Crystal Structure of VioE, a Key Player in the Construction of the Molecular Skeleton of Violacein*

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Violacein and the indolocarbazoles are naturally occurring bisindole products with various biological activities, including antitumor activity. Although these compounds have markedly different molecular skeletons, their biosynthetic pathways share the same intermediate “compound X,” which is produced from L-tryptophan via indole-3-pyruvic acid imine. Compound X is a short-lived intermediate that is spontaneously converted to chromopyrrolic acid for indolocarbazole biosynthesis, whereas VioE transforms compound X into protodeoxyviolaceinic acid, which is further modified by other enzymes to produce violacein. Thus, VioE plays a key role in the construction of the molecular skeleton of violacein. Here, we present the crystal structure of VioE, which consists of two subunits, each of which forms a structure resembling a baseball glove. Each subunit has a positively charged pocket at the center of the concave surface of the structure. Mutagenesis analysis of the surface pocket and other surface residues showed that the surface pocket serves as an active site. We have also solved the crystal structure of a complex of VioE and phenylpyruvic acid as an analogue of a VioE-substrate complex. A docking simulation with VioE and the IPA imine dimer, which is proposed to be compound X, agreed with the results from the mutational analysis and the VioE-phenylpyruvic acid complex structure. Based on these results, we propose that VioE traps the highly reactive substrate within the surface pocket to suppress CPA formation and promote protodeoxyviolaceinic acid formation caused by proximity and orientation effects.

The natural bisindole violacein, which is produced by Chromobacterium violaceum (1, 2) and Janthinobacterium lividum (3), is a violet pigment with various biological activities, including antibacterial and antiviral activities as well as a cytotoxic effect against several tumor cell lines (4). Staurosporine, a member of the indolocarbazole alkaloid family, shows strong antitumor activity through the inhibition of protein kinases (5). Recently, biosynthesis of these two natural products revealed that they share a common pathway in which homologous enzymes catalyze similar reactions (Fig. 1 and Refs. 6–10). In the common biosynthesis process, L-tryptophan is oxidized to indole-3-pyruvic acid (IPA)4 imine, which is then converted into the short-lived intermediate product “compound X” (6, 8, 11). This compound has been proposed to be an IPA imine dimer (6). After this process is completed, compound X is spontaneously converted into chromopyrrolic acid (CPA), a precursor of staurosporine (6, 8). In vivo and in vitro studies of the violacein biosynthetic pathway suggest that the novel enzyme VioE catalytically rearranges the skeleton of compound X to produce the short-lived intermediate product, protodeoxyviolaceinic acid (PVA), which is finally converted into violacein by other violacein biosynthesis enzymes (11). PVA is unstable in the presence of O2, and it is quickly converted into a dead end product prodeoxyviolacein (PDV) by autoxidation (Fig. 1). Furthermore, VioE does not catalyze the conversion of CPA into PVA and PDV (12). VioE is thus regarded as a key enzyme that “switches” the biosynthetic pathway through an intramolecular 1,2-shift of an indole ring. Interestingly, even though it catalyzes a major conversion of the molecular scaffold of the substrate, VioE does not require coenzymes or metals.

Although a number of biochemical studies have examined the mechanisms underlying violacein biosynthesis, the detailed reaction mechanism of the VioE-mediated conversion of compound X to PVA remains to be elucidated. To address this issue, we have determined the crystal structure of VioE. This enzyme adopts a dimer structure with a positively charged pocket on the surface of each subunit. Mutagenesis analysis of the surface pocket as well as other surface residues suggested that the pocket serves as the active site of VioE. To examine the catalytic mechanism of VioE, we solved the crystal structure of the complex of VioE and phenylpyruvic acid (PPA) as a stable mimic of the VioE-substrate complex. In addition, we performed a docking simulation for VioE and the IPA imine dimer.

§ The abbreviations used are: IPA, indole-3-pyruvic acid; CPA, chromopyrrolic acid; PVA, protodeoxyviolaceinic acid; PDV, prodeoxyviolacein; PPA, phenylpyruvic acid; DTT, dithiothreitol; Ni-NTA, nickel-nitrilotriacetic acid; SeMet, selenomethionine.

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The atomic coordinates and structure factors (codes 2ZF3, 2ZF4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Experimental Procedures

Expression and Purification of VioE for Crystallization—The gene encoding C. violaceum VioE was amplified in a PCR using pETvioE (8) as a template and a pair of primers (forward, 5′-CGC-CATATGGAAAACCGGAACCGCC-3′; reverse, 5′-CGCG-GATCCCTACTAGCGCTTGGCGGCGAAG-3′) and cloned in the NdeI-BamHI site of the pET-15b vector (Novagen). Protein expression in Escherichia coli BL21(DE3) cells cultured in 2×YT medium was induced with 0.1 mM isopropyl-1-thio-D-galactopyranoside when the A600 nm reached 0.4. After a 16-h incubation at 28 °C, the cells were harvested by centrifugation at 4 °C (Hitachi, Himac CR 22G). The pellets were resuspended in suspension buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 20 mM imidazole, and 1 mM dithiothreitol (DTT)) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche Applied Science; 2 tablets/100 ml) and sonicated (Branson Sonifier 250). After centrifugation (35,000 × g, 60 min), the supernatants were collected and loaded onto Ni-NTA Superflow resin (Qiagen). After washing with 10 column volumes of suspension buffer, the buffer in the column was replaced with thrombin reaction buffer (20 mM Tris-HCl, pH 7.9, 150 mM NaCl, and 5 mM β-mercaptoethanol) containing thrombin protease (Novagen) to remove the His6 tag. After 16 h of incubation at 20 °C, VioE without the His tag was eluted using the suspension buffer. The VioE fractions were collected and further purified on a monQ HR 16/10 column (GE Healthcare) equilibrated with 20 mM Tris (pH 7.4) and 1 mM DTT; elution was performed with a 0–0.2 M NaCl gradient.

E. coli BL21(DE3)-Codonplus RIL-X cells were used to express SeMet-substituted VioE. The cells were grown in LeMaster medium supplemented with 25 mg/l-seleno-l-methionine (Wako Pure Chemicals) (13, 14).

Expression and Purification of VioE for the Enzymatic Assay—Wild-type VioE and the mutant protein variants used for the enzymatic activity assay were expressed as C-terminally hexahistidine-tagged fusion proteins as previously described (8). Recombinant protein purification was performed using Ni-NTA affinity chromatography and 100 μl of Ni-NTA (Qiagen) resin bed in a spin column container (Centri-Sep, Princeton Separations). The cells harvested from 4 ml of E. coli culture were washed with buffer A (50 mM KH2PO4, 300 mM NaCl, and 20 mM imidazole at pH 7.4). The cells were resuspended in 500 μl of buffer A and sonicated. After centrifugation (15,000 rpm, 20 min, 4 °C), 400 μl of supernatant was applied to the spin column, mixed with the Ni-NTA resin, and incubated for 10 min at 4 °C. After centrifugation (2,000 rpm, 1 min, 4 °C) to remove undesired proteins, the matrix was washed with an additional 600 μl of buffer A and recentrifuged. Then 500 μl of buffer B (50 mM KH2PO4, 300 mM NaCl, and 300 mM imidazole at pH 7.4) was added, and the samples were incubated for 10 min at 4 °C. After the incubation, the columns were centrifuged to elute the protein. The eluted proteins were dialyzed three times in buffer D (50 mM KH2PO4, 300 mM NaCl, and 0.1 mM DTT at pH 7.4) to yield the purified VioE samples. The concentrations of VioE were determined using the Bradford method (Protein assay kit I, Nippon Bio-Rad).

Crystallization, Data Collection, and Structure Determination—The purified VioE was dialyzed against 20 mM Tris (pH 7.4) and 1 mM DTT and then concentrated to 16 mg ml−1. VioE (14 mg ml−1) was crystallized using the sitting drop, vapor diffusion method. Single crystals were obtained using 6% PEG 4000 and 0.1 M citrate (pH 3.5) as a reservoir solution at 20 °C. To obtain a complex crystal with the IPA analogue PPA, the native VioE crystals were soaked with the harvest solution containing PPA (8% PEG 4000, 1 mM PPA, 5% Me2SO, and 0.1 M citrate at pH 3.5) for 24 h at 20 °C. The SeMet derivative was...
TABLE 1
Data collection and refinement statistics
The numbers in parentheses show the values for the highest resolution shell.

|                          | Native | SeMet-SAD | +PPA |
|--------------------------|--------|-----------|-------|
| **Data collection**       |        |           |       |
| Space group              | P2, 2, 2 | P2, 2, 2 | P2, 2, 2 |
| Unit cell parameters (Å) | a = 84.1 | a = 83.4 | a = 83.4 |
|                          | b = 88.4 | b = 91.7 | b = 91.0 |
|                          | c = 153.9 | c = 157.9 | c = 157.0 |
| Beamline                 | SPRing-8 | SPRing-8 | SPRing-8 |
| Wavelength (Å)           | 1.0000 | 0.97907 | 1.0000 |
| Resolution range (Å)     | 50.00–2.00 | 50.00–2.10 | 30.00–2.18 |
| Total number of reflections | 564,970 | 1,029,008 | 416,327 |
| Number of unique reflections | 77,895 | 71,444 | 63,652 |
| Completeness (%)         | 100.0 (100.0) | 100.0 (99.9) | 97.1 (81.2) |
| Rmerge (%)               | 19.9 | 20.6 | 24.5 |
| I/σ                      | 11.9 (4.67) | 10.6 (6.4) | 15.6 (4.0) |
| Mosaicity (%)            | 0.47 | 0.23 | 0.30 |
| FOM* after SOLVE         | 0.33 |
| FOM* after RESOLVE       | 0.64 |

**Refinement**

| Refinement | Range (Å) | 40.0–2.0 | 30.0–2.18 |
|------------|-----------|----------|-----------|
| Rmerge (%) | 19.9 | 20.6 |
| Rfree (%)  | 24.5 | 25.4 |

**Root mean square deviations from ideal**

| Bond length (Å) | 0.010 |
| Bond angle (%)  | 1.360 |

**Ramachandran plot**

| Most favored regions (%) | 92.2 |
| Additional allowed regions (%) | 7.8 |
| Generously allowed regions (%) | 0.0 |
| Disallowed regions (%) | 0.0 |

*FOM, figure of merit.

purified and crystallized in essentially the same way as the native protein.

Native, SeMet-labeled, and PPA complex crystals were cryo-protected in 30% xylitol, 8% PEG 4000, and 0.1 M citrate at pH 3.5. Data from the native crystal were collected to 2.0 Å, and anomalous data for the SeMet-substituted crystal were collected to 2.1 Å at the SPRing-8 beamlines BL41XU and BL44B2. The data were processed with HKL2000 (15). The experimental phases were determined using the single-wavelength anomalous diffraction method and the program SOLVE/RESOLVE (16). Because of poor isomorphism between the native and the SeMet-labeled crystals (Table 1), the initial structure was modeled using the electron density data derived from the anomalous data, and a partial model was generated using RESOLVE. The initial structure was then used as a search model for molecular replacement with the native data set and MOLREP from CCP4 (17). The native structure was refined with the native datasets using LAFILE (18), REFMAC5 from CCP4 (17), and CNS (19) and was modified using the molecular modeling program COOT (20). The complex structure with PPA was determined and refined using REFMAC5 from CCP4 (17) and CNS (19). As we were completing the model, TLS refinement with REFMAC5 (17) was also carried out for both the native and PPA complex structures. The crystallographic and refinement statistics are summarized in Table 1. The figures were drawn with PyMOL (21). The atomic coordinates were deposited in the Protein Data Bank.

**Mutagenesis Analysis**—Twenty VioE mutant proteins were constructed: Y17F, S19A, W21A, C35S, F50A, N51A, P52A, R61A, E66A, K77A, R84A, F109A, R140A, K143A, M160A, T162A, S170A, R172A, R172Q, and F174A. The mutations were introduced using a QuickChange mutagenesis kit (Stratagene) and the pETvioE plasmid (8) as a template. The F174A mutant protein was not used for further analysis because of aggregation during the purification process.

In the presence of StaD and VioE, IPA imine is converted into PDV via compound X and PVA (Fig. 1). Thus, the activities of wild-type and mutant VioE were measured by examining the formation of PDV using analytical HPLC. The reaction solution contained 300 nM VioE, 20 nM StaD (9), 1 mM IPA, 100 mM (NH₄)₂SO₄, and 0.1 mM DTT in 100 mM sodium phosphate buffer at pH 7.0. After incubation at 30 °C for 30 min, the enzymes were inactivated by the addition of 1% trifluoroacetic acid. Reaction mixtures were extracted three times with ethyl acetate, and the extracts were analyzed using HPLC at 610 nm, which is the maximum absorption wavelength of PDV.

HPLC analysis was performed using an HP1100 system (Hewlett Packard) and a Cosmosil column (inner diameter, 4.6 mm; length, 250 mm; Nacalai Tesque). The temperature was set at 30 °C, and the flow rate was 1 ml/min. The solvent was acetonitrile and 0.1% trifluoroacetic acid in H₂O. Elution was performed for 30 min using a 20–90% linear gradient of acetonitrile. The amount of PDV was calculated from the area under the elution profile monitored at 610 nm.

**Docking Simulation**—Docking of the IPA imine dimer to the protein moiety of the VioE-PPA complex was performed using the AUTODOCK 4.0.1 program (22). The initial structure of the IPA imine dimer was generated using Monomer Library Sketcher in CCP4 (17) and Chem3D Ultra 10.0. Compound X has three stereoisomers (RR, SS, and RS) because it has two equivalent chiral carbon atoms. All of these stereoisomers were examined in the docking study. All of the bonds connected to a benzylic β carbon were allowed to rotate, and VioE was modeled as a rigid protein. All default parameters except for the number of genetic algorithm runs were used for the simulation. The grid size was set to 40 × 40 × 40 Å³. Fifty Lamarckian genetic algorithm runs were performed, and each one provided a single solution (i.e. one predicted binding mode). Cluster analysis was performed at the end of the simulation. Solutions that were within a 1-Å root mean square deviation of each other were grouped in the same cluster, and the clusters were ranked according to their lowest energy member. The SS stereoisomer of compound X and chain C of the VioE-PPA complex resulted in the lowest energy binding mode, when compared with all of the other binding modes.

**RESULTS AND DISCUSSION**

**Structural Determination**—We have obtained an initial structural model using the single-wavelength anomalous diffraction method and a 2.1 Å resolution anomalous data set obtained with a SeMet-labeled crystal. Structural refinement was performed with a 2.0 Å resolution data set obtained from a native crystal (an example of a typical electron density map is shown in Fig. 2A). Consistent with previous reports (6, 8), VioE in the crystal formed a homodimer that does not include any coenzymes (Fig. 2, B and C). Each asymmetric unit contained three dimers (chains A + B, chains C + D, and chains E + F; Fig. 2B).
Crystal Structure of VioE

FIGURE 2. Overall structure of VioE. A, an F_obs – F_calc, omit electron density map of the residues that are involved in the formation of the concave surface of chain A contoured at the 2.5σ level. Main chains are drawn as thin cyan tubes. Tyr17, Phe50, Phe109, and Arg172 are shown as ball-and-stick models. Cyan, red, and blue indicate carbon, oxygen, and nitrogen atoms, respectively. B, VioE molecules in an asymmetric unit are shown as ribbon models. Chains A, B, C, D, E, and F are shown in cyan, light green, light blue, red, yellow, and magenta, respectively. The distorted loops of chain F are denoted in black and with an arrow. C, the overall structure of the VioE dimer, composed of chain A and chain B, is shown as a ribbon model. The dotted line indicates a noncrystallographic 2-fold axis. D, the overall structure of a subunit of VioE (chain A) shown as ribbon model to illustrate the primary structure from the N terminus to the C terminus (blue to red).

With the exception of chain F, all of the chains were essentially identical to each other with root mean square deviations of 0.52–0.95 Å for the Ca atoms. The structure of chain F was different in that two loop regions (the β4–β5 loop and the β6–α2 loop; Fig. 2B) were closer to the center of the monomer. This structural deviation in chain F was probably a crystallographic artifact caused by contacts with the identical region of the symmetry-related chain C molecule in the crystal (data not shown). Indeed, the distortion of chain F was not observed in the SeMet-labeled crystal, nor was it seen in the crystal of the complex with PPA, presumably because of the larger unit cell volumes and the longer distances between the chain F and the symmetry-related chain C molecules in these crystals (data not shown). Here, we primarily focus on chain A from the native structure and chain C from the VioE-PPA complex structure, because their electron density maps are clearer than those of the other chains.

Overall Structure of VioE—Each monomer of VioE assumes a configuration that resembles an opened baseball glove. The substrate fits into the “pocket” of the glove like a baseball as will be described in detail in a later section. An 11-stranded antiparallel β sheet (β1–β11) comprises the majority of the VioE structure; i.e., the β strands form a somewhat bent, single-layer sheet (Fig. 2, C and D). Three short helical structures (α1, α2, and a 310 helix) are located between β3 and β4, between β6 and β7, and in the C-terminal loop, respectively. Overall, the β sheet forms a concave surface in the center of the subunit.

The other side of the subunit forms the dimer interface, which buries 3,400–3,500 Å² of surface area from both subunits (Fig. 2C). The two subunits of the dimer are related by a noncrystallographic 2-fold axis that is almost parallel to β2 and β3 (Fig. 2C). The dimerization surfaces of the two subunits, which are complementary to each other, interact via hydrophobic and polar interactions (not shown). Unlike most β-barrel proteins, each subunit of VioE does not contain an obvious hydrophobic core, which suggests that VioE is unstable as a monomer. Therefore, the dimerization is likely to be required for the stabilization of the structure of VioE, and the large dimer interface serves as a structural core.

Structural Similarities with Other Proteins—Searching a data base using SSM (23) revealed several bacterial homologues of VioE: the bacterial lipoprotein localization factors LolA and LolB, lipoprotein LppX from Mycobacterium tuberculosis (25), and an N-terminal domain of the σ² factor regulatory protein RseB (26) (Fig. 3). These four proteins also have a baseball glove-like scaffold consisting of an 11-stranded antiparallel β sheet. In addition, the topologies of the β strands in these proteins are almost identical to that of VioE, although their sequences show low degrees of homology (identities < 17%).

The structural features of LolA and LolB that were distinct from those of VioE included the α-helices attached to the concave surface of the β sheet, which result in a narrow hydrophobic cavity between the β sheet and the α-helices (24). This narrow cavity may facilitate the lipid binding that is essential for the functions of these proteins (24). LppX contains several helices on the edges of the concave surface, resulting in a larger hydrophobic pocket that can bind complex lipids, such as phthiocerol dimycocerosates (25). For RseB, which does not require a pocket that can bind to small molecules, the concave surface is occupied by several loops and helices.

Because VioE and these proteins do not appear to be genetically or functionally related, the origin of the structural similarities is not clear. VioE and the homologous proteins, however, bind to small molecules because they carry out their functions, and one advantage of this structure is that the baseball glove fold includes a concave surface for the binding of exogenous ligands. Assuming that evolution has modified the loops and helices to optimize the size and shape of the β sheet for each substrate, the observed glove-like topology provides a suitable scaffold for enzymes and transporters, which hold molecules in place as they carry out their functions.

The Positively Charged Surface Pocket—VioE binds a structurally unidentified substrate (compound X in Fig. 1), which is produced from two IPA imine molecules by VioB. As previously shown (6, 8), the substrate itself spontaneously forms CPA in the absence of VioE (Fig. 1). Compound X has been predicted to have two carboxyl groups and two indole rings (8), because both
the precursor of the substrate and CPA have two carboxyl groups and two indole rings. Thus, electrostatic and hydrophobic interactions are expected to play a role in the binding between compound X and VioE as has been observed between CPA and StaP, an enzyme in the staurosporine biosynthetic pathway that converts CPA into an indolocarbazole core (27). These observations suggest that the active site of this enzyme should contain a positively charged residue(s).

To locate any relevant, positively charged residues at the active site, alanine-scanning mutagenesis was used to examine the basic residues on the surface of the protein (R61A, K77A, R84A, R140A, K143A, and R172A; the positions of the residues are shown in Fig. 5A). As shown in the left panel of Fig. 4, a substantial loss of enzymatic activity was only observed for the mutation at Arg<sup>172</sup> (8% of the wild-type activity). Arg<sup>172</sup> is located in the triangular surface pocket (~15 Å long and 4 Å deep) near the center of the surface of the monomer. Several polar residues, including Tyr<sup>17</sup>, Ser<sup>19</sup>, and Cys<sup>35</sup>, surround Arg<sup>172</sup>, resulting in a positively charged base of the pocket (Fig. 5, A and B). A number of hydrophobic (Trp<sup>13</sup>, Trp<sup>21</sup>, Ile<sup>46</sup>, Phe<sup>50</sup>, Pro<sup>52</sup>, Met<sup>64</sup>, Leu<sup>110</sup>, Pro<sup>111</sup>, Met<sup>160</sup>, and Phe<sup>174</sup>) and several hydrophilic (Glu<sup>66</sup>, Thr<sup>162</sup>, and Ser<sup>170</sup>) residues form the relatively hydrophobic edge of the pocket (Fig. 5B). Thus, alanine-scanning mutagenesis suggests that the surface pocket acts as an active site, which is consistent with the fact that many of the residues located in the pocket are well conserved among the VioE family proteins from <i>J. lividum</i> or <i>Pseudoalteromonas tunicate</i> D2.

We then examined 12 additional residues that are located in the pocket using site-directed mutagenesis (Y17F, S19A, W21A, C35S, F50A, N51A, P52A, E66A, F109A, M160A, T162A, and S170A). Moreover, to further explore the role of Arg<sup>172</sup>, glutamine was introduced at position 172 to remove the positive charge. The activities of these mutant proteins are summarized in the right panel of Fig. 4. The R172Q mutation also affected the activity of the enzyme, which again suggests that Arg<sup>172</sup> is involved in the active site. Among the other variants, mutations at Ser<sup>19</sup>, Phe<sup>50</sup>, and Glu<sup>66</sup> reduced the activity to less than 50% of the wild-type activity. Smaller but still significant losses of activity were observed for the N51A and Y17F mutant proteins. The activities of the other mutant enzymes (W21A, C35S, P52A, F109A, M160A, T162A, and S170A) were comparable with that of the wild-type enzyme (~80% relative activity).

As shown in Fig. 5B, the mutated residues that reduced the activity of VioE (Tyr<sup>17</sup>, Ser<sup>19</sup>, Phe<sup>50</sup>, and Asn<sup>51</sup>, Glu<sup>66</sup>, and Arg<sup>172</sup>) are clustered on the left edge and the bottom (the center in the figure) of the triangular pocket. Therefore, the results suggested that the triangular surface pocket and, in particular, the left side of the pocket in Fig. 5B serve as a substrate binding and/or catalytic site.

Potential VioE-Substrate Interactions—The substrate of VioE is a short-lived intermediate that is spontaneously converted into CPA. Thus, it is very difficult to obtain a crystal...
structure of VioE complexed with its substrate. To overcome this difficulty and gain insights into the interactions between VioE and the substrate, we used PPA as a molecular probe and solved the crystal structure of the VioE-PPA complex; similar to compound X, PPA has both aromatic and pyruvate moieties. Fig. 5C shows the structure and omit electron density map for the surface pocket of the VioE-PPA complex. The significant electron density indicates that two molecules of PPA bind in the surface pocket. Overall, the structure of VioE is not altered upon PPA binding. The side chain of Arg172 in the pocket, however, extends from the bottom of the pocket, making the surface pocket substantially smaller than that of apo-VioE (Fig. 5C). These structural features were observed for all of the chains in the crystal structure of VioE-PPA complex.

All of the residues that were suggested to be involved in the enzyme activity (Fig. 4; Tyr17, Ser19, Phe60, Asn51, Glu66, and Arg172) are located close to the bound PPA molecules (Fig. 5, B and C). Weaker but similar electron densities in the surface pocket as well as the reorientation of Arg172 were also observed for complexes with IPA or p-hydroxyphenyl pyruvic acid (structure not shown). These findings suggest that the VioE-PPA complex can be regarded as a stable mimic of the VioE-substrate complex and that the substrate occupies a similar position in the pocket.

To examine the possibility that the IPA imine dimer, a potential substrate for VioE proposed by Balibar and Walsh (6), can be accommodated within the surface pocket, we carried out a docking simulation using the protein moiety of the VioE-PPA complex. Fig. 5D shows the lowest energy binding conformation. Our results demonstrate that the IPA imine dimer is able to fit into the surface pocket. The two indole rings point down into the pocket, where they interact with Glu66, Phe50, and Tyr17. Furthermore, the carboxyl and imino
IPA imine molecules at the benzylic VioB or its homologous enzymes, is a coupling reaction of two imino into PDV or CPA via an IPA imine dimer (Scheme 2 in Ref. 6). The first step of the process, which can be accelerated by imine into PDV or CPA via an IPA imine dimer (Scheme 2 in Ref. 6). The first step of the process, which can be accelerated by

As observed for the VioE-PPA complex, this binding mode is consistent with the results from the mutational analysis. It should also be noted that two indole rings are almost superimposable onto the phenyl rings of PPA bound to VioE. Therefore, the structure shown in Fig. 5D, which details the extensive polar and hydrophobic interactions, represents a likely binding mode of VioE to the substrate.

**Implications about the Catalytic Mechanism**—In vitro studies have revealed that VioB produces the VioE substrate, which is spontaneously converted into CPA or enzymatically transformed into PVA by VioE (6, 8, 11). Balibar and Walsh (6) proposed a possible mechanism for the conversion of the IPA imine into PDV or CPA via an IPA imine dimer (Scheme 2 in Ref. 6). The first step of the process, which can be accelerated by VioB or its homologous enzymes, is a coupling reaction of two IPA imine molecules at the benzylic C atom of PPA, resulting in compound X. VioE converts the species into another intermediate with a cyclopropyl ring. A decarboxylation reaction with a concomitant reaction that opens the ring results in a 1,2-shift of the indole moiety. Finally, the pyrrole ring is formed to complete the violacein skeleton. For the spontaneous conversion into CPA, two imino groups in the IPA imine dimer are coupled to form the pyrrole ring of CPA. Thus, VioE suppresses the spontaneous formation of CPA as well as inducing the rearrangement of the indole ring. Formation of the intermediate with the cyclopropyl ring would explain these functions of VioE.

Based on the mutational assay, the crystal structures of VioE and the PPA-VioE complex, and the in silico docking simulation, we propose a mechanism for PVA production by VioE as well as spontaneous CPA production (Fig. 6). In the absence of VioE, the two imino groups of compound X are closely associated to allow the intramolecular reaction required for CPA formation to proceed (6, 8, 9). Upon substrate binding, Arg^{172} moves to the edge of the pocket as was observed in the VioE-PPA complex structure. The relocated Arg^{172} residue forms a polar interaction with the substrate. Tyr^{17} and Ser^{19} also contribute to the substrate recognition site. Glu^{66} at the edge of the pocket likely interacts with the NH group of the indole moiety. These polar residues are important for substrate binding rather than the acid/base catalysis, because mutation of any these residues did not completely eliminate the activity of VioE. Phe^{50}, which is located next to Glu^{66}, forms π–π or hydrophobic interactions with the indole moiety. These polar and aromatic interactions keep the two imino groups away from each other to suppress CPA formation. The high reactivity of the short-lived substrate, the conformation of which is restrained in the surface pocket, allows VioE to direct the reaction to produce PVA. In other words, VioB produces the highly reactive intermediate species, whereas VioE is a key enzyme that regulates the intramolecular reaction of the intermediate species through proximity and orientation effects.

**Conclusions**—In this report, we have presented the first structure of VioE, which dimerizes and has a positively charged surface pocket on each side of the dimer. We used structure-
based mutational assays and a crystal structure of a complex of VioE and a substrate analogue to demonstrate that the positively charged pocket serves as an active site for PVA formation. Although determining the structure of the VioE-substrate complex is difficult because of the instability of the substrate, the present work provides the first structural model of the molecular-skeletal conversion catalyzed by VioE, in which a short-lived compound serves as a critical intermediate. The present findings will help to elucidate the underlying rearrangement mechanism, which should be helpful for the design of systems that allow the biosynthesis of novel indole-containing products.

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Addendum—While our manuscript was being revised, Hoshino and co-workers (11) reported new findings regarding the violacein biosynthesis pathway. We have therefore updated our article according to their work.

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