44484</td>
<td>tryptophan halogenase</td>
</tr>
<tr>
<td>prnB</td>
<td>361</td>
<td>BambDRAFT_1576 (88), EOA44485</td>
<td>pyrrolnitrin biosynthesis enzyme</td>
</tr>
<tr>
<td>prnC</td>
<td>567</td>
<td>halogenase PrnC (95), EOA44486</td>
<td>halogenase</td>
</tr>
<tr>
<td>prnD</td>
<td>363</td>
<td>phenylpropionate dioxygenase (90), ZP_00470796</td>
<td>aryamine oxygenase</td>
</tr>
<tr>
<td>prnE</td>
<td>411</td>
<td>putative transmembrane antiporter (82), EOA45996</td>
<td>transmembrane transporter</td>
</tr>
<tr>
<td>prnF</td>
<td>186</td>
<td>putative electron transfer flavoprotein (62), CAG69429</td>
<td>flavin reductase</td>
</tr>
<tr>
<td>prnS</td>
<td>172</td>
<td>sigma-24 (FecI) (50), YP_425971</td>
<td>RNA polymerase sigma (RpoS)</td>
</tr>
<tr>
<td>prnR</td>
<td>314</td>
<td>putative FecR (37), ABC21685</td>
<td>regulatory protein</td>
</tr>
</tbody>
</table>

FIG. 2. Proposed pyrrolnitrin biosynthetic gene cluster. (A) Genetic organization of the proposed pyrrolnitrin biosynthetic gene cluster including prnF, encoding flavin reductase. (B) Proposed functions for individual ORFs.
flavin reductase to supply the reduced flavin for the PrnD component. Accordingly, four ORFs were named \textit{prnE},\textit{ prnF}, \textit{prnS}, and \textit{prnR}, respectively. As to the three ORFs in front of \textit{prnA} in the pyrrolnitrin biosynthetic gene cluster, their deduced products also showed similarities to proteins of known functions (Fig. 2B). Although there is a possibility that \textit{orf1} and \textit{orf2} are involved in the pyrrolnitrin biosynthetic gene cluster as a resistance gene and an oxidoreductase gene, the deduced product of \textit{orf3}, pyruvate dehydrogenase, is apparently unrelated to pyrrolnitrin biosynthesis. In addition, \textit{prnF} is much closer to \textit{prnD} than \textit{orf2} in the genome. Further promoter scan analysis strongly suggested the presence of a promoter in front of \textit{prnA}, indicating that \textit{prnD} and \textit{prnF} are very likely to be in the same operon while \textit{prnD} and \textit{orf2} are not. Thus, based on these bioinformatics studies, we considered \textit{PrnF} a better candidate reductase for \textit{PrnD} than ORF2.

**Characterization of the \textit{prnF} gene encoding a flavin reductase.** The \textit{prnF} gene encodes a polypeptide of 160 amino acids, with a calculated molecular mass of 17,101 Da and an overall GC content of 62%, which is similar to that of the chromosomes of \textit{Pseudomonas} species (60 to 66%) (27). The deduced \textit{prnF} gene product contains the pfm\textsubscript{161} flavin reductase-like domain present in flavin reductases and various monooxygenase components. In the C-terminal region of \textit{PrnF}, a highly conserved GDH motif, which plays a role in NAD(P)H binding in Fre (16), was found.

A homology search revealed that the deduced \textit{prnF} gene product showed 31.5, 28.6, 26.4, 25.6, and 25.5% amino acid identity with \\textit{PheA2} (8, 20), SnaC (39), VlmR (32), ActVB (18), and SpaC (12, 46), respectively. The deduced product of \textit{prnF} belongs to a family of flavin:NAD(P)H reductases, the majority of which are part of two-component monooxygenase systems. VlmR is a flavin reductase that functions in a two-component enzyme system to provide isobutylamine N-hydroxylase with reduced flavin and is involved in the synthesis of valanimycin. ActVB is also a flavin: NADH reductase that participates in the last step of actinorhodin biosynthesis. In analogy to these two-component flavin monooxygenases, \textit{PrnF} and \textit{PrnD} are proposed to form a two-component oxygenase system in which \textit{PrnF} supplies the reduced flavin that \textit{PrnD} needs to function (Fig. 1).

**Heterologous expression of \textit{prnF} gene and identification of flavin:NADH reductase.** In order to check its proposed function, \textit{prnF} was cloned in the T5 RNA polymerase-based plasmid pQE\textsubscript{30}Xa to give pQE\textsubscript{30}Xa-\textit{prnF} and heterologously expressed in \textit{E. coli} BL21(DE3). The pQE\textsubscript{30}Xa vector encodes an N-terminal His tag and a 16-amino-acid spacer that contains a factor Xa cleavage site (GSGSGSGGERPQNYGT). Analyses carried out with the extracts of \textit{E. coli} BL21(DE3) harboring pQE\textsubscript{30}Xa-\textit{prnF} revealed the presence of a high level of flavin: NADH oxidoreductase activity compared with the control extracts of \textit{E. coli} BL21(DE3) cells harboring the plasmid pQE\textsubscript{30}Xa. To ascertain that \textit{FAD} reduction in the presence of \textit{NADH} was carried out specifically by the \textit{PrnF} protein and not by another enzyme induced in the host cell as a consequence of the overexpression of the \textit{prnF} gene, the \textit{PrnF} enzyme was purified. Total protein extracts from \textit{E. coli} BL21(DE3) transformed with pQE\textsubscript{30}Xa-\textit{prnF} or with pQE\textsubscript{30}Xa as a control were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 19-kDa protein, which was in agreement with the predicted molecular mass for the \textit{PrnF} protein, could be identified in total and soluble extracts only from cells harboring pQE\textsubscript{30}Xa-\textit{prnF} and induced by IPTG (see Fig. S1 in the supplemental material). This protein was assigned as \textit{PrnF}, and it accounted for ~5% of total protein based on the specific activity in the cell extracts. The enzyme was purified by Ni NTA affinity chromatography about 25-fold to near-homogeneity. The N-terminal His\textsubscript{6} tag was then removed by factor Xa. The factor-Xa-treated \textit{PrnF} protein was separated from the fusion protein and His\textsubscript{6} tag peptide fragment using an Ni NTA column. The purified \textit{PrnF} reductase was colorless, and the UV-visible spectrum showed no evidence of any chromogenic cofactor. Oxidation of NAD(P)H to NAD(P) was monitored by the decrease in absorbance at 340 nm. In the presence of \textit{FAD}, \textit{PrnF} oxidized \textit{NADH} with a \textit{k}_{cat} value of 65 \text{s}^{-1} (\textit{k}_{cat} = 3.2 \mu\text{M} for \textit{FAD} and 43.1 \mu\text{M} for \textit{NADH}). \textit{NADPH} was not accepted for oxidation by \textit{PrnF}. These findings strongly supported the assumption that the reductase activity observed in crude extracts of \textit{E. coli} BL21(DE3) harboring pQE\textsubscript{30}Xa-\textit{prnF} corresponded to that of the \textit{PrnF} protein. By using gel filtration chromatography, the molecular mass of native \textit{PrnF} was determined to be ~39 kDa, showing that the protein is homodimeric in solution. The characterization of \textit{PrnF} as a NADH-dependent flavin reductase then allowed for the investigation of its participation in the two-component oxygenase reaction.

**Formation of aryl nitro compound by \textit{PrnD} in the presence of \textit{PrnF}.** Although we have observed that \textit{PrnF} was able to produce reduced \textit{FAD} in vitro in the absence of \textit{PrnD}, it was necessary to investigate whether such activity could participate in the oxygenation of arylamines. An assay was performed with \textit{FAD}, \textit{NADH}, and purified \textit{PrnD} and \textit{PrnF}. \textit{pABA} was used as the substrate to detect the appearance of new products derived from that arylamine compound (23, 24). Analysis by HPLC revealed a new product peak that was formed when \textit{PrnF}-\textit{PrnD} was incubated with the substrate \textit{pABA}, \textit{FAD}, and \textit{NADH}. The product coeluted with an authentic standard of \textit{pNBA} with the same retention time (3.86 min). This compound was further identified as \textit{pNBA} by high-resolution (HR) electron ionization mass spectrometry \textit{(M^{+} calculated, 152.0586; found, 152.0587)}. This product was absent if heat-denatured \textit{PrnD} was used or if \textit{pABA} was omitted from the enzyme assay. This indicated that the product was derived enzymatically from \textit{pABA}. In the absence of \textit{PrnF}, \textit{PrnD} showed almost no oxygenating activity. However, rates of product formation by \textit{PrnD} \textit{(k}_{cat} = 11.3 \text{min}^{-1}; \textit{k}_{cat}/\textit{K}_{m} \textit{pABA} = 398 \mu\text{M}) were dramatically enhanced with the addition of \textit{PrnF}. The observation that \textit{PrnD} activity was absolutely dependent on the presence of \textit{PrnF} supports the hypothesis that both components are required for arylamine oxygenation. When the ratio of \textit{PrnF} to \textit{PrnD} was varied, \textit{PrnD} activity exhibited saturation above a 0.1:1 ratio. This might be ascribed to the fact that \textit{PrnF} \textit{(k}_{cat} = 65 \text{ s}^{-1}) has much higher activity than \textit{PrnD} \textit{(k}_{cat} = 11.3 \text{ min}^{-1})}. Subsequent kinetic characterization of \textit{PrnF} in the presence of \textit{PrnD} was conducted at this ratio of protein components.

**Substrate specificity.** \textit{PrnF} enzyme activity was measured using various electron acceptors in place of \textit{FAD} at fixed concentrations of the second substrates. When flavin compounds were added as electron acceptors, FMN, riboflavin, and lumi-
TABLE 1. Substrate specificities of the flavin:NADH reductase (PrnF)∗

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Second substrate</th>
<th>(k_{\text{cat}} \text{ (s}^{-1}))</th>
<th>(K_m \text{ (\muM)})</th>
<th>(k_{\text{cat}}/K_m \text{ (\muM}^{-1} \text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>NADH</td>
<td>65.0 ± 8.2</td>
<td>3.2 ± 0.6</td>
<td>20.3 ± 3.45</td>
</tr>
<tr>
<td>FMN</td>
<td>NADH</td>
<td>72.4 ± 9.0</td>
<td>28.6 ± 3.6</td>
<td>2.53 ± 0.27</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>NADH</td>
<td>68.2 ± 7.7</td>
<td>38.3 ± 5.4</td>
<td>1.78 ± 0.25</td>
</tr>
<tr>
<td>Lumiflavin</td>
<td>NADH</td>
<td>66.5 ± 9.4</td>
<td>58.4 ± 7.8</td>
<td>1.14 ± 0.16</td>
</tr>
<tr>
<td>NADH</td>
<td>FAD</td>
<td>62.6 ± 7.6</td>
<td>43.1 ± 5.7</td>
<td>1.45 ± 0.19</td>
</tr>
<tr>
<td>NADPH</td>
<td>FAD</td>
<td>32.9 ± 5.1</td>
<td>94.3 ± 10.2</td>
<td>0.35 ± 0.03</td>
</tr>
</tbody>
</table>

∗ Enzyme activity was measured as described in Materials and Methods at various concentrations of substrates in 50 mM Tris-HCl (pH 7.0) at 30°C. \(K_m\) is the Michaelis constant for the organic substrate.

Flavin were 12, 9, and 6% as effective as FAD, respectively (Table 1). In addition to flavin compounds, the enzyme acted on artificial electron acceptors, such as methylene blue and ferricyanide, but these compounds were less than 5% as effective as FAD. This behavior appears to be uncommon for flavin reductases that do not contain a flavin as a prosthetic group, since they reduce FMN, FAD, and riboflavin with similar efficiencies (10, 12, 20, 30). As an electron donor or the second substrate, PrnF preferred NADH over NADPH. The fact that FAD is a true substrate rather than a tightly bound cofactor for the enzyme was demonstrated by the complete reduction of an excess of FAD by the enzyme in the presence of excess NADH (10-fold greater than the corresponding \(K_m\)) were fitted with the Michaelis-Menten equation to give a \(K_m\) value for FAD of 39.2 \(\mu M\) and a \(k_{\text{cat}}\) value of 70.6 s\(^{-1}\) (23). A 12-fold increase in the \(K_m\) value for FAD in the presence of PrnD and the pABA substrate suggests that the reaction mechanism of PrnF is modified.

Protein-protein interaction between PrnF and PrnD. To verify whether direct contact between the flavin reductase PrnF and the oxygenase PrnD is necessary, the two protein components were separated by a dialysis membrane permeable to compounds smaller than 12 kDa (12). Under these conditions, oxygenation of pABA occurred only with about 5% (4.9 U/mg protein compared to 95 U/mg protein) of the efficiency seen under normal conditions. In addition, the binding parameters of FAD-bound PrnF for PrnD were determined. The increase of FAD-bound PrnF emission at 530 nm was examined upon titration with increasing concentrations of PrnD. The fluorescence emission of FAD-bound PrnF was increased by increasing concentrations of PrnD. Upon excitation of FAD-bound PrnF at 450 nm, the binding curve of PrnD to PrnF demonstrated saturation binding (Fig. 4). The number of binding sites was resolved by fitting the binding curve to a Hill plot, as described in Materials and Methods. The Hill plot yielded an \(n\) value of 1.20, i.e., one binding site with a \(K_d\) value of 56 nM. Taken together, measurement of the increase of FAD-bound PrnF fluorescence by PrnD binding indicated that PrnF bound PrnD with nanomolar affinity at one binding site.
The complex formation of PrnD and PrnF was further demonstrated by gel filtration studies. PrnD and PrnF were mixed together in Tris-HCl buffer (pH 7.0) and subjected to gel filtration chromatography. Gel filtration studies showed that PrnD comigrated with PrnF as a complex in solution. The apparent molecular mass of the PrnD-PrnF complex appeared to be approximately 120 kDa, as assessed from the elution time of the protein complex peak compared with that of the protein standards, confirming a 1:1 stoichiometry for binding of the two proteins, PrnD (86 kDa) and PrnF (39 kDa), to form a catalytic complex.

In combination with the change in the kinetic parameter in the presence of PrnD, it can be concluded that direct protein-protein interaction between the reductase PrnF and the oxygenase PrnD is necessary for electron transfer or oxygenation of arylamine. All these data suggest that PrnF reduces FAD to FADH₂ for the PrnD oxygenase and identified the PrnF flavin reductase as the best candidate for coupling with the PrnD oxygenase component and that the protein-protein interaction between the PrnD and PrnF components is unusual. Indeed, the N-oxygenating activity of PrnD was only slightly affected even after it was separated from the ORF2 oxidoreductase by a membrane, indicating the reduced flavin can be released from the ORF2 oxidoreductase and then diffused into the active site of PrnD.

**DISCUSSION**

Given the prominence of arylamine oxygenation among natural aromatic products, there has been intense interest in understanding the mechanisms by which an arylhydroxylase functional group is incorporated during natural product biosynthesis (4, 19, 24, 42). The first flavin-dependent arylamine oxygenase was recently characterized from a strain producing pyrrolnitrin, *P. fluorescens* Pf-5 (23). Despite the many representatives of this N-oxygenase class and its role in the biosynthesis of important N-oxygenated aromatic natural products, only PrnD has been characterized (23, 24). Our previous work on PrnD has shown several basic features of this oxygenase (23, 24). First, a separate flavin reductase is required to catalyze the initial reduction of flavin by NAD(P)H. Second, it depends on flavin cofactors in the reduced state. A third feature is the requirement for molecular oxygen. In the current work, we established the requirement for a separate flavin reductase to provide reduced FADH₂ for the PrnD oxygenase and identified the PrnF flavin reductase, in analogy with two-component flavin monooxygenase systems (18, 20, 32).

Based on the analysis of the genome sequence of *P. fluorescens* Pf-5, a flavin reductase-encoding gene (*prnF*) was proposed as encoding a flavin reductase coupling with the arylamine N-oxygenase (PrnD) involved in pyrrolnitrin biosynthesis. The identified *prnF* gene was cloned from *P. fluorescens* Pf-5 and overexpressed in *E. coli*, and it was confirmed that this gene product, PrnF, exhibited flavin reductase activity. Since the *prnF* gene is located in close proximity to the gene encoding PrnD on the *P. fluorescens* Pf-5 chromosome (Fig. 2), it appears likely that the function of the PrnF protein is to provide reduced FAD to PrnD. This hypothesis is supported by the fact that a mixture of PrnF and PrnD efficiently catalyzes the oxygenation of arylamine to the arylhydroxyl compound. Since
O¹⁸ incorporation experiments strongly support that hydroxylamine and nitroso compounds are the intermediates in pyrrolnitrin biosynthesis (24), it is reasonable to assume that both the prnF and prnD gene products are involved in the consecutive monooxygenation for pyrrolnitrin biosynthesis as a novel two-component monooxygenase system. In addition, coexpression of prnF with the N-oxygenation gene prnD from P. fluorescens Pf-5 was critical for high N-oxygenating activity, resulting in about 15-fold-higher N-oxygenating activity than that of E. coli BL21 carrying only prnD. The low N-oxygenation activity of E. coli BL21 carrying only prnD was attributed to the low level and inefficiency of the native flavin reductases existing in E. coli, such as Frc (11) and NADPH-sulfite oxidoreductase (7), two reported housekeeping flavin reductases in E. coli.

Flavin frequently acts as an electron carrier between NADH and O₂. In two-component flavin oxygenases, FADH₂ shuttles electrons from a reductase that oxidizes NAD(P)H to a second protein. However, the use of two proteins, one to generate FADH₂ and the other to catalyze oxidation of the substrate, creates the liability that diffusing FADH₂ will be intercepted by O₂ adventitiously. Thus, reduced flavin transfer between the flavin reductase and the oxygenase component should be tightly controlled. Direct flavin transfer from the flavin reductase to the monooxygenase enzyme through protein-protein interactions would protect the flavin from oxidation. Only two examples of protein-protein interactions in the two-component oxygenase family have been identified, between Vibrio harveyi luciferase and NADPH-prefering flavin reductase (Frp) (26) and between E. coli SsuD and SsuE (1). Thus, the PrnF/PrnD two-component system is the third example exhibiting protein-protein interactions for the transfer of reduced flavins.

The flavin:NAD(P)H reductases can be classified into several families according to their sequence similarities and biochemical properties. One of these families (class I) is comprised of the flavoprotein reductases that contain a tightly bound flavin as a prosthetic group, e.g., the sulfite reductase from E. coli (7) and the Frp reductase from Vibrio harveyi (25). Another family (class II) is represented by those enzymes that do not contain a flavin as a prosthetic group and thus cannot be considered flavoproteins. Instead, they use flavins as substrates, with rather broad substrate specificity. The PrnF flavin reductase from P. fluorescens Pf-5 does not show the yellow color and fluorescence typical of flavin-containing enzymes, suggesting that this enzyme belongs to class II. A homology search shows that PrnF has significant sequence identity (up to 31.5%) with the class II flavin reductase components of the two-component oxygenases PheA2, SnaC, VlmR, ActVB, and HpaC. These proteins and PrnF all exist as homodimers and utilize flavins as substrates rather than as cofactors. However, PrnF is unique in that it prefers FAD and is specific for NADH. In addition, PrnF is believed to transfer electrons to the oxygenase component via protein-protein interactions. No sequence similarities were found between any other known flavoproteins and PrnF.

In summary, the prnF gene encoding a flavin:NADH reductase was cloned from the pyrrolnitrin producer P. fluorescens Pf-5 and overexpressed in soluble form. The purified PrnF protein exhibited the expected flavin reductase enzymatic activity, thereby confirming the identity of the protein. The proximity of the prnF gene to the prnD gene, coding for amino-

pyrrolnitrin oxygenase, on the P. fluorescens Pf-5 chromosome and evidence from enzymology and bioinformatics experiments strongly suggest that these two genes are components of a two-component arylamine oxygenase system. Compared to other well-characterized oxygenases, this PrnD/PrnF system has several unique features. (i) This system catalyzes the unusual arylamine oxidation. With the completion of this work, this system now represents the only characterized example of oxidoreductases involved in the conversion of arylamines to aryl nitro compounds. (ii) Compared to other reductases involved in two-component oxygenases, PrnF is unique in that it prefers FAD and is specific for NADH. (iii) PrnF transfers electrons to PrnD through protein-protein interactions via reduced flavins, representing the third example involved in such an electron transfer mechanism. The successful overexpression and characterization of the PrnD/PrnF two-component oxygenase allows us to complete the functional characterization of all the genes for arylamine oxygenation in P. fluorescens Pf-5 and now sets the stage for more-detailed investigations of this novel two-component arylamine oxygenase system, such as X-ray crystallography and protein-protein interaction studies.

ACKNOWLEDGMENTS

This research was supported by a grant from the Office of Naval Research (N00014-02-1-0725) and partially supported by the Institute of Biomedical Science and Technology, Konkuk University.

REFERENCES


