The Violacein Biosynthetic Enzyme VioE Shares a Fold with Lipoprotein Transporter Proteins

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VioE, an unusual enzyme with no characterized homologues, plays a key role in the biosynthesis of violacein, a purple pigment with antibacterial and cytotoxic properties. Without bound cofactors or metals, VioE, from the bacterium Chromobacterium violaceum, mediates a 1,2 shift of an indole ring and oxidative chemistry to generate prodeoxyviolacein, a precursor to violacein. Our 1.21 Å resolution structure of VioE shows that the enzyme shares a core fold previously described for lipoprotein transporter proteins LolA and LolB. For both LolB and VioE, a bound polyethylene glycol molecule suggests the location of the binding and/or active site of the protein. Mutations of residues near the bound polyethylene glycol molecule in VioE have identified the active site and five residues important for binding or catalysis. This structural and mutagenesis study suggests that VioE acts as a catalytic chaperone, using a fold previously associated with lipoprotein transporters to catalyze the production of its prodeoxyviolacein product.

Violacein 5 (Fig. 1) is a purple pigment that gives tropical bacterium Chromobacterium violaceum its characteristic coloring (1–5). Violacein 5 has potential medical applications as an antibacterial (6–8), anti-tryptanocidal (8–10), anti-ulcerogenic (11), and anti-cancer drug (8, 12–17). The molecule, made of l-tryptophan and molecular oxygen (18–21), was originally thought to be synthesized by the action of just four enzymes VioA, VioB, VioC, and VioE, a bound polyethylene glycol molecule suggests the location of the binding and/or active site of the protein. Mutations of residues near the bound polyethylene glycol molecule in VioE have identified the active site and five residues important for binding or catalysis. This structural and mutagenesis study suggests that VioE acts as a catalytic chaperone, using a fold previously associated with lipoprotein transporters to catalyze the production of its prodeoxyviolacein product.

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† The Online version of this article (available at http://www.jbc.org/) contains supplemental Table S1 and supplemental Figs. S1–S7.

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5 The abbreviations used are: IPA, indole 3-pyruvic acid; PEG, polyethylene glycol.
bound cofactors or metals, reveals the similarity of VioE to the core structure of the lipoprotein carrier proteins LolA and LolB (32). Further, VioE resembles LolB in its binding of a PEG molecule in what appears to be the active site. To investigate whether the PEG molecule indeed identified the active site of VioE, we carried out site-directed mutagenesis on each of nine residues near the bound PEG molecule and further along the putative active site. Five of the altered proteins show significantly reduced production of prodeoxyviolacein compared with wild-type VioE. This work suggests that the fold of a lipoprotein carrier protein has been co-opted to a catalytic function, enabling VioE to bind a chiefly hydrophobic molecule in a hydrophobic cavity and to catalyze a 1,2 shift reaction and oxidative chemistry, producing prodeoxyviolacein, rather than the unshifted shunt product chromopyrrolic acid.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—Eight liters of LB medium containing 30 mg/liter of kanamycin and 34 mg/liter of chloramphenicol were inoculated with a starting culture of Rosetta™2(DE3)pLysS (Novagen) transformed with pET24b-VioE (26). The cultures were grown at 21 °C to an A_{600} of 0.6 and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside and grown for an additional 17 h at 21 °C at 250 rpm. Cell pellets (40 g) were frozen at −20 °C overnight, then sonicated with lysozyme in 300 mM NaCl, 25 mM Tris, 5 mM imidazole, pH 8.0, and then centrifuged at 50,000 × g to pellet insoluble material. The supernatant was incubated with nickel (II)-loaded Chelating Sepharose™Fast Flow (GE Biosciences) for 40 min at 4 °C. Unbound material was removed, and then the column was washed with 25 column volumes of 300 mM NaCl, 25 mM Tris, 20 mM imidazole, pH 8.0. Bound protein was then eluted with 300 mM NaCl, 25 mM Tris, 200 mM imidazole, pH 8.0. This sample was then loaded onto a HiLoad Superdex™75 prep grade column (Amersham Biosciences) pre-equilibrated with 50 mM NaCl, 20 mM Tris, pH 8.0. Fractions containing VioE were pooled and concentrated to 17 mg/ml in 10% glycerol, 50 mM NaCl, 20 mM Tris, pH 8.0. Protein was then frozen in liquid nitrogen and stored at −80 °C. VioE was crystallized at room temperature from this sample using the hanging drop vapor diffusion method by incubating 1.5 μl of protein with 1.5 μl of precipitant solution (composed of 25% (v/v) PEG 400, 20% (w/v) PEG 3350, 0.1 M MgCl₂, and 0.1 M Tris, pH 8.5) over a 0.5-ml precipitant well solution. Crystals appeared overnight and grew larger over subsequent days. Native crystals were flash frozen in liquid nitrogen, without additional cryoprotection, for x-ray data collection.

Selenomethionine protein was prepared by inoculating minimal medium (6 g/liter Na₂HPO₄, 3 g/liter KH₂PO₄, 1 g/liter NH₄Cl, 0.5 g/liter NaCl, 1 mM MgSO₄, 0.4% (w/v) glucose, 50 mg/liter kanamycin, and 34 mg/liter chloramphenicol) with a starting culture of Rosetta™2(DE3)pLysS transformed with

![FIGURE 1. Biosynthesis of violacein and indolocarbazoles.](image)

Indole 3-pyruvic acid (r = NH imine form, r = O ketone form) is generated biosynthetically from L-tryptophan by the action of the enzyme VioA or a homologue, such as RebO. In both the violacein and related indolocarbazole biosynthetic pathways, two molecules of IPA imine are dimerized to generate an as yet uncharacterized intermediate, which has been proposed to be 2 (26).
TABLE 1
Data collection and refinement statistics

|                               | Native                  | Selenomethioninea     |
|-------------------------------|-------------------------|-----------------------|
| Wavelength (Å)                | 0.9795                  | 0.9792                |
| Space group                   | P2 2 2                  | P2 2 2                |
| Unit cell (Å)                 | a = 53.5, b = 82.5, c = 90.6 | a = 53.4, b = 82.3, c = 90.6 |
| Resolution (Å)b               | 50-1.21 (1.25-1.21)     | 50-1.86 (1.93-1.86)   |
| ComPLETENESS (%)b             | 0.066 (0.258)           | 0.060 (0.226)         |
| Unique reflections            | 90.6 (54.3)             | 95.0 (58.7)           |
| Reduction (%)b                | 111,465                 | 61,353                |
| l/σ (c)                       | 13.9 (3.2)              | 6.9 (3.8)             |
| Average B-factors (Å2)        | 35.7 (3.4)              | 27.8 (5.1)            |

Refinement statistics
Resolution range (Å) | 50–1.21
R_invariants (%)c   | 16.8
R_free (%)d         | 19.5
No. nonhydrogen atoms
Protein             | 3183
Water               | 680
PEG                 | 23
Average B-factors (Å2)
Protein             | 15.0
Water               | 32.0
PEG                 | 44.2
Root mean squared deviation bond length (Å) | 0.006
Root mean squared deviation bond angle (°) | 1.209
Ramachandran plot (% residues)
Most favored        | 91.6
Additionally allowed | 8.4
Generously allowed   | 0
Disallowed          | 0

a For this data set, Bijvoet pairs were not merged during data processing.

b The values in parentheses indicate the highest resolution bin.

c \( R_{\text{invariants}} = \frac{\sum |I_{\text{calc}}(hkl) - I_{\text{obs}}(hkl)|}{\sum I_{\text{calc}}(hkl)} \), where \( I_{\text{calc}}(hkl) \) is the intensity of the \( i^\text{th} \) measured reflection, and \( <I_{\text{obs}}(hkl)> \) is the mean intensity for the reflection with the mille index \( (hkl) \).

d \( R_{\text{free}} = \frac{\sum |I_{\text{calc}}(hkl) - I_{\text{obs}}(hkl)|}{\sum I_{\text{calc}}(hkl)} \); \( R_{\text{free}} \) is calculated identically, using 5% of reflections omitted from refinement.

Crystal Structure of VioE

pET24b-VioE and growing cultures at 37 °C. At an A_600 of 0.6, the cultures were supplemented with 100 mg/liter each l-lysine, l-threonine, and l-phenylalanine and 50 mg/liter each l-isoleucine, l-leucine, l-valine, and l-selenomethionine (33) and grown for an additional 20 min. Protein expression was then induced with 0.1 mM isopropyl \( \beta \)-D-thiogalactopyranoside, and the cultures were grown for an additional 20 h at 21 °C at 250 rpm. Selenomethionine protein was purified identically to native protein, except that all of the buffers, excluding the final storage buffer, were supplemented with 5 mM \( \beta \)-mercaptoethanol. A purified sample of the selenomethionine protein was assayed for incorporation of selenium using liquid chromatography-mass spectrometry, and strong peaks were found from a 23,028-atomic mass unit species, corresponding to the incorporation of four seleniums, with weak peaks found from a 23,104-atomic mass unit species, corresponding to the incorporation of four oxidized seleniums. A small number of crystals with defects were produced in the same condition as the native protein, and these were used in streak seeding experiments to obtain crystals appropriate for x-ray data collection. Single selenomethionine-VioE crystals were flash frozen directly into liquid nitrogen for x-ray data collection.

Data Collection and Processing—The data were collected at the Advanced Photon Source (Argonne, IL), beamline NE-CAT 24ID-C, with the Quantum 315 detector in a cryostream of 141.6 K. For the native crystal, the data were collected in two sweeps, one of 180° and one of 360°. A 180° sweep was first carried out in 1° oscillation steps to obtain data to 1.8 Å resolution. Next, a 360° sweep was carried out in 0.5° oscillation steps to full resolution. A small selenomethionine VioE crystal was used for a fluorescence scan to obtain the appropriate wavelength for an anomalous dispersion data collection. A larger selenomethionine VioE crystal was then used to carry out a single-wavelength anomalous dispersion data collection at the selenium peak wavelength of 0.9792 Å, with a 360° sweep in 0.5° oscillation steps. All of the data were indexed and integrated in DENZO and scaled in SCALEPACK (34). The data processing statistics are shown in Table 1. Data to 1.21 Å resolution for the native and to 1.86 Å resolution for the selenomethionine derivative were used, despite the low completeness in the highest resolution bins (54.3 and 58.7%, respectively). The values of other statistics in these highest resolution bins (including \( R_{\text{sym}} \), \( l/\sigma \), and redundancy) suggest that the data are of high quality to these resolutions.

Structure Building and Refinement—Six selenium sites were found using SHARP (35); these include three selenomethionines from each molecule of the two in the asymmetric unit and do not include the N-terminal methionines, which are disordered. The overall figure of merit (acentric) of data to 1.87 Å was calculated by SHARP to be 0.438. The native and selenomethionine data were scaled in SHARP to generate a single file used for all initial building and refinement. 2.0-Å resolution experimental maps from the SHARP output, solvent flattened with SOLOMON (36), were used to build manually the entire main chain of the protein, with placement of most side chains. This model was then used in iterative rounds of CNS (37) refinement and manual adjustment of the model in COOT (38) to the full 1.21 Å resolution of the native data, to generate a nearly complete structure with placement of a first set of water molecules. Iterative rounds of refinement were then continued in Refmac (39) with manual adjustment of the model in COOT, using only native data (with identically flagged reflections).
Crystal Structure of VioE

Alternate conformations of side chains, additional water molecules, and PEG molecules were incorporated at this stage. Refinement was then carried out with incorporation of anisotropic B-factors, and, in final rounds of refinement, with incorporation and refinement of hydrogen atoms. Crystallized VioE contains 199 residues, including the C-terminal His$_{6}$ tail with two linking residues. In both chains A and B of VioE, residues 1–4 are disordered; in chain A, residues 190–199 are additionally disordered, and in chain B, residues 195–199 are absent. The refinement statistics are listed in Table 1.

Calculation of Buried Surface Area—The program AREAIMOL, from the CCP4i suite of programs (40), was used to calculate the accessible surface area for chain A alone, chain B alone, and the dimer, using a 1.4 Å probe radius. The buried surface area was taken as the sum of the accessible area for chain A and chain B, minus the accessible area for the dimer. The accessible area of chain A is 10,300 Å$^2$, chain B is 10,700 Å$^2$, and the dimer is 17,400 Å$^2$.

Sedimentation Velocity Experiments—Sedimentation velocity experiments were carried out in a Beckman Coulter Optima XL-I analytical ultracentrifuge using a Beckman An60Ti rotor and a Beckman XL-A Monochromator in the MIT Biophysical Instrumentation Facility. Absorbance data were collected during a velocity sedimentation run at 20.0 °C at 42,000 rpm. Protein samples were prepared as for crystallization, except that VioE eluting from a nickel (II)-loaded Chelating Sepharase$^{\text{TM}}$ Fast Flow (GE Biosciences) column was loaded onto a HiLoad Superdex$^{\text{TM}}$ 75 prep grade column (Amersham Biosciences) pre-equilibrated with 200 mM NaCl, 25 mM Tris, pH 8.0. VioE from the peak gel filtration fraction was then diluted in the identical buffer to concentrations of 8.6 and 2.3 μM, and the samples were loaded into one side of a sample cell, with buffer (200 mM NaCl, 25 mM Tris, pH 8.0) loaded into the other side of each sample cell. SedInterp (41) was used to derive values based on the experimental conditions and protein sequence for the density, partial specific volume, and mass extinction coefficient at 280 nm. These values were used to analyze the absorbance data from the sedimentation velocity experiments using SEDANAL (42).

Gel Filtration—The oligomeric state of purified VioE was additionally assayed via gel filtration using a HiLoad Superdex$^{\text{TM}}$ 75 prep grade column (Amersham Biosciences) pre-equilibrated with 200 mM NaCl, 25 mM Tris, pH 8.0. Gel filtration standards (Bio-Rad) were used to calibrate this column.

Mutagenesis and Mutant Protein Preparation—All VioE mutants were generated using splicing by overlap extension method (43) with the primers listed in supplemental Table S1. Two overlapping fragments (“a” and “b”) were generated from two initial PCRs, using 1 μl of miniprepper pET24b-VioE as a template, with 4 μM of each primer, 5% Me$_2$SO, and 2X Phusion HF master mix (Invitrogen). After PCR purification using a GFX kit (GE Healthcare), the “a” and “b” fragments were mixed together and further amplified using the 5’-forward first primer from the “a” fragment reaction and the 3’-reverse second primer from the “b” fragment; all other components in the PCR were identical to those used in the first round of amplification. All of the PCRs were carried out using the following cycle parameters: 98 °C for 2 min, followed by 30 cycles of 98 °C for 15 s, 57 °C for 30 s, and 72 °C for 1.5 min, followed by cooling to 4 °C. The PCR products were once again purified using the GFX kit and then subjected to digestion with Ndel/Xhol for 3 h. The resulting DNA was purified and ligated into a similarly digested pET24b vector using Quick Ligase (New England Biolabs). The ligation reactions were then transformed into chemically competent TOP10 cells plated on LB-agar-kanamycin plates. The resulting colonies were miniprepped, and the plasmids were sequenced at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute. The plasmids were then transformed into BL21(DE3) cells, and protein was prepared as described previously (26), using a dialysis rather than a gel filtration step to transfer protein into the final storage buffer.

Activity Assays—Activity assays were carried out, as described previously (26), by incubation of L-tryptophan (500 μM) with VioA (5 μM), VioB (5 μM), VioE (5 μM), and 50 units of catalase in 75 mM glycine, pH 9.25. The reactions were quenched after 1 h with 20% (v/v) Me$_2$SO and 200% (v/v) methanol, and, after removal of precipitated protein, the samples were analyzed via high pressure liquid chromatography, as described previously (26). Prodeoxyviolacein 3 peaks at 590 nm were integrated with 32 Karat Software (version 5.0, Build 1021, Beckman Coulter, Inc.). All of the activity assays were run in triplicate and compared with authentic standards of prodeoxyviolacein 3.

Circular Dichroism—Circular dichroism spectra of native and mutant proteins were collected on an Aviv model 202 CD Spectrometer (software by Aviv, version 3.00C) at the MIT Biophysical Instrumentation Facility from 190 to 250 nm with a 1-nm wavelength step, an averaging time of 3 s, and a settling time of 0.33 s. The samples were processed in a 0.1-cm-path length rectangular quartz cuvette. All of the proteins, which were stored in a buffer solution composed of 50 mM NaCl, 20 mM Tris, pH 8.4, and 10% glycerol, were diluted in degassed, double distilled water to 17 μM in 400-μl samples. A buffer solution, also diluted in degassed, double distilled water, with a final composition of 0.2% glycerol, 1 mM NaCl, and 0.4 mM Tris, pH 8.4, was analyzed between each sample, and the average values over 10 buffer samples were used for background subtraction before generation of a final graph.

RESULTS

VioE crystallizes as a dimer, with each concave side of the curved β-sheet facing away from the interface (Fig. 2A) and with an average of 1800 Å$^2$ of buried surface area per monomer. The large dimeric interface of the two chains is chiefly hydrophobic (63% of interacting residues) with water largely excluded (only 28 molecules of water in the interface) and with 17 hydrogen bonds that pair polar atoms with one another across the interface. To investigate the oligomeric state in solution of VioE, a sedimentation velocity experiment was carried out at each of two concentrations (8.6 and 2.3 μM). The results of the sedimentation velocity experiment are consistent with a dimer in solution (supplemental Fig. S1). The sedimentation velocity experiments are in contrast to the result from preparative scale gel filtration, which suggested that VioE eluted as a monomer, based on the gel filtration standards (supplemental
Fig. S2). However, given the results of sedimentation velocity analysis, the large size of the dimeric interface, the predominance of hydrophobic residues, the general exclusion of water, and the presence of specific interactions distributed across much of the interface, the dimer appears to be the preferred oligomeric state of purified VioE.

In each monomer, strand 1 begins in the center of the protein and initiates an anti-parallel $\beta$-sheet, building from $\beta_1$ to $\beta_6$...
In all panels, secondary structural elements are labeled in 3D, and are shown in each of which are within 12 Å from the bound PEG molecule. Residues that were not targeted for mutagenesis continue from the C terminus of bringing the two halves of the structure together. A loop containing Tyr39, which is also positioned between close off the exposed side of the LolA structure.

The putative lid and extended active site of VioE. A, the proposed closed lid region of LolA is shown (Protein Data Bank code 1UA8), with Arg43 mediating a series of hydrogen-bonding interactions that close off the exposed side of the LolA structure. B, in VioE, the structurally superimposed residue to Arg43 is Tyr39, which is also positioned between β2 and β3 and also makes hydrogen bonding contacts to residues shown in blue. C, side chains within 6Å of the PEG molecule of chain B are shown. Alternate conformations were refined for Ser19, Met160, and Ser170. D, mutated residues are shown within the context of surrounding residues, each of which are within 12 Å from the bound PEG molecule. Residues that were not targeted for mutagenesis are shown in gray. Those in pink, when mutated, had no significant effect on the production of deoxyviolacein, whereas those in green, when mutated, had significantly reduced production of deoxyviolacein. In all panels, secondary structural elements are labeled in red.

Based on primary sequence, there are three putative homologues of C. violaceum VioE from related species (supplemental Fig. S3), all of which are uncharacterized proteins.

The only partially characterized enzyme with any kind of sequence identity is the enzyme RifG (25, 44) (supplemental Fig. S4). RifG, a homologue of a dehydroquinocate synthase from Amycolatopsis mediterranei S699 (44), is proposed to function together with other enzymes from the Rif operon in the formation in 3-amino-5-hydroxybenzoic acid (44), specifically in the cyclization of 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (45). However, the similarity of RifG to VioE is quite poor (E value = 0.055, with 31 identical residues, 8 clustered in the region between β6 and β7 of VioE), the sizes of the proteins differ dramatically (191 residues for VioE, and 351 residues for RifG), and the relevance of the similarity of RifG to VioE is unclear.

The story is different when one considers three-dimensional structure; VioE does have close structural homologues. VioE is structurally homologous to the uncleaved β-barrel, core structure of both LolA (root mean square deviation of 1.73 Å over 124 residues) and LolB (root mean square deviation of 1.67 Å over 98 residues) (Fig. 2, C–I, and supplemental Fig. S5) (32). Unlike VioE, LolA and LolB are not enzymes. They are well characterized periplasmic lipoprotein transporters in E. coli. LolA binds lipoproteins released in an ATP-dependent manner by the ABC transporter LolCDE from the periplasmic side of the inner membrane and shuttles these lipoproteins through the periplasmic space, depositing them with LolB, a membrane-anchored protein, which incorporates them into the outer membrane (46).

Lola and LolB have “lid” regions, composed of α-helices (32, 47). Although the lid of LolB is thought to always be open (32), in LolA, these helices are thought to act as gates, controlling the access of hydrophobic cargo, the acyl chains of lipoproteins, to the uncleaved β-barrel, where they are thought to be sequestered during lipoprotein transport (32, 47, 48). These LolA α-helices are thought to open and close, as lipoproteins enter and depart the hydrophobic core (32, 47, 48). In LolA, Arg34, positioned between β2 and β3, makes hydrogen bonding contacts with backbone carbonyls on the α1 (Leu10) and α2 (Ile93 and Ala94) helices (Fig. 3A) (32); Arg34 is thus thought to play a key role in stabilizing the closed form of LolA. Mutation of Arg34 to Leu leads to the accumulation of LolA-lipoprotein complexes and eliminates transfer of lipoproteins from LolA to LolB (49), sug-
gesting that the closing of the lid via formation of hydrogen bonds to Arg\textsuperscript{33} may drive the energy-independent transfer of lipoproteins from LolA to LolB (32, 47, 48). LolB, which has a higher affinity for lipoproteins than LolA (50), lacks a residue playing this role; LolB is thought to be more stable in the "open" conformation, where its hydrophobic core is more accessible to lipoproteins (32).

VioE lacks the three longer \( \alpha \)-helices of LolA and LolB, having only two short \( \alpha \)-helices and one short \( \beta \) helical turn, but it also may have a similar lid region. Amino acids 5–10 traverse a similar space in the VioE structure as the \( \alpha \)-1-helix of LolA. Further, amino acids 105–121 of VioE, a loop region extending from \( \beta \)6 to \( \beta \)7 and containing the second \( \alpha \)-helix of VioE, traverse across similar space to that of the \( \alpha \)2 and \( \alpha \)3-helices of LolA and the \( \alpha \)3-helix of LolB. Finally, the structurally superimposed residue to Arg\textsuperscript{33} of LolA is Tyr\textsuperscript{39} of VioE. Tyr\textsuperscript{39}, which is also positioned between \( \beta \)2 and \( \beta \)3 of VioE (like Arg\textsuperscript{33} of LolA), is in position to hydrogen bond with the indole nitrogen from Tyr\textsuperscript{39} of VioE (Fig. 3). This suggests that the site of PEG binding also represents a binding site for a molecule, in the case of VioE. PEG is not well ordered in the structure, its positioning is the structure (supplemental Fig. S6). Despite the fact that the maps show diffuse electron density, indicating flexibility in the binding of the PEG molecules (Fig. 3), there is no significant change in prodeoxyviolacein \( \beta \) product during catalysis. Additionally, mutation of Ser\textsuperscript{170}, which is out further from the bound PEG molecule (Fig. 3), among others. To investigate the importance of particular residues to catalysis and to investigate whether the PEG molecule had indeed identified the active site of VioE, a series of nine site-directed mutants were generated, and their activities were tested (Fig. 4). To verify that each mutant was properly folded, each mutant was examined with CD spectrometry and compared with wild-type VioE. We found that each mutant shows a similar CD spectrum to that of wild-type VioE (supplemental Fig. S7), suggesting that all mutants are properly folded and that mutations reveal the importance of particular residues to binding or catalysis and not a general effect on the proper folding of the protein. All of the targeted side chains were mutated to alanine.

Two groups of mutants were generated. The first group was in the immediate area of the PEG molecule (within 6 Å), and includes Arg\textsuperscript{172}, closest to the PEG molecule (Fig. 3C). Mutation of Arg\textsuperscript{172} resulted in a significant decrease in prodeoxyviolacein \( \beta \) production (Fig. 4). Similarly, mutation of either Tyr\textsuperscript{17} or Ser\textsuperscript{19}, each between 3 and 5 Å from the bound PEG molecule (Fig. 3C) resulted in a significant decrease in prodeoxyviolacein \( \beta \) production (Fig. 4). Additionally, mutation of Asn\textsuperscript{51}, between 5 and 8 Å from the bound PEG molecule (Fig. 3C), in contrast to the sharp quality of electron density throughout most of the structure (supplemental Fig. S6). Despite the fact that the PEG is not well ordered in the structure, its positioning is intriguing. As was the case for LolB, it is tempting to speculate that the site of PEG binding also represents a binding site for a substrate, an acyl chain, in the case of VioE, or a substrate molecule, in the case of VioE.

Surrounding the PEG molecule in the VioE structure are a series of hydrophobic residues (Fig. 3C): Trp\textsuperscript{13}, Phe\textsuperscript{37}, Ile\textsuperscript{46}, Phe\textsuperscript{50}, Pro\textsuperscript{12}, Phe\textsuperscript{109}, Leu\textsuperscript{110}, Met\textsuperscript{160}, and Phe\textsuperscript{174}. Any putative substrates of VioE would be hydrophobic; both IPA \( \beta \)1 (the substrate of VioB) and prodeoxyviolacein \( \beta \)3 (the product of VioE) are poorly soluble in water, and it is unlikely that any molecules spanning the chemical space between IPA \( \beta \)1 or prodeoxyviolacein \( \beta \)3 would be well soluble in water. These hydrophobic residues surrounding the PEG molecule in the putative VioE active site should form an ideal binding pocket for the hydrophobic substrate molecule, any intermediates, and the prodeoxyviolacein \( \beta \) product during catalysis.

Within 6 Å of the bound PEG molecule are also a small number of polar and charged residues, Tyr\textsuperscript{17}, Ser\textsuperscript{19}, Thr\textsuperscript{31}, Cys\textsuperscript{35}, Asn\textsuperscript{51}, Ser\textsuperscript{170}, and Arg\textsuperscript{172}, which might have relevance in binding the polar parts of the substrate molecule or in catalysis (Fig. 3C). Further from the bound PEG molecule, within 12 Å, are additional polar or charged residues, including Ser\textsuperscript{21}, Cys\textsuperscript{44}, Ser\textsuperscript{64}, Glu\textsuperscript{66}, Lys\textsuperscript{77}, and Lys\textsuperscript{79} (Fig. 3D), among others. To investigate the importance of particular residues to catalysis and to investigate whether the PEG molecule had indeed identified the active site of VioE, a series of nine site-directed mutants were generated, and their activities were tested (Fig. 4). To verify that each mutant was properly folded, each mutant was examined with CD spectrometry and compared with wild-type VioE. We found that each mutant shows a similar CD spectrum to that of wild-type VioE (supplemental Fig. S7), suggesting that all mutants are properly folded and that mutations reveal the importance of particular residues to binding or catalysis and not a general effect on the proper folding of the protein. All of the targeted side chains were mutated to alanine.
nificantly alter prodeoxyviolacein 3 production (Fig. 4). These mutations thus delineate the active site adjacent to the bound PEG molecule (Fig. 3D).

The second group of amino acids mutated is further away from the PEG molecule (>8 Å) but still in the general area of the concave β-sheet where substrate might bind. Glu<sup>66</sup>, positioned a distance of 9 Å from Arg<sup>172</sup> (Fig. 3D), was a primary target, because its distance from Arg<sup>172</sup> is within range of the size of putative substrates and products; for instance, the distance in prodeoxyviolacein 3 from the ketone oxygen to the furthest indole nitrogen is ~7 Å. Mutation of Glu<sup>66</sup> resulted in a significant decrease in prodeoxyviolacein 3 production (Fig. 4). Two nearby residues to Glu<sup>66</sup>, Met<sup>64</sup> and Lys<sup>77</sup>, were then targeted for mutagenesis. Although the average production of prodeoxyviolacein 3 was increased for both mutants relative to wild-type, based on the standard deviation, the increase was nonsignificant for both mutants (Fig. 4). This group therefore delineates a second part of the putative active site of VioE that includes charged residue Glu<sup>66</sup> (Fig. 3D).

DISCUSSION

The unexpected result that VioE adapts a fold normally associated with lipoprotein carrier proteins to carry out an enzymatic function suggests that a crucial part of its function is, like a lipoprotein carrier protein, to bind a hydrophobic molecule. In the case of VioE, the binding of a hydrophobic substrate in a particular conformation may effectively chaperone the production of a desired product. The conversion that VioE facilitates involves oxidative chemistry, but the most unusual aspect of the reaction, a 1,2 shift of an indole ring, is a reaction that is not known to be catalyzed by other characterized enzymes. Other shift reactions have been characterized (51–54), and each of the responsible enzymes utilize iron as a cofactor (as a heme or non-heme iron), whereas VioE requires no cofactors or metals. The lack of cofactors of VioE might mean that this indole shift reaction is mechanistically more facile than other observed, enzyme-mediated shift reactions. Perhaps the main role of VioE is to stabilize its substrate in a favorable conformation to enable the 1,2 shift reaction to occur through classic acid-base or oxidative chemistry, and the fold adapted by VioE works well for this. Other enzymes thought to use stabilization of an alternate, higher energy orientation of a substrate to promote catalysis include the peptidyl-prolyl isomerases (55, 56), although a precise delineation of the mechanisms of these enzymes is still under investigation. One proposed substrate for VioE is the Cβ-Cβ benzyllycoupled iminophenylpyruvate dimer 2 (Fig. 1 and Ref. 26), a molecule that could collapse spontaneously to chromoppyrrolic acid 4 (26). Stabilization of this molecule (or a related intermediate) by VioE in an orientation suitable for promotion of a 1,2 indole shift might reroute it toward prodeoxyviolacein 3.

Our site-directed mutagenesis studies have identified a number of residues likely to be involved in binding polar parts of the substrate molecule or in catalysis: Tyr<sup>17</sup>, Ser<sup>19</sup>, Asn<sup>31</sup>, Glu<sup>66</sup>, and Arg<sup>172</sup>. Although mutation of any of these residues does not completely abolish VioE activity, the significant decrease in activity by each of these mutants implies that they play a role in enzymatic acceleration of prodeoxyviolacein 3 production by VioE. Our findings do suggest that the PEG molecule cocrystallized with VioE has indeed identified the active site of the enzyme. However, it appears that the active site extends beyond the site of PEG binding to include the side chain of Glu<sup>66</sup>. An extended active site is not surprising given the larger size of the known product molecule.

In conclusion, our crystal structure of VioE has revealed that an unusual enzyme, a cofactorless, metal-free protein known to catalyze an unusual 1,2 shift reaction, also adapts an unusual fold, that of a lipoprotein carrier protein. This finding indicates that the main role of VioE is to sequester a hydrophobic molecule from the milieu of the cell. Once bound, VioE likely uses acid-base chemistry, probably coupling transformations to spontaneous oxidative processes, to carry out its reaction, without the need for metals or cofactors. Further studies of this intriguing enzyme, including elucidation of its as yet unknown substrate, will help unravel how each residue identified in this study enables VioE to reroute an unknown intermediate away from spontaneous formation of chromoppyrrolic acid 4 and toward production of the violacein 5 scaffold.

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