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VioE, a prodeoxyviolacein synthase involved in violacein biosynthesis, is responsible for intramolecular indole rearrangement

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Abstract—Indole-3-pyruvic acid is transformed to prodeoxyviolacein by the novel enzyme VioE, which is involved in the violacein biosynthetic pathway in *Chromobacterium violaceum* ATCC12472. VioE catalyzes the decarboxylation and indole-ring rearrangement of a nascent compound that is produced from indole-3-pyruvic acid and NH_4^+ by the action of chromopyrrolic acid synthase (VioB or StaD), and ultimately the reaction yields prodeoxyviolacein.

Violacein is a blue-violet pigment produced by *Chromobacterium violaceum*. Previous studies on violacein biosynthesis by using isotope-labeled precursors showed that this pigment is biosynthesized from two tryptophan molecules.[1] The C-3 site of each tryptophan is coupled to form a pyrrole ring, and subsequent decarboxylation and 1,2-shift of the indole ring afford the violacein chromophore. This is a unique example of enzymatic decarboxylation and intramolecular indole rearrangement, and the process that most similar to this one is the phenyl ring rearrangement which results in the interconversion of flavanone and isoflavone in plants.[2] In addition to violacein, *C. violaceum* also produces chromopyrrolic acid (CPA).[3] Analogous to the biosynthesis of violacein, that of CPA also occurs via a coupling reaction involving two tryptophan molecules; however, decarboxylation and the 1,2-shift of the indole ring following pyrrole ring formation do not occur. Recently, we reported that CPA is a key intermediate in the biosynthesis of indolocarbazole compounds, which are antitumor alkaloids mainly produced by actinomycetes.[4,5] We and several other groups have conducted genetic studies on indolocarbazole biosynthesis, and these studies revealed that CPA is biosynthesized from two indole-3-pyruvic acid (IPA) molecules by the action of hemoprotein StaD or its homolog RebD.[6–8] The entire violacein biosynthetic gene cluster has been cloned; it comprises five genes, namely, *vioA*, *vioB, vioC, vioD*, and *vioE*, and spans 8 kb.[9] The *vioB* gene is a homolog of *staD* (37% identity in a deduced amino acid sequence). Besides its involvement in violacein chromophore formation, the *vio* cluster is also suggested to be responsible for CPA production in *C. violaceum*. Heterologous expression of the *vio* cluster in *Streptomyces albus* or *Escherichia coli* revealed that *vioE* is involved in violacein chromophore formation, and it induces an intramolecular 1,2-shift of the indole ring at the 5-hydroxyindole side.[10] However, bioconversion experiments on CPA supported the hypothesis that CPA is not an intermediate of violacein biosynthesis.[10] Thus, two questions arise: one is with regard to the enzymatic reaction catalyzed by VioE, and the other is the role of VioB in violacein biosynthesis. We conducted an in vitro study on VioB and VioE and revealed that VioE directly biosynthesized prodeoxyviolacein via decarboxylation and a 1,2-shift of the indole ring of an unidentified intermediate that was formed from two molecules of IPA by the action of VioB (Scheme 1).

Keywords: Biosynthesis; Violacein; Chromopyrrolic acid; *staD*; *rebD*; *vioB*; *vioE*; Indole shift; Indolocarbazole.
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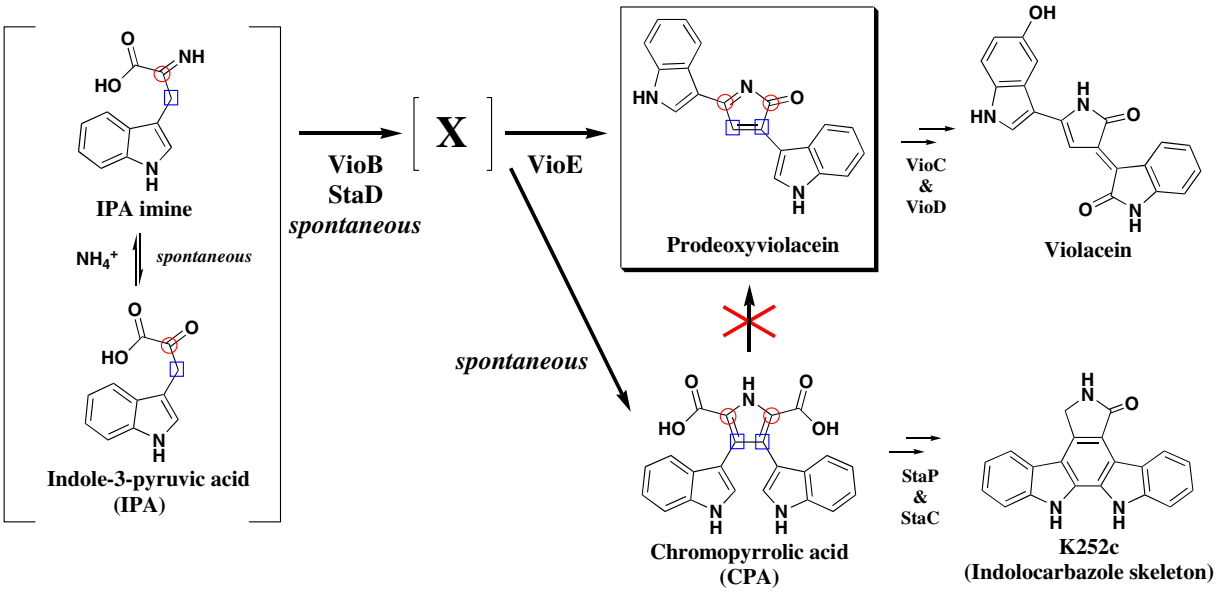

Scheme 1. Proposed pathway of violacein and indolocarbazole biosynthesis. The unidentified compound X, whose existence was predicted in this study, is indicated as X. The ^{13}C isotope-labeled positions observed in the isotope feeding experiments conducted by Hoshino et al. are indicated by circles and squares in the structures of IPA, CPA, and prodeoxyviolacein.[1,3]

The VioE and VioB proteins with hexameric histidine (His) tags at their C terminals were overexpressed in *E. coli* by using the pETVioE and pETVioB expression vectors, respectively.[†] We purified VioE and VioB by using an Ni-NTA column (Profinity IMAC, Bio-Rad) and used these proteins for further enzymatic characterization (Fig. 1).

From the deduced amino acid sequences of VioE and VioB, their molecular weights (M_w) were calculated to be 21.8 and 111.2 kDa, respectively. The native M_w of VioE was estimated to be 42.7 kDa by gel filtration chromatography using Superdex 75 10/300 GL (Amersham Bioscience Co.); this indicated that VioE is a dimer. VioB is also identified as a dimer because the native M_w of VioB was 254.8 kDa, as determined by chromatography using Superdex 200 10/300 GL (Amersham Bioscience Co.).

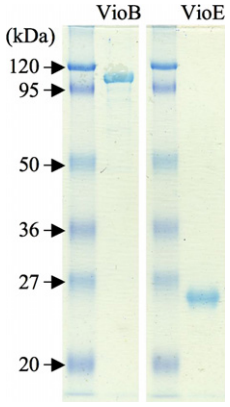

Figure 1. SDS-PAGE of purified VioB and VioE.

The purified VioB enzyme (30 nM) was incubated with 1 mM IPA and 100 mM $(NH_4)_2SO_4$ at 30 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM DTT. After the addition of 1% TFA, the reaction product was analyzed by HPLC. A new reaction product whose retention time and UV–Vis spectrum are consistent with those of authentic CPA was formed (Fig. 2A). We can conclude that like

[†] *Construction of pETVioE and pETVioB, and the expression of recombinant VioE and VioB in E. coli*. By PCR, the *vioE* gene was generated as a 576-bp DNA fragment by using chromosomal DNA from *C. violaceum* and two oligonucleotide primers, that is, 5′-CCG CAT ATG GAA AAC CGG GAA CCG CCG CTG-3′ and 5′-CGG CTC GAG GCG CTT GGC GGC GAA GAC GGC-3′. The underlined bases indicate the *Nde*I and *Xho*I restriction enzyme sites, respectively. The PCR-generated *vioE* fragment was cloned into the *Nde*I and *Xho*I sites of pET26b(+) (Novagen) to generate pETVioE in which the *vioE* gene was expressed under the control of the T7 RNA polymerase promoter. VioE was overexpressed in LB medium supplemented with kanamycin (50 μg/ml) at 24 °C. The cells were grown until $OD_{600} = 0.6$, and 100 μM IPTG was then added to the culture to induce *vioE*; the cells were grown for an additional 15 h. By PCR, the *vioB* gene was generated as a 2997-bp DNA fragment by using chromosomal DNA from *C. violaceum* and two oligonucleotide primers, that is, 5′-TCG GGA AAC ATA TGA GCA TTC TGG ATT TTC-3′ and 5′-TTC CTC GAG GGC CTC TCT AGA AAG CTT TCC-3′. The underlined bases indicate the *Nde*I and *Xho*I restriction enzyme sites, respectively. The cloning of *vioB* was identical to that of *vioE*. VioB was overexpressed in LB medium supplemented with kanamycin (50 μg/ml) at 24 °C. Cell growth continued until $OD_{600} = 0.6$, and 100 μM IPTG and 0.5 mM δ-aminolevulinic acid were then added to the culture to induce *vioB*; the cells were grown for an additional 15 h.

StaD and RebD, VioB is also a CPA synthase.[‡] The K_m value for IPA was calculated to be 450 μM from Hanes-Woolf plots of the kinetic data.[§]

The incubation of IPA and NH_4^+ with VioE afforded a new reaction product that showed a retention time of 13.3 min on HPLC (Fig. 2B).[¶] The new peak at 13.3 min exhibited a UV-Vis spectrum characteristic of prodeoxyviolacein; on LC/MS analysis, the molecular ion $[M+H]^+$ appeared at m/z 312.2, while $[M+H]^-$ appeared at m/z 310.0. Authentic prodeoxyviolacein showed the same retention time and M_w.[‖] We therefore concluded that VioE is involved in the decarboxylation and the intramolecular 1,2-shift of the indole ring, which ultimately yields prodeoxyviolacein.[‡] However, the catalytic efficiency of VioE alone is too low to determine the kinetic profile. Our previous studies revealed that a small amount of CPA was spontaneously produced by simple incubation of IPA with NH_4^+ and that StaD enhances CPA formation.[6] These results suggested the possibility that prodeoxyviolacein is formed by a coupling reaction of VioE and CPA synthase. Therefore, we coincubated VioE and StaD[6] with IPA and NH_4^+ as the substrates.[††] The amount of prodeoxyviolacein

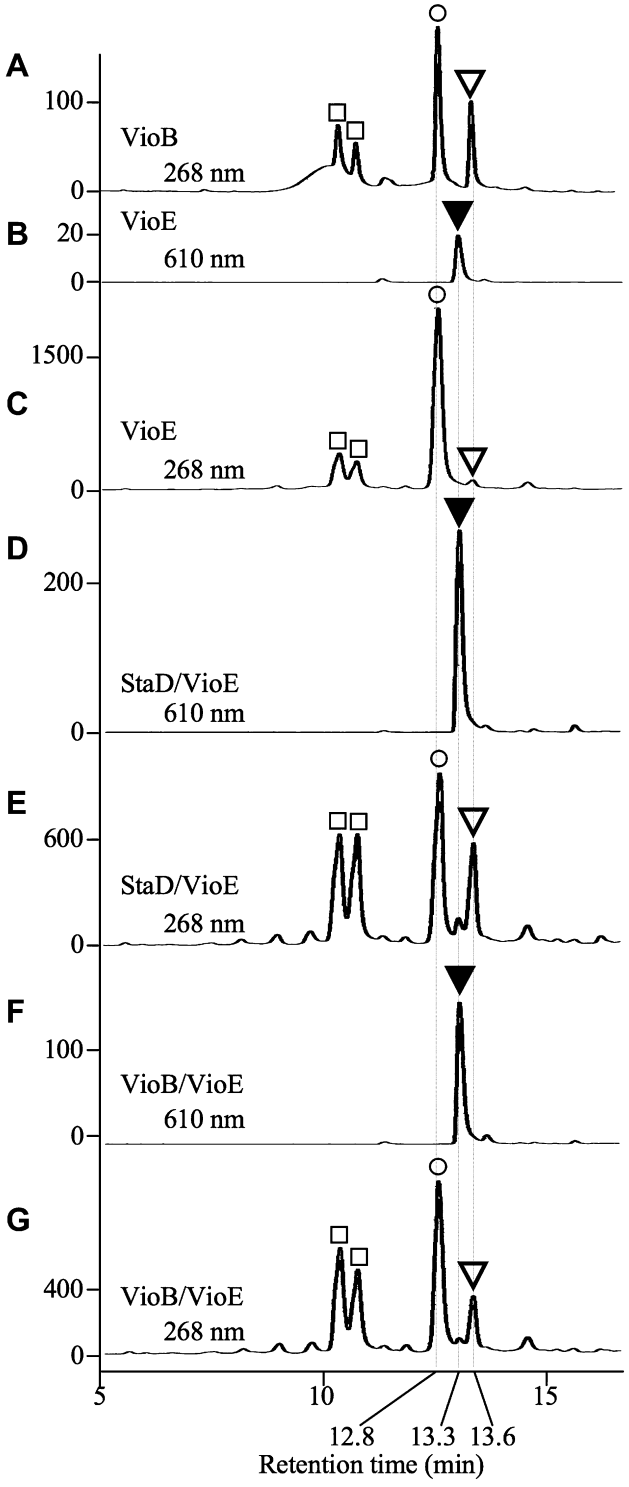

Figure 2. HPLC analysis of the VioE, StaD, and VioB enzyme reactions. HPLC profile of the reaction mixture with (A) VioB alone, (B, C) VioE alone, (D, E) VioE and StaD, and (F, G) VioE and VioB. Prodeoxyviolacein (retention time, 13.3 min) is indicated by a filled arrow head; CPA (13.6 min), by a blank arrow head; IPA (12.8 min), by a circle; and its degradation products, by a square. HPLC analysis was performed using an HP1100 system (Hewlett Packard) and a COSMOSIL column (i.d., 4.6 mm; length, 250 mm; Nacalai Tesque). The temperature was 30 °C, and the flow rate was 1 ml/min. The solvent was acetonitrile and 0.1% TFA in H_2O. Elution was performed for 30 min by using a 20–90% linear gradient of acetonitrile, and CPA and prodeoxyviolacein were detected at 268 nm (A, C, E, and G) and 610 nm (B, D, and F), respectively.

[‡] While this manuscript was under preparation, Balibar and Walsh reported an in vitro study on violacein biosynthesis, and they also speculated the existence of compound X as a VioE substrate.[11] However, the characteristics of VioB and VioE are slightly different from those observed in our results. Balibar and Walsh showed that VioB is unable to form CPA from IPA and NH_4^+. This is probably due to a difference in the reaction pH because they examined the enzyme reaction at pH 9.5. They also did not confirm the enzymatic activity of VioE in the formation of prodeoxyviolacein from IPA and NH_4^+.

[§] To determine the K_m value, various concentrations of IPA (50–500 μM) and 100 mM $(NH_4)_2SO_4$ were incubated with VioB (60 nM) for 5 min at 30 °C in 100 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM DTT. After incubation, VioB was inactivated by the addition of 1% TFA, and the formation of CPA was analyzed using HPLC.

[¶] The purified VioE protein (6.4 μM) was incubated with 1 mM IPA and 100 mM $(NH_4)_2SO_4$ at 30 °C for 90 min in 100 mM sodium phosphate buffer (pH 8) containing 0.1 mM DTT. After incubation, VioE was inactivated by the addition of 1% TFA, and it was extracted by ethyl acetate treatment repeated three times. The extract was evaporated, dissolved in DMSO, and analyzed by HPLC at 268 nm (for CPA) and 610 nm (for prodeoxyviolacein).

[‖] *Preparation of authentic prodeoxyviolacein.* The reaction mixture (2 l) was extracted twice with an equal volume of ethyl acetate and concentrated under reduced pressure to yield the crude product. After the residue was dissolved in $CHCl_3$, it was purified using a silica gel column (Silica Gel 60, Nacalai Tesque). The residue was eluted twice with a stepwise gradient of CH_3Cl–MeOH (100:1–20:1, 200 ml). The collected fractions were combined and evaporated for NMR analysis. ^1H NMR data for prodeoxyviolacein (DMSO-δ_6, containing a trace of TFA, 400 MHz): 12.48 (br s, 1H), 9.87 (s, 1H), 8.62 (m, 1H), 8.56 (m, 1H), 8.34 (m, 1H), 8.24 (s, 1H), 7.77 (m, 1H), 7.61 (m, 1H), 7.55 (m, 2H), 7.39 (m, 2H).

[††] Purified VioE (6.4 μM) and VioB or StaD (50 nM) were incubated with 1 mM IPA and 100 mM $(NH_4)_2SO_4$ at 30 °C for 90 min in 100 mM sodium phosphate buffer (pH 8) containing 0.1 mM DTT. After the addition of 1% TFA, the reaction product was extracted by ethyl acetate treatment repeated three times, and the extract was analyzed by HPLC at 268 nm (for CPA) and 610 nm (for prodeoxyviolacein).

produced by VioE increased due to the presence of StaD, and prodeoxyviolacein accumulated with CPA as a byproduct (Fig. 2D and E). When instead of StaD, an equal mole of the VioB protein was added, the amounts of prodeoxyviolacein and CPA formed were lower than those produced in the VioE/StaD system (Fig. 2F and G).[††] To investigate the possibility of complex formation between VioE and CPA synthase, biochemical pull-down experiments were conducted using hexameric His-tagged VioE and untagged StaD. The purified proteins were mixed and applied onto an Ni-NTA column. The untagged StaD was not coeluted with the tagged VioE. In addition, gel filtration with a mixture of native VioE and VioB did not reveal any complex elution peaks. These results showed that no stable complex is formed between VioE and CPA synthase. Further, CPA did not act as a VioE substrate (data not shown). Our results are consistent with those of the CPA bioconversion experiment.[10]

Based on the results, we propose a violacein biosynthesis pathway from IPA to prodeoxyviolacein, as shown in Scheme 1. The actual VioE substrate is an unidentified nascent compound X, which is generated by a VioB- or StaD-catalyzed coupling reaction with two IPA molecules and NH_4^+. It is likely that the actual synthesis product of VioB and StaD is not CPA but the unidentified compound X, which is then spontaneously converted to CPA. Our previous studies revealed that CPA was spontaneously formed by simple incubation of IPA with NH_4^+, and the k_{cat}/k_{uncat} value for StaD at an IPA concentration of 0.25 mM was 101.2.[6] This spontaneous chemical reaction on incubation of IPA with NH_4^+ would also yield the nascent compound X and then yield CPA in a manner identical to the VioB- or StaD-catalyzed reaction; however, the reaction efficiency of chemical and enzymatic reactions is very different. In this study, we show that VioE alone can transform IPA and NH_4^+ to afford prodeoxyviolacein. During this transformation, VioE uses chemically produced compound X. If VioE is not present, compound X is immediately transformed to CPA via a chemical reaction. VioE cannot catalyze the entire amount of compound X that is formed, and the remaining amount is spontaneously converted to CPA. This might be the reason why *C. violaceum* produces CPA as well as violacein.[3]

Acknowledgments

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