An NADPH:FAD Oxidoreductase from the Valanimycin Producer, *Streptomyces viridifaciens*

CLONING, ANALYSIS, AND OVEREXPRESSION

Ronald J. Parry‡ and Wenyiing Li

From the Department of Chemistry, Rice University, Houston, Texas 77005-1892

The valanimycin producer *Streptomyces viridifaciens* contains a two-component enzyme system that catalyzes the oxidation of isobutylamine to isobutylhydroxylamine. One component of this enzyme system is isobutylamine hydroxylase, and the other component is a flavin reductase. The gene (*vlmR*) encoding the flavin reductase required by isobutylamine hydroxylase has been cloned from *S. viridifaciens* by chromosome walking. The gene codes for a protein of 194 amino acids with a calculated mass of 21,265 Da and a calculated pI of 10.2. Overexpression of the *vlmR* gene in *Escherichia coli* as an N-terminal His-tag derivative yielded a soluble protein that was purified to homogeneity. Removal of the N-terminal His-tag from the overexpressed protein by thrombin cleavage also produced a soluble protein. Both forms of the protein exhibited a high degree of flavin reductase activity, and the thrombin-cleaved form functioned in combination with isobutylamine hydroxylase to catalyze the conversion of isobutylamine to isobutylhydroxylamine. Kinetic data indicate that the overexpressed protein utilizes FAD and NADPH in preference to FMN, riboflavin, and NADH. The deduced amino acid sequence of the VlmR protein exhibited similarity to other several flavin reductases that may constitute a new family of flavin reductases.

The antibiotic valanimycin (Fig. 1) is a naturally occurring azoxy compound isolated from the fermentation broth of *Streptomyces viridifaciens* MG456-hF10 (1). In addition to antibacterial activity, valanimycin exhibits potent antitumor activity against *in vitro* cell cultures of mouse leukemia L1210, P388/S (doxorubicin-sensitive), and P388/ADR (doxorubicin-resistant) (1). Valanimycin is a member of a growing class of natural products that contain the azoxy group. This group now includes the pyranoside; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids

The bacterial strains, phages, and plasmids are listed in Table I.

<table>
<thead>
<tr>
<th>Media and Bacteriological Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> strains were grown in LB medium at 37 °C. Selection was made with 100 μg of ampicillin or 30 μg of kanamycin per ml of LB agar or other medium.</td>
</tr>
</tbody>
</table>

DNA Methods

Plasmid DNA was purified with a QIAprep Spin Plasmid Kit. DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction Kit. PCR products were separated on agarose gels and purified from the gel. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures. Automated DNA sequencing was performed with an Applied Biosystems DNA sequencer at the Molecular Genetics Core Facility, University of Texas Houston Medical School, using universal and synthetic oligonucleotide primers.

* This work was supported by National Institutes of Health Grant GM35818 and The Robert A. Welch Foundation Grant C-729. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Chemistry, Rice University, 6100 Main St., MS-60, Houston, TX 77005-1892. Tel.: 713-527-8101 (ext. 2446); Fax: 713-285-5155.

1 The abbreviations used are: IBAH, isobutylamine N-hydroxylase; PCR, polymerase chain reaction; dNTP, deoxynucleotide 5′-triphosphate; hFR, the N-terminal His-tag form of NADPH:FAD oxidoreductase expressed from pET28vlmA1R; tFR, the form of NADPH: FAD oxidoreductase obtained by removal of the His-tag from hFR with thrombin; kb, kilobase pairs; Bis-TriA, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)-propane-1,3-diol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis.
Cloning and Overexpression of NADPH:FAD Oxidoreductase

**Table I**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant properties</th>
<th>Source or Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong> E. coli DH5α</td>
<td>F− d80lacZΔM15 ΔlacZYA-argF(U169 deoR recA1 endA1 hsdR17 Δrκ− mκ−) phoB supE44 λ− thi-1 gyrA96 relA1</td>
<td>Life Technologies, Inc.</td>
</tr>
<tr>
<td>DH10B</td>
<td>F− merA Δ(varr-bsd-RMS-mer-BC) d80lacZΔM15 ΔlacX74 deoR recA1 araD139</td>
<td>Life Technologies, Inc.</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F− ompT hsdS(rB’ mB’) galD recA23 lacIq ΔlacZΔM15 QS-85</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong> pET-24a (+)</td>
<td>Multicloning site vector; Kan’, T7lac promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET-28b (+)</td>
<td>Multicloning site vector; Kan’, T7lac promoter</td>
<td>Novagen</td>
</tr>
<tr>
<td>pPROEXHTa</td>
<td>Multicloning site vector; Amp’, trc promoter</td>
<td>Life Technologies, Inc.</td>
</tr>
<tr>
<td>pB634E3</td>
<td>7-kb EcoRI-EcoRI insert from phage 6.3.4 in pBluescriptII SK(−); Amp’</td>
<td>(15)</td>
</tr>
<tr>
<td>pB634E3SacI</td>
<td>3-kb EcoRI-SacI insert from pB634E3</td>
<td>This study</td>
</tr>
<tr>
<td>pET24 (vlmRN1RC1)</td>
<td>0.6-kb Ndel-XhoI vlmR gene insert in pET24a (+); Kan’, T7lac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pET28 (vlmRN1RC1)</td>
<td>0.6-kb Ndel-XhoI vlmR gene insert in pET28b (+); Kan’, T7lac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pPROEX (vlmRN3RC3)</td>
<td>0.6-kb blunt end-HindIII vlmR gene insert in pPROEXHTa; Amp’, trc promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Transformations**

Transformations were carried out using commercially available competent *E. coli* cells. The procedures followed the protocols recommended by the manufacturers.

**PCR Protocols**

**Protocol A**—A 50-μl PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3 at 25 °C), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 μM each dNTP, 0.5 μM each PCR primer, DNA template, and 2 μg DNA polymerase (1.25 units). PCR tubes (0.65 ml) containing all the components except the polymerase were incubated at 94 °C for 0.5 min in a PCR personal cycler (Biometra). The PCR was initiated by addition of the polymerase to the PCR tubes. The temperature program was as follows: 94 °C, 0.5 min; 94 °C, 0.5 min; 65 °C, 1 min; 72 °C, 3 min; 30 cycles; 72 °C, 7 min.

**Protocol B**—A 100-μl PCR reaction mixture contained 20 mM Tris-HCl (pH 8.8 at 25 °C), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100, 100 mg/ml bovine serum albumin, 200 μM each dNTP, 0.5 μM each PCR primer, DNA template, and 3.75 units of cloned Pfu DNA polymerase (Stratagene). PCR tubes (0.65 ml) containing all the components except the polymerase were incubated at 95 °C for 0.5 min in a PCR personal cycler (Biometra). The PCR was initiated by addition of the polymerase to the PCR tubes. The temperature program was as follows: 95 °C, 0.5 min; 95 °C, 0.5 min; 60 °C, 0.5 min; 72 °C, 3 min; 28 cycles; 72 °C, 8 min.

**Sequence Analysis**

DNA sequence assembly and restriction site analysis were performed with Sequencher, Macintosh version 3.0. Sequence analyses were performed with the Wisconsin GCG Package, version 8.1-UNIX, including MAP, BESTFIT, ISOELECTRIC, BLAST, MOTIFS, CODONPREFER-ENCE, and PILEUP. Pattern searches were also carried out with PSCAN at the ISREC-Server.² The computations for BLAST were performed at the NCBi using the BLAST network service. Alignment of amino acid sequences was achieved as follows. First, an initial multiple sequence alignment of the sequences was created with PILEUP. The alignment was then manually adjusted according to information obtained from the BLAST searches. The aligned sequences thus obtained were displayed using SeqVu, Macintosh version 1.01.

**Construction of Expression Plasmids**

pET24 (vlmRN1RC1)—Two PCR primers (RN1, 5′-ATAAT ATG ACC CCC TCG GCG GCT GCC AC-3′; and RC1, 5′-TGCG CTC GGA GGT GAT AGC AGC C-3′) were designed to allow the introduction of an Ndel site on the upstream side of the gene and an Xhol site on the downstream side. A DNA fragment (0.6 kb) containing the vlmR gene was amplified by PCR (protocol A) from pB634E3SacI using RN1 and RC1 as primers. The PCR product was purified from an agarose gel and then digested with Ndel and Xhol. The resulting fragment was isolated from an agarose gel and cloned into Ndel-Xhol-digested plasmid vector pET24a (+). The resulting recombinant plasmid pET24 (vlmRN1RC1) was amplified in *E. coli* DH5α cells, and the insert was sequenced to ensure that the correct construction had been obtained. The pET24 (vlmRN1RC1) construct was then introduced into *E. coli* BL21(DE3) for expression of the vlmR gene.

pPROEX (vlmRN3RC3)—Two PCR primers (RN3, 5′-ATG ACC CCC TCT TGT GCT GCC TGG C-3′; and RC3, 5′-TTT AGG CTT CTA CTC AAG GGA GGT GAT AGC AGC C-3′) were designed to allow the introduction of an HindIII site on the downstream side. In

² http://ulrec3.unil.ch/software/profilescan.html; sensitivity was set to “include borderline matches.”
RN3, 4 bases were changed (underlined) so that the codons for the first eight amino acids corresponded to those preferred by E. coli. A DNA fragment (0.6 kb) containing the vlmR gene was amplified by PCR (protocol B) from pBS34E3SacI containing RN3 and RC3 as primers. The purified PCR product was then digested with HindIII, and the resulting fragment was isolated from an agarose gel. The purified restriction fragment was cloned into Etet-HindIII-digested pPROEXHta vector. The resulting recombinant plasmid pPROEX(vlmRN3RC3) was amplified in E. coli DH5α cells, and the insert was sequenced to ensure that the correct construction had been obtained. The pPROEX(vlmRN3RC3) construct was then introduced into E. coli DH10B for expression of the vlmR gene.

**Heterologous Expression of vlmR in E. coli**

Expression of VlmR from pET24(vlmRN1RC1) and pPROEX-(vlmRN3RC3) was carried out following the guidelines of the manufacturer of the expression vectors. Expression of VlmR from pET28(vlmRN1RC1) used the following procedure. LB broth (100 ml, 30 μg/ml kanamycin) was inoculated with 4 ml of frozen stock of E. coli BL21(DE3) cells harboring pET28(vlmRN1RC1) and incubated at 37 °C at 300 rpm for about 1.5 h. The culture was stored at 4 °C overnight. The next day, the cells were collected by centrifugation (4,000 × g, 10 min) and resuspended in LB broth (5 ml, 30 μg/ml kanamycin). Four ml of this cell suspension was used to inoculate LB broth (4 × 200 ml, 30 μg/ml kanamycin in four 1-liter flasks). The broth was incubated at 37 °C and 300 rpm for about 2 h to reach an A600 of about 0.7. IPTG (8 ml of a 100 mM solution) was then added and the culture was incubated at 37 °C and 300 rpm for an additional 3 h. The cells were collected by centrifugation and stored at −20 °C.

**Protein Purification**

All of the steps in the purification protocol were performed at room temperature, except for the initial sonication and centrifugation which were carried out at 4 °C. Cells (2.8 g wet weight) from 800 ml of a culture of BL21(DE3) containing pET28(vlmRN1RC1) were suspended in about 60 ml of ice-cold buffer D (20 mM Bis-Tris HCl, 100 mM NaCl, pH 8.0, 10% w/v glycerol) and sonicated for 4 min (70% power, Branson 350). Prepurified argon was introduced into the solution by means of a needle through the septum, and a second needle inserted into the septum was used for aspiration. Prepurified argon was introduced into the solution by means of a needle through the septum, and a second needle inserted into the septum was used for aspiration. The hFR fraction was then eluted with 3 ml of buffer G. The concentration of FAD in the hFR fraction is calculated to be 15.2 μM FAD, and 99 μM FAD reductase derived by thrombin cleavage (tFR) was eluted with 32 ml of buffer G.

**Inhibition of the FAD Reductase Activity by Crude Cell Extract**

Crude cell extract from E. coli BL21(DE3) containing pET28-(vlmRN1RC1) (1 ml, protein concentration, 3.2 mg/ml) was loaded onto a CentriCon-10 concentrator (10,000 Da cutoff) and spun at 3000 × g for 1 h. About 200 μl of filtrate was obtained, and the protein was retained in the upper chamber. Assays were then carried out using 0.35 μg/ml of the presence of 5 μl of either the filtrate or the concentrated protein solution. The filtrate did not inhibit the reductase activity, whereas the concentrated protein solution completely inhibited the reductase activity (less than 2% residual activity). Addition of 5 μl of the unconcentrated crude cell extract to the assay mixture also completely inhibited reductase activity.

**Test for FAD Loss during TALON Affinity Chromatography of hFR**

To 5 ml of a crude cell extract from E. coli BL21(DE3) containing pET28(vlmRN1RC1) with a protein concentration of 3.2 mg/ml, 68 μl of a solution of FAD (14.7 mM) in buffer D was added to give a final FAD concentration of 200 μM. After being incubated at 25 °C for 3 h, the mixture was loaded onto a TALON column (1-ml bed volume, 10 mm inner diameter) equilibrated with buffer D. The column was washed with 2 ml of buffer D, 5 ml of buffer E, and 0.5 ml of buffer G. The hFR fraction was then eluted with 3 ml of buffer G. The hFR concentration was determined to be 28.6 μM (0.67 mg/ml), and the A280 was found to be 0.1718. Assuming that the enzyme-bound FAD has the same absorption coefficient as free FAD (13.3 mM−1 cm−1), the concentration of FAD in the hFR fraction is calculated to be 15.2 μM.

### Spectral Measurements

Absorption spectra were measured with a Hewlett-Packard 8452A Diode Array Spectrophotometer equipped with a thermostated cell holder. Enzyme activities were determined with a 5-s cycle time and 0.5-s integration time. The concentrations of substrates and cofactors were determined spectrophotometrically using the following absorption coefficient data: NADH, 6.22 mM−1 cm−1 (340 nm); NADPH, 6.22 mM−1 cm−1 (340 nm); FAD, 11.3 mM−1 cm−1 (450 nm), 4.78 mM−1 cm−1 (340 nm); FMN, 12.2 mM−1 cm−1 (450 nm), 5.45 mM−1 cm−1 (340 nm); riboflavin, 12.2 mM−1 cm−1 (450 nm), 5.85 mM−1 cm−1 (340 nm).

### Determination of Hydrogen Peroxide Concentration

The sample solution (0.5 ml) was mixed with 0.5 ml of a solution containing 2.5 mM 4-aminophenylpyrine and 170 mM phenol in a 1.5-ml cuvette, and a background absorption spectrum was taken. Hydrogen peroxide (0.5 μl) (5.25 units/μl) Sigma, Type X) was added, and the mixture was incubated for 3 min at 25 °C. The A520 was measured to determine the hydrogen peroxide concentration in the mixture using a value for ε520 of 6.38 mM−1 cm−1 (16).

### Enzyme Assay

All enzyme assays were carried out at 25 °C. NADPH oxidation activity was measured by monitoring the initial rate of the decrease in absorbance at 340 nm. The standard assay mixture (1 ml) contained 20 mM Bis-Tris-HCl, pH 7.5, 40 μM FAD, 100 μM NADPH, and the enzyme sample. One unit of enzyme activity was defined as amount of the enzyme required to oxidize 1 μmol of NADPH per min. The ability of the FAD reductase to support the N-hydroxylation of IBA was assayed in the presence of a large molar excess of the over-expressed N-hydroxylase. The assay was performed at 30 °C for 30 min in 100 μM of 100 mM Bis-Tris-HCl buffer, pH 7.5, in a 1.7-ml microcentrifuge tube. The assay mixture contained 100 mM NaCl, 10 mM FAD, 4 mM NADH, 10 mM isobutylamine, 0.4 units/μl catalase, 1.40 μM overexpressed isobutylamine N-hydroxylase, and 80 mM TFR. After the incubation, the concentration of isobutylamine-N-hydroxylase was determined to be 168 μM by HPLC analysis (15).

### FAD Reductase Activity and Effect of Molecular Oxygen

An incubation mixture (3 ml) containing 20 mM Bis-Tris-HCl, pH 7.5, 41 μM FAD, and 99 μM NADPH was placed in a cuvette (3.5 ml, 1-cm light path), equipped with a magnetic stirbar and rubber septum. Prepurified argon was introduced into the solution by means of a needle through the septum, and a second needle inserted into the septum was
connected to a tube serving as the outlet. The end of the outlet tube was immersed in mineral oil. The incubation mixture was flushed with argon for 4 min to remove oxygen. The UV-visible spectrum was then determined (Fig. 2, curve A). A solution of tFR (30 µl, 2.1 µg, 3.0 units) was injected into the cuvette to initiate the enzymatic reaction, and the mixture was stirred at room temperature under argon. The yellow color disappeared immediately. After 4 min, another spectrum was determined (Fig. 2, curve B). This spectrum indicated that the concentration of the remaining FAD was 4 µM, and that of the remaining NADPH was 66 µM. Oxygen gas was then bubbled into the incubation mixture for 2 min, whereupon the yellow color returned. The UV-visible spectrum of the reoxidized solution (Fig. 2, curve C) indicated that the concentration of FAD was 41 µM, whereas that of the remaining NADPH was 4 µM. By using the peroxidase assay, the hydrogen peroxide concentration in the reoxidized incubation mixture was found to be 89 µM.

Identification of the Flavin Component of the Reductase

About 0.5 mg of the His-tag fusion protein expressed in E. coli DH10B from pPROEX(vlmRN3RC) was suspended in a mixture of 100 µl of 20 mM sodium phosphate buffer, pH 7.5, and 0.9 ml of methanol. The suspension was heated at 95 °C for 15 min. After centrifugation, the supernatant was dried with a Speed-Vac concentrator. Water (10 µl) was added to the residue, and the solution was analyzed by TLC on a K6F silica gel plate using the upper layer of an n-butyl alcohol:glacial acetic acid:water (4:1:5) mixture as the developing solvent. Aqueous solutions of FAD, FMN, and riboflavin were spotted as references, and the compounds were visualized with UV light. Under these conditions, FAD, FMN, and riboflavin exhibited ε450 values of 0.06, 0.16, and 0.43, respectively. The enzyme-derived sample gave a single fluorescent spot with an ε450 of 0.06, indicating that the flavin present in the recombinant flavin reductase is FAD.

Determination of the Stoichiometry and Dissociation Constant for Binding of FAD to tFR

Method 1—A 1.5-ml mixture of tFR (0.44 mg/ml, 20.5 µM) and FAD (116.6 µM) in buffer F was dialyzed against 500 ml of buffer D for 2 days at 25 °C using a Pierce Slide-A-Lyzer 10,000 dialysis cassette. The protein concentration of the resulting solution was determined to 0.83 mg/ml, i.e. 23.7 µM. Using the solution outside the dialysis cassette as background, the UV-visible spectrum of the protein solution was determined (Fig. 3, curve B). The spectrum was similar to that of free FAD (Fig. 3, curve A) and gave an A450 of 0.161. Assuming that the enzyme-bound FAD has the same absorption coefficient as free FAD (11.3 mM⁻¹ cm⁻¹), the concentration of enzyme-bound FAD is calculated to be 14.2 µM. Thus 60% of the tFR-bound FAD. Therefore, the concentration of free FAD was calculated to be 0.3 µM, and Kd was about 0.5 µM⁻¹.

Method 2—A 2-ml mixture of tFR (0.44 mg/ml, 20.5 µM) and FAD (100 µM) was incubated at 25 °C for 30 min. The mixture was loaded into a CentriCon-10 concentrator (10,000 M_r cutoff) and spun at 3000 × g for 15 min. On the basis of the assumption that the enzyme-bound FAD has the same absorption coefficient as free FAD, the concentrations of FAD in the filtrate and retentate were determined from the absorbance at 450 of 11.3 mM⁻¹ cm⁻¹. The spectrum of tFR-bound FAD was normalized using ε450 of 11.3 mM⁻¹ cm⁻¹. The spectrum of tFR-bound FAD was normalized using ε450 of 11.3 mM⁻¹ cm⁻¹. 

FAD has the same absorption coefficient as free FAD, the concentrations of FAD in the filtrate and retentate were determined from the A450 to be 90.0 and 125.9 µM, respectively. Thus the concentration of enzyme-bound FAD was calculated to be 93% of the tFR-bound FAD. Therefore, 95% of the tFR-bound FAD at a saturating concentration of FAD, suggesting that 1 mol of FAD was bound per mol of tFR monomer.

Method 3—One-half a milliliter of a stock solution of tFR in buffer D (12.64 µM) was mixed with 0.5 ml of an FAD stock solution in buffer D, and the mixture was incubated at 25 °C for 20 min. Five such mixtures were made in triplicate from different FAD stock solutions (5, 6, 7, 9, 10, 11).

![Figure 2](http://www.jbc.org/)  
**Fig. 2. Reduction of FAD catalyzed by tFR.** Curve A, UV-visible spectrum of a solution of FAD (41 µM) in 20 mM Bis-Tris-HCl buffer, pH 7.5, before addition of enzyme; curve B, 4 min after addition of tFR (3.0 units) under anaerobic conditions; curve C, 2 min after readmission of oxygen.

![Figure 3](http://www.jbc.org/)  
**Fig. 3. UV spectra of free (curve A) and tFR-bound (curve B) FAD, and difference spectrum (curve C).** The spectrum of tFR-bound FAD was normalized using ε450 of 11.3 mM⁻¹ cm⁻¹. Δε = ε (free FAD) − ε (tFR-bound FAD).
and 18 μM). The final concentrations of tFR and FAD ([E] and [FAD]) were calculated from those of the stock solutions and confirmed by measurement of the \( A_{250} \). Each mixture (15 total) was loaded onto a CentriCon-10 concentrator (10,000 M cutoff) and spun at 4000 × g for 3 min so that about 0.15 ml of filtrate was obtained. The concentration of FAD in the filtrate ([FAD]) was determined from the \( A_{250} \) and the concentration of enzyme-bound FAD ([FAD]_b) as [FAD] = [FAD]_b. Assuming that the total concentration of FAD binding sites for tFR is \([BS]_t\), then \([FAD]_t\) = \([BS]_t\) - \([FAD]_b\). Scatchard analysis ([FAD]_b plotted against [FAD]_t/FAD) (Fig. 4) gave a \( K_b \) of 0.70 ± 0.05 μM and a [BS] of 6.35 ± 0.1 μM. Since the concentration of tFR was 6.32 μM, the ratio of FAD binding sites to tFR was 1.0:1. This suggests that 1 mol of tFR contains 1 FAD binding site.

Method 4—The binding of FAD to tFR was also examined by a stopped-flow study with an MC 200 Monochromator equipped with a MilliFlow Stopped Flow Reactor (SLM Instruments, Inc.). One syringe contained a solution of tFR (4 mg/ml) in buffer D, and a solution of FAD in buffer D was placed in the other syringe. The stopping syringe was set at 0.15 ml. The two solutions were rapidly mixed in a 1:1 volume ratio, and the fluorescence intensity at 530 nm (excitation wavelength 450 nm) was monitored as a function of time. The integration time was 0.3 ms. For each FAD concentration, 10 reactions were carried out, and the data were averaged. To determine the value of the observed binding constant \( k_{obs} \), the equation \( F' = \Delta F - \Delta F \times \exp(-k_{obs} \times t) + F_{0} \) (where \( F \) is fluorescence intensity at time \( t \), \( \Delta F \) is fluorescence intensity at infinite time; \( \Delta F_{0} \) is fluorescence intensity difference between the fluorescence at infinite time and at time \( 0 \)) was used for nonlinear fitting.

**Spectral Properties of the FAD-Reductase Complex**

tFR in buffer F containing glycerol was desalted and the buffer exchanged with buffer D by using a PD-10 column. The resulting tFR solution had a concentration of 22.9 μM (0.49 mg/ml). Two ml of the tFR solution was mixed with 6 μl of 7.22 mM FAD solution to give a FAD final concentration of 21.66 μM. A 21.66 μM FAD solution in buffer D was prepared, and spectra of the FAD/tFR, tFR, and FAD solutions were determined using buffer D as background. The fluorescence measurements were carried out with an Amino-Bowman Series 2 Luminescence Spectrometer using identical conditions for all three solutions. CD spectra for all three solutions were measured with a Aviv Circular Dichroism Spectrometer (model 62ADS). All the spectra were taken at 25 °C.

**Native Molecular Weight Determination**

The native molecular weight for tFR was determined by using a Superose 6 FPLC gel filtration column with 50 mM sodium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. The column was calibrated by using Bio-Rad gel filtration standards.

**Other Methods**

SDS-PAGE was performed according to the method of Laemmli (17). A sample of total E. coli proteins was obtained by suspending the cells in SDS-gel loading buffer and incubating the mixture at 90 °C for 5 min. Isoelectric focusing (pH range 9–11) was performed according to the method of Robertson et al. (18). After electrophoresis, the gels were immersed in staining solution (0.25% w/v) Coomassie Brilliant Blue R250 in methanol:distilled water:acetic acid (4:5:4:5:10) and gently agitated for 10 min. The gels were then destained with washing solution (staining solution minus Coomassie Brilliant Blue R250) until the protein bands showed clearly. Protein concentrations were determined by the method of Bradford (19) using bovine serum albumin as the standard.

**RESULTS**

**Identification and Cloning of the vlmR Gene—Plasmid pB634E3 containing the vlmH gene (15) was digested with SacI to give three fragments that were cloned into pBluescript II SK(-) to give pB634ESac1, pB634ESac2, and pB634ESac3.**

**Overexpression of the vlmR Gene—To establish that the vlmR gene encodes a flavin reductase, the gene was cloned into the expression vector pET-24a (+). The recombinant vector containing the vlmR gene, pET24(RN1RC1), was introduced into E. coli BL21(DE3) and expression was induced with IPTG.**

**Downloaded from http://www.jbc.org/ by guest on June 23, 2017**

---

**Fig. 4.** Scatchard plot of FAD binding to tFR. Error bars indicate the standard deviation from three assays.

**Fig. 5.** Observed binding rate constant for binding of FAD to tFR as a function of FAD concentration. Error bars indicate the standard error for 10 determinations.
Cloning and Overexpression of NADPH:FAD Oxidoreductase

**Figs. 6, 7, and 8.**

**FIG. 6.** The vlmR coding region contained in pB634E3Sac1.

**FIG. 7.** Nucleotide sequence of the vlmR gene. The first base of the first codon is numbered 0.

**FIG. 8.** SDS-PAGE analysis of VlmR overexpression, purification, and thrombin cleavage. Lanes 1 and 7, molecular mass markers: ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), aprotinin (6,500); lane 2, total protein from *E. coli* BL21(DE3) harboring pET28(vlmRN1RC1) before IPTG induction; lane 3, total protein from *E. coli* BL21(DE3) harboring pET28(vlmRN1RC1) 3 h after IPTG induction; lane 4, crude extract from *E. coli* BL21(DE3) harboring pET28(vlmRN1RC1) 3 h after IPTG induction; lane 5, TALON fraction (hFR); lane 6, protein (tFR) obtained after thrombin treatment.

**Purification of the Flavin Reductase**—The VlmR protein expressed from pET28(vlmRN1RC1) was purified from the crude cell extract in one step, using a TALON affinity column (Table II). The purified fusion protein constituted about 35% of the total soluble protein. The purified fusion protein was shown to possess very high flavin reductase activity and was able to provide reduced flavin to IBAH for IBA hydroxylation. The N-terminal His-tag was then removed by thrombin cleavage. The cleavage conditions were controlled so that only about 70% of the fusion protein was digested to avoid possible second site cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. Control experiments indicated that the high molecular weight fraction of the crude extract from the same strain strongly inhibits flavin reductase activity.

**Molecular Mass and Isoelectric Point**—The subunit molecular mass of the tFR was determined to be ~23 kDa by SDS-PAGE. The native molecular mass of the tFR was measured to be ~36 kDa by gel filtration, which suggests that the protein exists as a homodimer. The pl of tFR was determined by isoelectric focusing to be 10.0. The nature of the thrombin cleavage site in the pET-28b (+) vector is such that three amino acids (Gly-Ser-His) will remain at the N terminus of the flavin reductase obtained from the pET28(vlmRN1RC1) construct in the apo form of the VlmR protein and a calculated isoelectric point of 10.2.

**Substrate Specificity**—The His-tag form of the flavin reductase expressed from the pPROEXHTa vector in *E. coli* DH10B exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. The His-tag peptide fragment was then removed by thrombin cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation.

The subunit molecular mass of the flavin reductase was determined to be ~23 kDa by SDS-PAGE. The native molecular mass of the flavin reductase was measured to be ~36 kDa by gel filtration, which suggests that the protein exists as a homodimer. The pl of the flavin reductase was determined by isoelectric focusing to be 10.0. The nature of the thrombin cleavage site in the pET-28b (+) vector is such that three amino acids (Gly-Ser-His) will remain at the N terminus of the flavin reductase obtained from the pET28(vlmRN1RC1) construct in the apo form of the flavin reductase. The His-tag form of the flavin reductase expressed from the pPROEXHTa vector in *E. coli* DH10B exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. The His-tag peptide fragment was then removed by thrombin cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. Control experiments indicated that the high molecular weight fraction of the crude extract from the same strain strongly inhibits flavin reductase activity.

**Molecular Mass and Isoelectric Point**—The subunit molecular mass of the flavin reductase was determined to be ~23 kDa by SDS-PAGE. The native molecular mass of the flavin reductase was measured to be ~36 kDa by gel filtration, which suggests that the protein exists as a homodimer. The pl of the flavin reductase was determined by isoelectric focusing to be 10.0. The nature of the thrombin cleavage site in the pET-28b (+) vector is such that three amino acids (Gly-Ser-His) will remain at the N terminus of the flavin reductase obtained from the pET28(vlmRN1RC1) construct in the apo form of the flavin reductase. The His-tag form of the flavin reductase expressed from the pPROEXHTa vector in *E. coli* DH10B exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. The His-tag peptide fragment was then removed by thrombin cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. Control experiments indicated that the high molecular weight fraction of the crude extract from the same strain strongly inhibits flavin reductase activity.

**Substrate Specificity**—The His-tag form of the flavin reductase expressed from the pPROEXHTa vector in *E. coli* DH10B exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. The His-tag peptide fragment was then removed by thrombin cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. Control experiments indicated that the high molecular weight fraction of the crude extract from the same strain strongly inhibits flavin reductase activity.

**Molecular Mass and Isoelectric Point**—The subunit molecular mass of the flavin reductase was determined to be ~23 kDa by SDS-PAGE. The native molecular mass of the flavin reductase was measured to be ~36 kDa by gel filtration, which suggests that the protein exists as a homodimer. The pl of the flavin reductase was determined by isoelectric focusing to be 10.0. The nature of the thrombin cleavage site in the pET-28b (+) vector is such that three amino acids (Gly-Ser-His) will remain at the N terminus of the flavin reductase obtained from the pET28(vlmRN1RC1) construct in the apo form of the flavin reductase. The His-tag form of the flavin reductase expressed from the pPROEXHTa vector in *E. coli* DH10B exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. The His-tag peptide fragment was then removed by thrombin cleavage. The thrombin-treated flavin reductase (tFR) was separated from the fusion protein and His-tag peptide fragment using a TALON column (Table II and Fig. 8). The purified tFR also exhibited very high flavin reductase activity, and it was able to provide reduced flavin to IBAH for IBA hydroxylation. Control experiments indicated that the high molecular weight fraction of the crude extract from the same strain strongly inhibits flavin reductase activity.
that contained no FAD after purification.

tem and a longer expression time (5.5 h) also produced hFR
the holo form. An experiment using the same expression sys-
paper form. Since the reductase was expressed at a high level, it
zyme-bound FAD is removed during the purification process,
noted a linear dependence on NADH over a concentration range
between free FAD and tFR-bound FAD exhibits negative peaks at
fluorescence emission at an excitation wavelength of 450 nm,
tFR exhibits no UV absorption between 300 and 800 nm, no
ations,
k
Km
Substrate Second substrate kcat Km kcat/Km
NADPH
40 mM FAD
s-1 µM
s-1/µM
62
32 ± 2
1.9
>300
1.8 × 10-3
FAD
100 mM NADPH
51
3.6 ± 0.4
14.2
FMN
45
8.4 ± 1
5.4
Riboflavin
6.7
11.1 ± 2
0.6

a

b

Used 0.173 µg (133 units/mg) of tFR in a 1-ml reaction volume.
Used 1.73 µg (133 units/mg) of tFR in a 1-ml reaction volume and an
NADH concentration of 50–300 µM.

Formation of Apo-reductase—The crude cell extract from
E. coli BL21(DE3) pET28 (vlmRN1RC1) displayed a faint yellow
color, but purified hFR was colorless and exhibited no UV
absorption between 300 and 800 nm. To examine the possible
loss of bound FAD during purification, a large molar excess of
FAD was added to the crude cell extract before TALON affinity
chromatography. After chromatography, the concentration of
hFR was found to be 28.6 µg (133 units/mg) of tFR in a 1-ml reaction volume. Consequently, it appears that only a portion of en-
zyme-bound FAD is removed during the purification process,
which suggests that most of the reductase is expressed in the
apo form. Since the reductase was expressed at a high level, it
is conceivable that the host E. coli might not be able to produce
sufficient FAD to convert all of the overexpressed enzyme into
the holo form. An experiment using the same expression sys-

tFR complex (Fig. 10) displays a large positive peak at 370 nm and a large
negative peak of 455 nm.

Sequence Homology Studies—A FASTA data base search did
not identify any proteins with significant similarities to VlmR.
Searches of the PROSITE data base with MOTIFS and PSCAN
also did not reveal any significant matches between the VlmR
protein and motifs in the data base. A BLAST search revealed
significant similarities between the VlmR protein and a small
number of other proteins. A multiple sequence alignment of the
VlmR protein with the related proteins identified by the
BLAST search is presented in Fig. 11. The alignments between
VlmR and these proteins are found throughout the amino acid
sequences. Some of the aligning proteins are known to be flavin
reductases, and others are hypothetical proteins of unknown
function. The alignments exhibited by these hypothetical pro-
tains suggest that these proteins are likely to be flavin reduc-
tases. Sequence comparisons between VlmR and a number of
potentially related proteins using the BESTFIT program re-
vealed that the closest match between VlmR and proteins of
known function was with the SnaC protein which is an NADH:
FMN oxidoreductase from Cheletobacter heintzii (22). Other notable matches included the ActVB protein
from Streptomyces coelicolor (23) and the NmoB/NtaB protein
from S. viridifaciens (24–26), both of which are NADH:
FMN oxidoreductases.

DISCUSSION

The vlmR gene, which encodes an NADPH:FAD oxidoreduc-
tase, has been cloned from S. viridifaciens MG456-hF10 by
chromosome walking and overexpressed in soluble form in E.
coli. The resulting protein exhibited a high degree of flavin reductase activity in the presence of NADPH. Overexpression of the VlmR protein in E. coli proved to be problematic until it was discovered that the presence of a leader sequence coding for a His-tag at the 5'-end of the gene allowed expression to occur. The difficulty in expression of the vlmR gene may be related to the fact that it is translationally coupled to the open reading frame (ORF2) that lies immediately upstream (Fig. 6).

Since the vlmR gene is located in close proximity to the gene encoding isobutylamine N-hydroxylase (vlmH) on the S. viridifaciens chromosome (Fig. 6), it appears likely that the function of the VlmR protein is to provide reduced FAD to isobutylamine N-hydroxylase. This expectation is supported by preliminary evidence indicating that a mixture of VlmR and isobutylamine N-hydroxylase will efficiently catalyze the hydroxylation of isobutylamine to isobutylhydroxylamine. Since precursor incorporation experiments strongly support the intermediacy of isobutylamine and isobutylhydroxylamine in valanimycin biosynthesis (12, 13), it is reasonable to assume that both the vlmR and vlmH gene products are involved in valanimycin biosynthesis.

### Multiple sequence alignment of VlmR and related proteins identified by the BLAST search

<table>
<thead>
<tr>
<th>Protein 1</th>
<th>Protein 2</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviation</td>
<td>Length (aa)</td>
<td>Compared range (aa)</td>
</tr>
<tr>
<td>Svf.VlmR</td>
<td>194</td>
<td>1–194</td>
</tr>
<tr>
<td>Chi.NmbO/NtaB</td>
<td>134</td>
<td>1–134</td>
</tr>
<tr>
<td>Scc.ActVB</td>
<td>122</td>
<td>1–122</td>
</tr>
<tr>
<td>Ssc.SnaC</td>
<td>132</td>
<td>1–132</td>
</tr>
<tr>
<td>Sys.Hyp1</td>
<td>124</td>
<td>400–573</td>
</tr>
<tr>
<td>Sys.Fp</td>
<td>597</td>
<td></td>
</tr>
<tr>
<td>Vfl.Fr</td>
<td>218</td>
<td>1–218</td>
</tr>
<tr>
<td>Vfl.Fr</td>
<td>218</td>
<td>1–218</td>
</tr>
<tr>
<td>Vfr</td>
<td>237</td>
<td>1–237</td>
</tr>
</tbody>
</table>

### Table IV

**Comparison between VlmR and related proteins with BESTFIT program**

<table>
<thead>
<tr>
<th>Gap weight, 3.0, length weight, 0.1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviation</td>
</tr>
<tr>
<td>Svf.VlmR</td>
</tr>
<tr>
<td>Chi.NmbO/NtaB</td>
</tr>
<tr>
<td>Scc.ActVB</td>
</tr>
<tr>
<td>Ssc.SnaC</td>
</tr>
<tr>
<td>Sys.Fp</td>
</tr>
<tr>
<td>Vfl.Fr</td>
</tr>
<tr>
<td>Vfl.Fr</td>
</tr>
<tr>
<td>Vfr</td>
</tr>
</tbody>
</table>

### Notes

- Abbreviations and accession numbers for proteins are as follows: Svf.VlmR, the vlmR gene product; Chi.NmbO/NtaB, nitritotriacetate monoxygenase component B from C. heintzii ATCC 29600 (NmbL49438, g111920; NtaB U384911, g148206); Scc.ActVB, NADH:FAD oxidoreductase from S. coelicolor (X63449, g46811); Ssc.SnaC, NADH:FAD:FAD oxidoreductase from S. pristinaespiralis (X63449, g46811); Sps.SnaC, NADH:FAD oxidoreductase from S. pristinaespiralis (X63449, g46811); Eco.HpaC, coupling component of 4-hydroxyphenylacetate hydroxylase from E. coli (Z29081, g452842); Sys.Hyp1, hypothetical protein from Synechocystis sp. PCC6803 (D64000, g1001553); Scs.F1m3, flm3 region hypothetical protein 1 from Synechococcus sp. PCC7942 (L19521, g4089; g1001242); Eco.F152, f152 hypothetical protein from E. coli (AE000202, g1787242); Sys.Fmnp, potential FMN protein from Synechocystis sp. PCC6803 (D90900, g1651803); Sys.Hyp2, hypothetical protein from Synechocystis sp. PCC6803 (D90914, g1653555); Sys.Fp, putative flavoprotein from Synechocystis sp. PCC6803 (D64000, g1001242); Vfl.Fr, NADH:FAD oxidoreductase from V. harveyi (U08996, g478868); Vfr, NADPH:FAD oxidoreductase from V. harveyi (D14674, g408912).
- aa, amino acids.
biosynthesis. However, definitive proof for the role of these genes in valanimycin biosynthesis will require that gene disruption experiments be performed.

A BLAST search indicated that VlmR is related to three proteins that are known to be NADH:FAD oxidoreductases: SnaC, ActVB, and NmoB/NtaB. These proteins and VlmR all exist as homodimers, bind flavins weakly, and utilize flavins as a substrate rather than as a cofactor. However, VlmR is unique in that it prefers FAD and NADPH and exhibits a PI of 10.0. Another unusual feature of VlmR is its strong preference for NADPH over NADH. This behavior is illustrated by the 1000-fold difference in the $k_{cat}/K_m$ values for the two substrates at an FAD concentration of 40 pM (Table II). It is conceivable that the larger $k_{cat}/K_m$ for NADPH is related to the fact that NADPH is more negatively charged than NADH and that VlmR will carry an excess of positive charges at biological pH due to its high PI.

Apart from these considerations, the purpose that might be served by the high PI of the VlmR protein is unclear. The calculated PI values for SnaC, ActVB, and NmoB/NtaB fall between 5 and 7.

The fluorescence emission spectrum and CD spectrum of FAD are altered significantly upon binding to tFR. The increase in the intensity of the emission spectrum of the FAD may be due to movement of the adenine ring away from the isoalloxazine ring since stacking between these two rings quenches the fluorescence of free FAD (27). The optical activity of tFR-bound FAD apparent from the CD spectrum presumably results from asymmetric interactions between the bound FAD and the protein.

Sequence analysis suggests that VlmR, SnaC, NmoB/NtaB, and ActVB belong to the same family of flavin reductases. The hypothetical proteins Hyp1 (28), Fm3 (29) and F1523 from *Synechocystis sp.*, *Synechococcus sp.*, and *E. coli*, respectively, also appear to be members of this same family. Another group of hypothetical proteins from *Synechocystis* sp. Fmnp (31), Fp (28), and Hyp2 (32), are characterized by a C-terminal region that aligns with the VlmR, SnaC family. The larger size of these proteins suggest they may be multifunctional proteins that contain a flavin reductase domain.

The HpaC protein from *E. coli* (33) also aligns with the VlmR, SnaC family. This protein is part of a two-component enzyme system that catalyzes the conversion of 4-hydroxyphenylacetate to 3,4-dihydroxyphenylacetate. It has been reported that no NADH oxidation activity was detected in a cell-free extract of an *E. coli* strain harboring a plasmid that carried the *hlr* gene to the gene (*vlmH*) encoding an NADPH:FAD oxidoreductase. In summary, a gene (*vlmH*) encoding an NADPH:FAD oxidoreductase has been cloned from the valanimycin producer *S. viridifaciens* and overexpressed in soluble form. The overexpressed protein exhibited the expected enzymatic activity, thereby confirming the identity of the protein. The proximity of the *vlmR* gene to the gene (*vlmH*) coding for isoalloxylamine N-hydroxylase on the *S. viridifaciens* chromosome as well as evidence from enzymology and precursor incorporation experiments makes it likely that these two genes are part of a gene cluster associated with valanimycin biosynthesis. The derived amino acid sequence of the VlmR protein exhibited similarity to several other flavin reductases that may constitute a new family of flavin reductases. The successful overexpression of both the N-hydroxylase and the flavin reductase components of the isoalloxylamine hydroxylase system in soluble and active form now sets the stage for a more detailed enzymatic investigation of this novel amine hydroxylase.

REFERENCES

17. Worthington Biochemical Corp., Freehold, NJ