Biosynthesis of Violacein, Structure and Function of L-Tryptophan Oxidase VioA from Chromobacterium violaceum*

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Violacein is a natural purple pigment of Chromobacterium violaceum with potential medical applications as antimicrobial, antiviral, and anticancer drugs. The initial step of violacein biosynthesis is the oxidative conversion of L-tryptophan into the corresponding variant H163A in the presence of L-phenylalanine indicated a potential role of His163 in substrate binding. The combined structural and mutational analyses lead to the detailed understanding of VioA substrate recognition. Related strategies for the in vivo synthesis of novel violacein derivatives are discussed.

The amino acid tryptophan (Trp) is a versatile metabolite that is shunted into several microbial secondary pathways. The oxidative dimerization of two Trp scaffolds is an essential step for the synthesis of the natural bisindole compounds violacein, rebeccamycin, and staurosporine. The water-insoluble purple pigment violacein is mainly produced by the bacteria Chromobacterium violaceum and Janthinobacterium lividum in tropical habitats where it might be responsible for the shielding against UV radiation (1–3). Various biological activities of violacein, including antibacterial, antiviral, as well as cytotoxic effects against several tumor cell lines, have been demonstrated (4). The related compounds rebeccamycin and staurosporine show strong antitumorigenic activity because of DNA topoisomerase or protein kinase inhibition (5, 6).

Synthesis of violacein, rebeccamycin, or staurosporine is based on a closely related initial pathway in which orthologous enzymes catalyze the oxidation of a Trp moiety into the related indole-3-pyruvic acid (IPA)2 imine by the enzymes VioA, RebO, or StaO (Fig. 1) (7–9). Subsequently, oxidative coupling of two imines by VioB, RebD, or StaD results in the formation of a short-lived compound that was proposed to be an IPA imine dimer (7, 10). For the synthesis of rebeccamycin and staurosporine, this reactive intermediate is spontaneously converted into chromopyrrolic acid (11–13). By contrast, violacein biosynthesis requires a key intramolecular rearrangement. The postulated IPA imine dimer is the substrate of VioE, which is catalyzing a [1,2]-shift of the indole ring to produce protodeoxyviolaceinic acid (7, 14). Fig. 1 gives a schematic overview about the related pathways as follows: common enzymatic reactions and the involved cofactors are highlighted (gray shading), subsequent steps for the synthesis of violacein (VioE, VioD, VioC, and one autocatalytic step) (3, 7), rebeccamycin (RebP, RebC, RebG, and RebM) (15, 16), and staurosporine (StaP, StaC, StaG, StaN, StaMA, and StaMB) (16) are indicated.

Synthesis of violacein starts with the FAD-dependent oxidation of L-Trp to IPA imine catalyzed by VioA. The VioA protein from C. violaceum shares a substantial degree of sequence conservation with RebO or StaO proteins (ranging from 18 to 22% identity; Clustal Omega (17)). Furthermore, sequence identity values of 14–22% were observed for the comparison of VioA with L-amino acid oxidases (LAAOs) (3, 18). LAAO-catalyzed two-electron oxidations are well studied from prokaryotic and

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‡ The abbreviations used are: IPA, indole-3-pyruvic acid; IAA, 3-(1H-indol-3-yl)-2-methylpropanoic acid; IEA, 2-(1H-indol-3-ylmethyl)prop-2-enoic acid; LAAO, L-amino acid oxidase; SXS, small angle x-ray scattering; NSD, normalized spatial discrepancy.

The atomic coordinates and structure factors (codes 5G3S, 5G3T, and 5G3U) have been deposited in the Protein Data Bank (http://wwwpdb.org/).
eukaryotic enzyme sources (19, 20). However, the synthesized imines are subsequently deaminated by virtue of an attacking water molecule into the respective \(\text{H}_2\text{Ko}2\text{A} \) (21, 22).

By contrast, violacein biosynthesis relies on the reactive IPA imine as a direct substrate of VioB. Furthermore, the postulated IPA imine dimer reaction product is also labile, which might reflect the need for an activated substrate for the unusual [1,2]-shift of the indole ring during VioE catalysis. However, present date in vitro investigations revealed that the direct interaction of VioA and VioB (or of VioB and VioE) is not an absolute prerequisite for protodeoxyviolaceinic acid synthesis (7).

In a recent publication, 50% FAD occupancy was determined for recombinantly purified VioA protein. Kinetic characterization of this protein was performed in a tandem peroxidase assay with an optimal pH of 9.25. Formation of the unstable IPA imine goes along with a reduced flavin on VioA, which is subsequently reoxidized by molecular oxygen leading to stoichiometric peroxide formation. The detection of hydrogen peroxide revealed \(k_{cat}\) and \(K_m(\text{Trp})\) values of 203 min\(^{-1}\) and 31 \(\mu\text{M}\) for VioA (7).

The heme-containing oxidase VioB catalyzes the coupling of two IPA imine molecules to form the postulated intermediate IPA imine dimer (7). For this molecule, only indirect experimental evidence is available due to the short-lived nature of this compound (often referred to as compound X in the literature) (7, 18). Catalase activity has been demonstrated for VioB, and it was proposed that the heme iron center is responsible for VioB catalysis (7).

In the absence of VioE, the off-pathway metabolite chromopyrrole acid is observed. This non-enzymatic chromopyrrole acid formation is observed without involvement of VioD. For staurosporine and rebeccamycin biosynthesis, the IPA imine dimer is spontaneously converted into chromopyrrole acid. Subsequent enzymes StaP, StaC, StaG, StaN, StaMA, StaMB or RebP, RebC, RebG, RebM then facilitate the synthesis of staurosporine or rebeccamycin.

FIGURE 1. Biosynthetic pathway of violacein (black), staurosporine (blue), and rebeccamycin (red). Orthologous enzymes VioA, StaO, or RebO and VioB, StaD, or RebD are involved in the initial pathway for the synthesis of violacein, staurosporine, and rebeccamycin, respectively (highlighted in gray). A Trp moiety (L-Trp) is converted into indole-3-pyruvic acid imine (IPA imine) and subsequently dimerized into a short-lived compound proposed as IPA imine dimer. Synthesis of rebeccamycin initially requires the chlorination of L-Trp to 7-Cl-Trp by virtue of RebH and RebF. Violacein biosynthesis then includes a VioE-dependent [1,2]-shift of the indole ring (curved arrow) resulting in protodeoxyviolaceinic acid. Subsequent VioD and VioC catalysis followed by a final autocatalytic oxidation step leads to the product violacein. A deoxyviolacein derivative is synthesized without involvement of VioD. For staurosporine and rebeccamycin biosynthesis, the IPA imine dimer is spontaneously converted into chromopyrrole acid. Subsequent enzymes StaP, StaC, StaG, StaN, StaMA, StaMB or RebP, RebC, RebG, RebM then facilitate the synthesis of staurosporine or rebeccamycin.

Under physiological conditions, the violacein biosynthetic pathway also results in the formation of deoxyviolacein (23). Deoxyviolacein, lacking the hydroxyl group at position 5, is synthesized without involvement of VioD, which might indicate a certain degree of flexibility for VioC substrate recognition (Fig. 1). (7)

In this study, recombinantly produced VioA from \(C.\ violaceaum\) is analyzed in a combined biochemical and x-ray crystallographic approach. Structure-based site-directed mutagenesis along with kinetic experiments in the presence of artificial substrates or active site inhibitors reveal the molecular mechanism of VioA.
Crystal Structure of VioA

Results

Production and Purification of VioA—The l-Trp oxidase VioA from *C. violaceum* was efficiently overproduced in *E. coli* as a soluble GST-VioA fusion protein (Fig. 2A, lane 1). The tagged protein was immobilized on a glutathione-agarose column. Subsequently, the target VioA protein was liberated by on-column cleavage with PreScission™ protease (Fig. 2A, lanes 2–7). Overall, 40 mg of the homogenous target protein was obtained from 1 liter of cell culture. SDS-PAGE analyses indicated a relative molecular weight of 45,000 (Fig. 2A, lanes 5–7, calculated molecular mass, 47,159 Da) and the integrity of purified VioA was confirmed by N-terminal sequencing.

UV-visible absorption spectroscopy of a purified VioA sample revealed characteristic absorption maxima at 387 and 457 nm (Fig. 2B, solid line), as is characteristic for FAD-containing flavoproteins (24). Spectroscopically, an overall FAD content of 55% was determined using the molar extinction coefficient of oxidized flavin, in agreement with the outcome of a recent publication (7). In addition, the FAD content of purified VioA was also estimated on the basis of single-turnover VioA activity experiments. Strict anoxic conditions were required to prevent the reoxidation of the resulting FADH2 cofactor due to electron transfer onto molecular oxygen. Purified samples of VioA at a concentration of 15 μM were incubated in the presence of varying l-Trp concentrations ranging from 0 to 10 μM. The recorded absorption spectra revealed a clear decrease of the characteristic FAD signal (Fig. 2B, compare solid line versus dotted lines). When the remaining FAD concentrations were
plotted against the applied L-Trp concentration, an FAD content of ~50% was estimated for the recombinantly purified VioA.

Tight binding of the FAD cofactor was demonstrated in an aerobic dialysis experiment. Under the employed turnover conditions, the FAD cofactor is constantly regenerated due to the presence of O₂. A VioA sample was incubated in the presence of a large volume of buffer containing 40 mM L-Trp. The resulting VioA dialysate after a subsequent buffer exchange step (to remove VioA educt and product) did not reveal a decreased FAD content (data not shown).

Inhibition of VioA and Synthesis of a Substrate- and Product-related Inhibitor—The identification of efficient active site inhibitors is a well established strategy for the detailed understanding of enzyme mechanisms. Inhibitor concentrations of up to 10 mM were analyzed in the coupled VioA activity assay (9) or alternatively in VioA substrate depletion kinetics (compare Fig. 3, A–C). Experiments in the presence of D-Trp revealed a moderate VioA inhibition (72% relative activity, 10 mM D-Trp). In the present literature, citrate and o-aminobenzoate have been described as efficient inhibitors of LAAOs (21, 25, 26). In agreement with these distantly related investigations, a relative activity of 15% in the presence of 10 mM citrate was observed. By contrast, in the presence of 10 mM o-aminobenzoate only a moderate inactivation indicated by a residual activity of 71% was determined (Fig. 3C). Because citrate does not closely resemble the L-Trp molecule, a substrate-related and a product-related L-Trp derivative was newly synthesized for subsequent inhibition experiments. In compound 3-(1H-indol-3-yl)-2-methylpropanoic acid (IAA), the amino group of L-Trp is replaced by a methyl group, whereas the non-chiral compound 2-(1H-indol-3-ylmethyl)prop-2-enoic acid (IEA) is carrying a methylene group, instead. Accordingly, the IEA molecule is sharing a closely related spatial orientation (due to the sp²-hybridized carbon) as the IPA imine reaction product of VioA (Fig. 3C). IEA and racemic IAA were synthesized from

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**FIGURE 3. Identification of VioA inhibitors.** D-Trp, citrate, o-aminobenzoate, IEA, and rac-IAA were analyzed as potential inhibitors in the coupled VioA horseradish peroxidase assay containing 0.2 μM VioA in the presence of 1 mM L-Trp (A). Alternatively, potential inhibitors were analyzed in an HPLC-based substrate depletion assay using 1 μM VioA and 500 μM L-Trp (B). A, representative inactivation kinetics monitoring the formation of the quinonimine dye at a wavelength of 505 nm. A VioA activity assay (solid line) and competition experiments in the presence of 10 mM D-Trp, o-aminobenzoate, citrate, IEA, and IAA, respectively, are displayed (gray line and consecutive dotted lines). B, representative substrate depletion assays analyzed on a Jasco HPLC system using μBondapak™C18 3.9 × 150 mm column. Reactions were incubated for 3 min at 30 °C. A control experiment in the absence of VioA (a), a standard VioA activity experiment (b), and inhibition experiments in the presence of 5 mM citrate (c), 5 mM IEA (d), or 5 mM rac-IAA (e) are displayed. C, relative activities of VioA inhibition experiments (coupled assay and depletions assay) using different inhibitor concentrations. The activity of the standard VioA assay in the presence of 1 mM L-Trp was set as 100%. Dashes indicate experiments not performed. Results are presented as means ± S.D. of three independent biological samples, measured as triplicates.
Crystal Structure of VioA

Identification of the Physiological VioA Dimer—Analytical size exclusion chromatography revealed a dimeric structure of VioA as indicated by a relative molecular mass of 94,000 ± 7,000 (Fig. 2D). Identical chromatographic results were also obtained in the presence of 500 μM IEA (Fig. 2D). Examination of potential VioA contacts in the ternary crystal structure (and in the binary structure) of VioA using the PISA server (Protein Interfaces, Surfaces and Assemblies) (33) revealed a globular dimer or alternatively an elongated dimer burying a surface area of 721.7 or 667.3 Å² (compare Fig. 2E, inset: top, globular dimer; middle, elongated dimer; bottom, monomer). Identical dimers were also observed for VioA-FADH₂.

Subsequently, small angle x-ray scattering (SAXS) experiments were performed to characterize the dimer of VioA in solution. This technique makes use of a dilute protein solution and allows for the reconstruction of a low resolution electron density map. Almost identical scattering curves for VioA and for VioA in the presence of 3.75 mM IEA were obtained. In Fig. 2E, the comparison of the experimental VioA scattering curve (black dots) with the theoretical scattering curves for the globular dimer (red), the elongated dimer (green), and for the VioA monomer (blue) is shown. A significantly higher quality of fit was obtained for the globular dimer (χ = 1.638) when compared with the elongated dimer (χ = 9.881) or the monomer (χ = 14.173). From SAXS measurements, an apparent molecular mass of 80,000–105,000 was determined, in agreement with the related gel filtration experiments (Fig. 2C). The ab initio model derived from the SAXS experiments described the shape of the globular dimer well (Fig. 2E, inset). These findings allowed us to clearly assign the globular dimer with a rather small interface as the physiological unit of VioA.

Overall Structure of VioA—Each VioA monomer is composed of 17 α-helices and 17 β-strands and folds into three distinct domains. The FAD-binding domain is composed of residues 4–38, 185–256, and 380–418 and forms a classical nucleotide-binding fold (with the P-loop sequence motif \(11\)GXGXXG(X)\(_3\)D\(_8\) (34)) of the glutathione reductase family 2. The substrate-binding domain includes residues 49–86, 177–180, and 262–366, and the helical domain contains residues 87–175 (Fig. 5, A, B, and G). The VioA dimer is associated via a discontinuous protein interface that involves amino acid residues of the FAD-binding and of the substrate-binding domain. The dimer assembly is based on two symmetry-related interfaces mainly mediated by residues Arg\(_{319}\), Ala\(_{323}\), Glu\(_{324}\), with His\(_4\), Arg\(_{235}\), Tyr\(_{206}\), Asp\(_{226}\), respectively. Among these, conserved residues Arg\(_{319}\), Tyr\(_{206}\), and Asp\(_{226}\) are engaged in a polar network, including a bidentate salt bridge interaction of Arg\(_{319}\) and Asp\(_{226}\). This "loosely associated" dimer forms a central cavity (along the 2-fold symmetry of the dimer) with a volume of \(~13 \times 10 \times 8 \) Å³ (Fig. 5A). From this cavity, the adenosyl moiety of the tightly bound FAD cofactor is accessible.

A Dali search of the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (35) indicated the strongest structural similarity to the LAAO from Calloselasma rhodostoma (Protein Data Bank code 1FBS). Both proteins share a sequence identity of only 14%. However, structural superposition revealed a root mean square deviation of solely

Indole and methyl 2-(bromomethyl)acrylate (1) following a procedure from Holzapfel et al. (27). Methyl 2-(bromomethyl) acrylate was obtained in two steps from methyl acrylate and paraformaldehyde, followed by bromination with PBr₅ (28, 29). Reduction of the methylene group was performed using magnesium in MeOH, and saponification of the corresponding esters 2 and 4 led to the desired products in good yields (Fig. 4) (30, 31). Kinetic experiments revealed a residual specific VioA activity of 61 and 53% in the presence of 1 mM IEA and IAA. At inhibitor concentrations of 10 mM, residual activities of 7 and 1% were determined. Results for the efficient inhibitors citrate, IEA, and IAA were independently confirmed in substrate depletion activity assays (Fig. 3). The results of these inhibition experiments were further substantiated in a direct biomolecular interaction study.

Microscale Thermophoresis—Thermophoresis is a phenomenon that describes the movement of molecules along a temperature gradient (also termed Ludwig-Soret effect) (32). In this study, we made use of microscale thermophoresis to analyze the direct binding of citrate, IEA, and IAA to VioA (labeled with the fluorescence dye NT-647-NHS). Fluorescence was measured at a spot in the capillary that was heated by an IR laser beam. Inhibitor binding was observed as a change in the thermophoretic properties of the fluorescently labeled protein due to complex formation with VioA at various ligand concentrations. The following dissociation constants were determined in agreement with the results of the above mentioned inhibition study: IAA 32.2 ± 10.3 μM = IEA 42.6 ± 13.6 μM = citrate 45.3 ± 7.8 μM. According to these results, the newly synthesized compounds IEA and IAA were considered as promising candidates for the co-crystallization of VioA.

Determination of the Binary and Ternary VioA Structure—The sitting-drop vapor diffusion technique was used to crystallize VioA, and the phase problem was resolved using single-wavelength anomalous diffraction data of a samarium nitrate-derivatized crystal. Electron density in the active center of the native VioA structure indicated the presence of the flavin cofactor (occupancy 100%). Crystals of VioA were also grown in the presence of IEA. The molecular replacement technique was used to solve the structure of the ternary VioA complex in the presence of the flavin and IEA. Table 1 summarizes the data collection and refinement statistics. The finalized models with a resolution of 1.8 and 2.4 Å (native structure and ternary complex) were deposited in the Protein Data Bank with accession numbers 5G3T and 5G3U.
2.7 Å (calculated via Dali server; 483 residues included). Most relevant structural differences are localized in the helical domain of VioA and include the deletion of two strands and one helical segment (following strand 5 of VioA) as well as the insertion of the short helix 1 (residues 167–170). Beside this, the substrate-binding domain of VioA is linked to the FAD-binding domain via loop region 367–375 (containing unresolved residues 369–371) and strand 16 (residues 376–378) instead of a helical segment found in LAAO. In Fig. 5G, the topology diagram (with labeled residues of each secondary structural element) is highlighting the FAD-binding domain (light blue), substrate-binding domain (blue), and the helical domain (dark blue).

**FAD Binding**—In the binary and ternary VioA structure, the prosthetic group FAD is bound and deeply buried in an identical extended conformation as observed for several LAAO enzymes (Fig. 5, A, B, H, and J) (21). The FAD molecule forms an array of polar contacts, including main chain atoms of amino acids Ser15, Met39, Arg46, Gly63, Arg64, and Leu208, and Met398, side chain atoms of residues Ser45, Asp38, Arg46, and Asp389, and also a series of hydrogen bonding interactions via spatially conserved water molecules (data not shown). A partially related polar network is observed in the structure of LAAO from C. rhodostoma. However, these interactions rely on the conservation of only three residues (Asp38, Arg46, and Arg64 VioA numbering) but otherwise make use of a closely related array of polar contacts involving several structurally conserved water molecules that are positioned via equivalent main chain atoms of non-conserved amino acids. As described for most flavoproteins, an Mg2+ ion is involved in ionic bonds to the diphosphate group of FADH2. The isoalloxazine ring of FAD points toward the substrate-binding domain. The dimethylbenzene ring is positioned in a partially hydrophobic pocket surrounded by highly conserved VioA residues Trp359, Val61, and Gly61. In both VioA complex structures, atom N5 of the isoalloxazine ring is in direct contact with a structurally conserved water molecule that is bound to Lys369 (Fig. 5, C and F). For the structure of LAAO from C. rhodostoma, the equivalent water molecule was discussed as a potential nucleophile for subsequent imine deamination (21). However, synthesis of violacein is strictly based on the formation of the IPA imine as the final product of VioA catalysis. Accordingly, a nucleophile function of the water molecule must be excluded for VioA. Instead, this water might be essential for the appropriate positioning of the isoalloxazine ring of FAD.

**Active Site of VioA**—The structure of VioA in the presence of the flavin cofactor and IEA reveals a detailed picture of VioA catalysis. In the final electron density map, the isoalloxazine ring adopts a bent conformation (Fig. 5E) as observed for many reduced flavoproteins, including the complex structure of LAAO from C. rhodostoma (36). For crystallization purposes, the VioA cofactor complex was always purified in the presence of the reducing agent DTT, which results in a reduced VioA-FADH2 sample. Crystal growth under oxic conditions then resulted in faintly yellow crystals indicative of the minor oxidation of the FAD complex. However, subsequent reduction of the prosthetic FAD group due to radiation damage during synchrotron data collection has been well described in the literature (37). Accordingly, the observed crystal structure was referred to as VioA-FADH2-IEA. For this structure, no differences of the overall flavin geometry were observed when compared with the binary VioA-FADH2 complex.
Crystal Structure of VioA
In the ternary structure, atom N5 of the prosthetic group resides out of the plane of the isoalloxazine ring and points toward the IEA ethene group. This inhibitor was synthesized as a potential transition state analog that closely resembles the IPA imine reaction product. Accordingly, the VioA:FADH₂:IEA structure might represent a snapshot in the course of VioA catalysis. The electron density of the IEA ligand clearly revealed the sp²-hybridized ethene group atoms of IEA. By contrast, the natural L-Trp substrate contains an sp³-Cα atom (compare Fig. 5E and the structure with a modeled L-Trp ligand depicted in Fig. 5F).

The substrate-binding site of VioA is located in a deeply buried cavity (Fig. 5, A, B, H, and J). IEA is accessible via a substrate entry channel that is delineated by residues Met155, Asp158, Ile159, Phe365, Cys395, and Trp397. The isoalloxazine ring of the cofactor is located at the re face of the inhibitor, and the imidazole moiety of IEA is extending away from the cofactor with the N1 atom pointing toward the entry path. The inhibitor’s carboxylate forms a salt bridge with the guanidinium group of Arg64 and is further engaged in hydrogen bonds with the hydroxyl of Tyr399 and with the imidazole ring of His163 (Fig. 5, C and D). A closely related carboxylate binding mode is also observed in the structure of LAAO (33). The C2 atom of IEA (representing the site of oxidative attack in the VioA substrate) is positioned at a distance of ~3.4 Å from the N5 atom of the isoalloxazine ring. Such distance often is observed for flavoenzymes performing a direct hydride transfer mechanism. The methylene group of IEA (as part of the imine mimic) points toward the planar indole ring of Trp397. The indole moiety of IEA resides in a hydrophobic pocket that is mainly formed by side chains of residues Tyr143, Ala145, Ile159, and Val363. IEA resides in a hydrophobic pocket that is mainly formed by side chains of residues Tyr143, Ala145, Ile159, and Val363. IEA resides in a hydrophobic pocket that is mainly formed by side chains of residues Tyr143, Ala145, Ile159, and Val363.

Possible Channel for the Oxidative Half-reaction of VioA—VioA catalysis is based on the FAD-dependent formation of the IPA imine (reductive half-reaction) in a process that is coupled to the O₂-dependent formation of H₂O₂ (oxidative half-reaction). Because of the tightly bound prosthetic flavin group, the second substrate O₂ must gain access to the active site of VioA. In the ternary VioA structure, the catalytic cavity is completely locked due to the binding of IEA. Accordingly, the deeply buried flavin appears to be inaccessible to O₂ via the substrate entrance channel. Inspection of the VioA protein surface (for both crystal structures) indicated a second water-filled channel (constriction radius ~2.0 Å) (Fig. 5I, right) giving direct access to the structurally conserved water molecule (3.1 Å) involved in a bridging interaction between atom N5 of the isoalloxazine and Lys269. This channel is mainly delineated by conserved residues Lys269 and Tyr397 and by the partially conserved residues Glu193, Tyr397, and Phe397. The observed channel might suggest a direct path for O₂ to the catalytic site. Indeed, the position of the conserved water molecule would be also appropriate for the binding of O₂ and the subsequent formation of H₂O₂. Analogously, a related LAAO structural analysis revealed a (spatially unrelated) substrate channel for the oxidative half-reaction (36).

Domain Movement of VioA—Structural comparison of the overall VioA:FADH₂:IEA and the VioA:FADH₂ complex reveals a significant domain movement (compare gray and blue representation in Fig. 5H). The structural alterations observed in the presence of IEA can be roughly described as a movement of the helical domain toward the FAD-binding domain, resulting in a partial closure of the substrate entry path as indicated by a 3.5-Å closer positioning of the Ca atoms of Met155 and Phe365. These overall movements result in slight alterations of the side chain conformation of active site residues Val363 and Trp397. Most significant changes are observed for residues His163 and Arg64, which are located in a 0.4-Å closer position in the VioA:FADH₂:IEA structure. The observed conformational alterations might be relevant for catalysis and/or for the appropriate timing of the IPA imine product release. The presented structures of VioA have been further explored in biochemical experiments to provide a solid basis for the understanding of the catalytic mechanism and the substrate recognition of VioA.

Substrate Specificity of VioA—A series of recently described activity assays (9) with the 20 canonical l-amino acids revealed solely the conversion of l-Trp. This is in agreement with our experiments in the presence of l-tyrosine, l-valine, l-proline, l-histidine, l-leucine, and l-alanine (detection limit <9.9 pmol min⁻¹ mg⁻¹, data not shown). Such efficient discrimination might be essential under physiological conditions, because VioA catalysis results in highly toxic peroxide formation. Our experiments using l-phenylalanine revealed a relative activity of 1.8% when compared with the natural substrate. To some extent, the benzyl group of l-phenylalanine might adopt a related spatial conformation in the active site of VioA under the employed in vitro assay conditions. Efficient discrimination of l-Trp was observed in agreement with a stereospecific hydride abstraction mechanism.

FIGURE 5. Crystal structure of VioA from C. violaceum. A, schematic representation of the physiological dimer of the VioA:FADH₂:IEA complex viewed along the 2-fold symmetry dimer axis (black lens). Individual VioA domains are colored light blue (FAD-binding domain), blue (substrate-binding domain), and dark blue (helical domain); the respective domains of the second protomer are rendered transparent. The co-crystallized FADH₂ (orange) and the inhibitor IEA (wheat) are shown as ball-and-stick model and the Mg²⁺ ion is displayed as a green sphere. The transparent van der Waals surface is depicted in light blue. B, schematic representation of an isolated VioA protomer from A with indicated N and C termini. C, close-up of the active site of VioA. The IEA inhibitor (wheat), FADH₂ (orange), and key VioA residues (blue ball-and-sticks) and the respective hydrogen bonding and salt bridge interactions are shown (dashed lines); water molecule is displayed as red sphere. D, IGLPLOT (66) representation of the active site showing non-polar and polar contacts (with indicated distances in green (Å)). E, conformation of the co-crystallized IEA and the flavo moiety of FADH₂. Portion of the 2F, − F, electron density map is shown as gray mesh at a contour level of 1.5 σ. F, the substrate l-Trp (violet) was modeled into the VioA active site. The distance between the hydrogen atom located at the sp³-hybridized Cα atom (light gray) and atom N5 of FAD (2.3 Å) is indicated (dashed line). G, topology diagram of VioA. Helices are depicted as cylinders and β-strands are arrows (color code as in A). Undefined regions are colored in gray and unresolved residues 369–371 are indicated by the dotted line. H, superposition of the binary (light gray) and ternary (dark blue) VioA structure (same orientation as in B). The ribbon representation is showing significant movement of the helical domain toward the FAD-binding domain. I, (center), a longitudinal section of the VioA monomer represented as van der Waals surface colored according to its surface electrostatics (−10 red to +10 blue) K/Te₂e. The left half is highlighting the substrate-binding pocket with the bound IEA molecule and the proposed substrate entry path (indicated by an orange asterisk). The right half additionally shows the FAD/FADH₂-binding pocket and the proposed channel for the second substrate O₂ (indicated by a yellow ×) required for the oxidative half-reaction of VioA. The orientation of this right half is roughly as in B and H.

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To further elucidate the substrate specificity of VioA, a series of four artificial substrates (modified at C1, C5, and C7; compare Fig. 7A) were analyzed in the coupled enzyme assay. For this purpose, 5-fluoro-Trp and 7-aza-Trp were synthesized starting from commercially available 5-fluorotryptophan and 7-aza tryptophan, respectively. First, a Mannich reaction to the corresponding amines was performed, followed by addition of methyl iodide (38). The resulting tetramethylammonium iodide salts were obtained with very good yields. Introduction of the amino acid side chain was realized using N,N-diphenyl-ethylglycine (39). Deprotection of the amino group and saponification led to the desired product in good yields (Fig. 6).

For compound 7-aza-Trp, a relative VioA activity of 23% was determined when compared with the natural substrate (Fig. 7, A and C). Interference of the 7-aza modification of the indolyl ring with the side chain of Ile159 might be responsible for the decreased activity. A significantly higher VioA activity of 76% was observed for 1-methyl-Trp in agreement with the ternary VioA structure (Fig. 7, A, C, and D). This modification does not result in any steric clashes because a substituent at N1 extends into the active site entry channel of VioA (Fig. 5, A, and C). This degree of flexibility for the side chain of residue Asp311 might be responsible for the broadened substrate specificity of VioA. These results are further supported by a recent investigation showing a relative activity of 86% in the presence of the artificial substrate 5-hydroxy-Trp (7). Basically, substrate discrimination only occurs in the presence of natural amino acids, whereas all chemically synthesized L-Trp derivatives were efficiently converted by VioA.

**Mutagenesis of Catalytic Residues of VioA—**In a structure-based mutagenesis approach, the impact of conservative (or alternatively of a more drastic) mutations was analyzed with respect to amino acids Arg64, His163, Lys269, Tyr309, Val163, and Trp397. The integrity of all site-directed mutant proteins was confirmed with thermal shift experiments, and the amount of co-purified FAD cofactor was determined (Fig. 7, B and C).

The bidentate salt bridge of the substrate’s carboxylate with the guanidinium group of Arg64, which is of prime importance because mutant proteins R64Q and R64S did not reveal any detectable VioA activity (Fig. 7C). The spatial positioning of this carboxylate by additional hydrogen bonding interactions to Tyr309 and His163 is further supported by residual activities of 5, 8, and 85% for variant proteins Y309A, H163N, and H163A (Fig. 7C). The moderately reduced activity of mutant H163A clearly indicates that VioA catalysis does not rely on an imidazole-mediated general acid/base mechanism.

For mutant W397A, no enzymatic activity was determined. The ternary VioA structure indicates the shielding of the substrate’s amino group via the planar indole ring system of Trp397 (Fig. 5, C and D). Obviously, this Trp plays a substantial role for the accurate positioning of the substrate as a prerequisite for subsequent enzyme catalysis. This hypothesis is in agreement with the observed residual activity of 63% for mutant W397Y (Fig. 7C). To some extent, the planar tyrosyl side chain might be able to cope with the spatial orientation of the indole side chain of Trp397.

In both VioA structures, Lys269 is in contact with N5 of the isoalloxazine ring of the cofactor via a bridging water molecule (Fig. 5, C and D). This indirect hydrogen bonding interaction of Lys269 is of central importance for the catalytic mechanism of VioA because mutant proteins K269Q and K269S revealed residual activities of solely 2 and 1% (Fig. 7C). This residue might be essential for the accurate positioning of the isoalloxazine ring. However, a potential role during the O2-dependent reoxidation of FADH2 must also be considered (40).

Val363 is part of the defined binding pocket that is relevant for the specific recognition of the hydrophobic indole ring of the substrate (Fig. 5, C and D). This is supported by our mutational analysis, because variants V363Q and V363A yielded relative activities of 17 and 57%. Replacing Val363 by a larger and more polar glutamine residue might result in a steric restriction of the hydrophobic active site. By contrast, the related alanine mutation might result in a widening of this pocket, reflected by an increased activity of 57% (Fig. 7C). Nevertheless, integrity of the overall hydrophobic active site pocket of VioA might be relevant to obtain full enzymatic activity.
Mutant Activities in the Presence of Non-natural Substrates—

As summarized in the table depicted in Fig. 7C, the activity of mutants R64Q, R64S, H163N, K269Q, K269S, Y309A, and W397A significantly decreased using artificial substrates 7-aza-Trp, 1-methyl-Trp, 5-methyl-Trp, and 5-fluoro-Trp so that values partially below the detection limit were determined. When the activity of the “more active” mutants H163A, V363Q, V363A, and W397Y in the presence of L-Trp was set as 100% (values in parentheses, respectively) the following order of substrate activities was obtained: 5-methyl-Trp (70–160%) / 5-fluoro-Trp (51–79%) / 1-methyl-L-Trp (24–77%) / 7-aza-Trp (7–22%). Basically, these mutant activities mirror the outcome of the wild type enzyme assays in the presence of the different artificial substrates. Nevertheless, the individually determined values do not only reflect the simple addition of a mutant and of an artificial substrate effect.

Site-directed mutagenesis of key residues often goes along with an altered specificity of the respective enzymes (41). Accordingly, active mutant proteins V363Q, V363A, H163A, and W397Y were further analyzed with respect to substrate specificity.

Widening the Substrate Specificity of VioA—VioA proteins H163N, V363Q, V363A, and W397Y were analyzed in the presence of L-tyrosine, L-valine, L-proline, L-histidine, L-leucine, and L-alanine and L-phenylalanine, respectively. None of these combinations resulted in any detectable enzymatic activity, indicative of a preserved VioA substrate specificity. By contrast, for variant H163A, discrimination of L-tyrosine, L-valine, L-proline, L-histidine, L-leucine, and L-alanine was shown, whereas a relative enzymatic activity of 5.4% was determined in the presence of L-phenylalanine, corresponding to a 3-fold increase with respect to the wild type enzyme (Fig. 7C). This indicates that His163 plays an essential role for the appropriate positioning of the (artificial) substrate in the active site of VioA. A slightly altered network for the recognition of the substrate’s carboxylate group might cause an improved binding of L-phenylalanine in the active site of VioA.

Discussion

VioA and orthologous enzymes StaO and RebO catalyze the FAD-dependent oxidation of a Trp scaffold within the biosynthesis of important natural bisindole compounds (Fig. 1). The catalytic cycle for the VioA-dependent IPA imine formation involves the reoxidation of the prosthetic group by O2 with formation of H2O2. The resulting imine reaction product is susceptible for subsequent deamination as it is observed for the related catalysis of LAAO enzymes (21, 42). By contrast, violacein biosynthesis requires efficient transfer of the labile intermediate from the active site of VioA to the subsequent enzyme of the pathway.

This investigation reveals a detailed picture for the understanding of VioA substrate specificity. Efficient discrimination of the canonical amino acids might be essential under in vivo conditions because the H2O2 “side product” and also unwanted
Crystal Structure of VioA

Imine products might be deleterious for the bacterial cell. However, in the presence of unnatural artificial VioA substrates, substantial flexibility due to the conversion of 7-aza-Trp, 1-methyl-Trp, 5-methyl-Trp, and 5-fluoro-Trp was indicated. Obviously, evolution of the VioA active site did not rely on clearly defined determinants for the recognition of the indole ring system.

Our structural investigation showed a well defined network for the specific interaction of the polar "head group" of the VioA substrate (Fig. 5, C and D). Such type of a fixed l-Trp only shows limited conformational flexibility at the Cα atom. The hydrophobic indole moiety is not specifically recognized in the hydrophobic part of the active site pocket. Obviously, conformational restraints at Cβ in combination with the rigid and planar indoyl substituent play a key role for VioA substrate recognition.

With respect to the overall biosynthetic pathway, compounds 7-aza-Trp, 1-methyl-Trp, 5-methyl-Trp, and 5-fluoro-Trp are interesting candidates for the in vivo synthesis of new violacein derivatives. As outlined in Fig. 1, variant Trp molecules with a modified indole ring at position 1, 5, or 7 are theoretically processed into the respective violacein (or deoxyviolacein) derivatives (compare numbering indicated for violacein and deoxyviolacein). Initial in vivo pathway reconstitution experiments in our laboratory already indicated the conversion of synthetic artificial Trp derivatives to new enzymatic products and intermediates. Structural elucidation of the novel compounds produced and investigations toward their possible biosynthetic pathway are ongoing. On the basis of these results, synthetic biology approaches for the engineering of variant violacein, rebeccamycin, or staurosporine pathways might be a promising strategy for the synthesis of new bisindole therapeutics. Successful engineering of staurosporine and rebeccamycin variant molecules has been demonstrated recently (43, 44).

The outcome of the combined structural and biochemical investigation allowed us to propose a detailed molecular mechanism of VioA catalysis. Enzyme kinetics of mutant proteins functionally identified Arg64, His163, Lys269, Tyr309, and Trp397 as essential residues of VioA catalysis. Inspection of the spatial orientation of these amino acids in both structures of VioA did not reveal an appropriate catalytic base for the specific deprotonation of the Cα-H-bond of the l-Trp substrate. This is inconsistent with an acid-base mechanism via a substrate carbanion. However, the Cα of l-Trp (as indicated by the bound IEA molecule) is well oriented with respect to a direct hydride transfer. In Fig. 5F, the Cα-H-bond (of the modeled substrate complex) is pointing toward the N5 atom of the cofactor exactly as observed for many related flavoenzymes performing a hydride transfer mechanism (21, 45, 46). Accordingly, the following Reaction sequence for VioA catalysis was postulated. The zwitterionic l-Trp substrate approaches the catalytic site via the substrate entry channel (compare orange asterisk in Fig. 5I, left and right). Subsequently, deprotonation of the α-amino group is required for the formation of the Michaelis complex. This proton abstraction might be supported by the optimal pH of 9.25 (7) and also by the active site architecture where the polar α-amino group points perpendicularly to the hydrophobic indole ring system of Trp397. Then, the exact orientation of Cα in the Michaelis complex facilitates the oxidative attack. This transition state might be further activated by distortion of the substrate. By analogy to the VioA-FADH2IEA complex, the tetrahedral Cα of the substrate might adopt a more planar geometry with partial sp2 character. Such a distorted geometry would not only reflect the sterical characteristics of the IPA imine reaction product but would also explain the observed competitive inhibition in the presence of the transition state analog IEA. Formation of this activated ternary complex is accompanied by protein domain movements as indicated by the structural transition from the VioA-FADH2 to the VioA-FADH2IEA complex. These conformational rearrangements result in a partial closure of the active site cavity and also include the repositioning of residues His163 and Arg64. These alterations might be a prerequisite for the direct transfer of the Cα-hydrate onto N5 of the isoalloxazine ring. This process is guided by the movement of the lone pair of electrons from the α-amino nitrogen to the Cα, leading to the formation of the protonated imine reaction product. To some extent, the ternary VioA complex also resembles a well protected enzyme-product complex in which the IPA imine is shielded from the deamination via attacking water molecules. Subsequently, channeling of the IPA imine only in the presence of the subsequent enzyme of the pathway would be an elegant solution to the metabolic problem. However, characterization of such a type of transient interaction might require the trapping of the overall VioA protein dynamics. With respect to this hypothesis, the conformational rearrangements of VioA might also be relevant for the appropriate timing of the release of the IPA imine reaction product.

The second half-reaction of VioA catalysis is responsible for the reoxidation of the prosthetic group by O2. For this purpose, a smaller entry channel gives direct access to the bridging water molecule that links Lys269 to the N5 atom of FADH2 (compare Fig. 5, C and D, and yellow × in Fig. 5I, right). The position of this water molecule (found in both structures) might also represent the site for H2O2 generation as a result of flavin reoxidation. Mutagenesis of Lys269 might indicate almost complete VioA inactivation due to a significant influence on the reductive and also on the oxidative half-reaction of VioA. A related binding position for the oxidative half-reaction of LAO from C. rhodostoma has been also described in the literature (36). Additional experimental work is required to further elucidate the oxidative half-reaction and the precise sequential order of VioA catalysis.

Experimental Procedures

Plasmid Construction and Site-directed Mutagenesis

The vioA gene from C. violaceum ATCC 12472 was amplified from genomic DNA (German Collection of Microorganisms and Cell Cultures (DSMZ)) using primers CAC GGA TCC ATG AAG CAT TCT TCC GAT ATC and GTA CTC GAG TCA CGC GGC GAT CGC CTG C (restriction sites underlined) and cloned into the BamHI and XhoI sites of pGEX-6P-1 (GE Healthcare), yielding the plasmid pGEX-vioA. Mutations in the vioA gene were introduced using the QuikChangeTM site-directed mutagenesis kit (Stratagene) using the following primers: CAC GGA TCC ATG AAG CAT TCT TCC GAT ATC and GTA CTC GAG TCA CGC GGC GAT CGC CTG C (restriction sites underlined). The amplified DNA fragments were cloned into the BamHI and XhoI sites of pGEX-6P-1 (GE Healthcare), yielding the plasmid pGEX-vioA. Mutations in the vioA gene were introduced using the QuikChangeTM site-directed mutagenesis kit (Stratagene) using the following primers: CAC GGA TCC ATG AAG CAT TCT TCC GAT ATC and GTA CTC GAG TCA CGC GGC GAT CGC CTG C (restriction sites underlined).
oligonucleotides (exchanged nucleotides in bold): R64Q, GAG CTG GGC GCG GGG CAG TAC TCC CCG CAG C and GCT GCG GGG AGT AAC CCG CGC CCA GTA C; R645, GAG CTG GGC GCG GGG TCT TAC TCC CCG CAG C and GCT GCG GGG AGT AAC ACC CGC CCA GTA C; H163N, CGA CAT CTT CGG CAA GAA CCC GGA AAT CCA GAG CG and CGC TCT GGA TTT CCG CGA CGA TGT CG; H163A, CGA CAT CTT CGG CAA GAA CCC GGA AAT CCA GAG CG and CGC TCT GGA TTT CCG CGA CGA TGT CG.

**Heterologous Production and Purification of VioA**

Recombinant VioA was produced in *E. coli* BL21 (ADE3) cells containing plasmid pGEX-vioA. An overnight culture was used to inoculate 500 ml of LB medium containing 100 µg ml⁻¹ ampicillin. Protein production was induced at an OD₆₀₀ of ~0.5 by addition of 50 µM isopropyl 1-thio-β-D-galactopyranoside. After 21 h at 25 °C and 180 rpm, cells were harvested by centrifugation (15 min, 3000 g) and washed with 25 ml of buffer 1 (50 mM Tris-HCl, pH 9.0, and 100 mM NaCl), and the vioA gene was added according to the manufacturer’s instructions. Cells were disrupted by a French press cell at 19,200 p.s.i. After 21 h at 25 °C, the cell-free extract was incubated for 2 h with 2 ml of Protino/H₂₃₀₄₁ and protein containing fractions were identified by SDS-PAGE. The target protein was stored in buffer 1 containing 5% (v/v) glycerol at 4 °C or at −20 °C. For subsequent crystallization experiments, VioA purification was performed in the presence of 2 mM DTT.

**Determination of Protein Concentration**

The concentration of purified VioA was determined at 280 nm using an extinction coefficient of ε₂₈₀ = 71,695 M⁻¹ cm⁻¹.

**N-terminal Amino Acid Sequence Determination**

Automated Edman degradation was used to confirm the identity of purified VioA (47–49).

**Thermal Shift Experiments**

The temperature of the unfolding transition midpoint was determined in thermal shift experiments as described elsewhere (50).

**FAD Cofactor Quantification**

The FAD content of purified VioA samples was quantified by UV-visible absorption spectroscopy using an extinction coefficient of ε₅₆₀ = 11,300 M⁻¹ cm⁻¹.

Alternatively, single turnover experiments were performed under anoxic conditions (oxygen partial pressure below 1 ppm, oxygen detector, Coy Laboratories, Grass Lake, MI) to estimate the amount of co-purified FAD. All buffers were prepared by repeat cycles of evaporation and nitrogen flushing. Purified samples of VioA at a concentration of 5 µM were supplemented with increasing concentrations of L-Trp (0–10 µM) in a total volume of 600 µl and incubated for 2 min at 22 °C. The absorption spectrum of all samples was measured using sealed cuvettes (550–250 nm). Employed concentrations of L-Trp where then plotted versus the photometrically determined FAD concentration to estimate the amount of copurified FAD in the original VioA sample.

**Synthesis of VioA Inhibitors and Trp-derivatives, General Remarks**

Commercially available starting material and solvents were purchased from Sigma, Fisher/Acros, Alfa Aesar, or Carl Roth and were used without further purification. Technical solvents were distilled before use. All reactions involving water-sensitive chemicals were carried out in heat gun-dried glass equipment with magnetic stirring under an argon atmosphere. TLC was performed on Polygram® SIL G/UV254 plates (Macherey-Nagel) with detection by UV (254 nm) or by immersion in aqueous solution containing sulfuric acid, ammonium molybdate tetrahydrate, and Cer(IV)sulfate tetrahydrate, followed by heating. Flash chromatography was performed on Silica Gel M60 (0.04–0.063 mm, 230–400 mesh ASTM) (Macherey & Nagel) under nitrogen pressure. NMR spectra were recorded on a Bruker DRX-400 spectrometer (¹H 400 MHz, ¹³C 101 MHz). Chemical shifts are reported in parts per million relative to tetramethylsilane in case of CDCl₃, and MeOD, and relative to trimethylsilylpropanoic acid in case of D₂O, as an internal standard (δ = 0). Coupling constants J are given in Hertz.

**Synthesis of Inhibitors IEA and IAA**

IEA (2-(1H-Indol-3-yl)methyl)prop-2-enolic Acid) (3)—To a stirred solution of ester 2 (750 mg, 3.48 mmol) in 10 ml of MeOH was added slowly a solution of KOH (590 mg, 10.52 mmol) in 3 ml of water and 5 ml of MeOH at 0 °C. The mixture...
was stirred for 30 min with cooling. After stirring for 24 h at room temperature, the solvent was evaporated under reduced pressure. The aqueous phase was acidified with HCl to pH 4 and extracted with ethyl acetate. After drying with MgSO₄ and filtration, the solvent was evaporated under reduced pressure. Purification on a silica gel column (pentane/ethyl acetate 2:1, \( R_f = 0.23 \)) gave the desired product as a brownish solid (541 mg, 2.69 mmol, 77%). \(^1\)H NMR (ppm) (400 MHz, CDCl₃): 11.00 (br.s., 1H), 7.97 (s, 1H), 7.55–7.51 (m, 1H), 7.35 (dd, \( J = 8.1, J' = 0.8, 1H \)), 7.19 (dd, \( J = 8.3, J' = 6.9, J'' = 1.4, 1H \)), 7.11 (dd, \( J = 7.8, J' = 7.1, J'' = 1.0, 1H \)), 7.02 (d, \( J = 2.3, 1H \)), 6.36–6.34 (m, 1H), 5.63–5.60 (m, 1H), 3.79–3.76 (m, 2H). \(^{13}\)C NMR (ppm) (100 MHz, CDCl₃): 172.7 (Cq), 138.9 (Cq), 136.4 (Cq), 134.4 (Cq), 128.0 (Cq), 127.2 (Cq), 122.9 (Cq), 121.2 (Cq), 119.4 (Cq), 119.0 (Cq), 112.7 (Cq), 111.1 (Cq), 27.2 (-CH₃).

**Methyl 3-(1H-Indol-3-yl)-2-methylpropanoate (4)—** Following the general method described by Youn et al. (30), magnesium turnings (180 mg, 7.41 mmol) were added to a solution of \( \text{KOH} \) (750 mg, 13.08 mmol, 0.23) gave the desired product as a brownish solid (541 mg, 2.69 mmol, 77%). \(^1\)H NMR (ppm) (400 MHz, CDCl₃): 11.00 (br.s., 1H), 7.97 (s, 1H), 7.55–7.51 (m, 1H), 7.35 (dd, \( J = 8.1, J' = 0.8, 1H \)), 7.19 (dd, \( J = 8.3, J' = 6.9, J'' = 1.4, 1H \)), 7.11 (dd, \( J = 7.8, J' = 7.1, J'' = 1.0, 1H \)), 7.02 (d, \( J = 2.3, 1H \)), 6.36–6.34 (m, 1H), 5.63–5.60 (m, 1H), 3.79–3.76 (m, 2H). \(^{13}\)C NMR (ppm) (100 MHz, CDCl₃): 172.7 (Cq), 138.9 (Cq), 136.4 (Cq), 134.4 (Cq), 128.0 (Cq), 127.2 (Cq), 122.9 (Cq), 121.2 (Cq), 119.4 (Cq), 119.0 (Cq), 112.7 (Cq), 111.1 (Cq), 27.2 (-CH₃).

**Synthesis of Trp-derivatives 7-Aza-Trp and 5-Fluoro-Trp**

7-Azagramine (7A) was synthesized according to the procedure of Pierce et al. (38). Purification by flash column chromatography (CHCl₃/MeOH/NH₄OH 90:10:1 → 40:10:1) yielded the product as a yellow solid (2.34 g, 13.34 mmol, 79%). Analytical data were in agreement with the literature (38).

5-Fluoroagamine (7F)—A solution of 5-fluoroindole (1.0 g, 7.40 mmol) in 1 ml of AcOH was added to a solution of dimethylyamine (567 mg, 1.42 ml, 12.58 mmol, 40% aqueous solution) and formaldehyde (333 mg, 0.9 ml, 11.09 mmol, 37% aqueous solution) in 5 ml of acetic acid at 0 °C under an argon atmosphere. The mixture was stirred without cooling for 3 h, poured onto 20 ml of ice-water, and adjusted to pH 11 with 2 M NaOH. The resulting suspension was extracted with diethyl ether and dried with MgSO₄, and the solvent was evaporated under reduced pressure. Column chromatography on silica (CHCl₃/MeOH/NH₄OH 90:10:1 → 40:10:1) gave the product (1.33 g, 6.92 mmol, 93%) as a beige-colored solid. \(^1\)H NMR (ppm) (400 MHz, CDCl₃): 8.47 (s, 1H), 7.36–7.31 (m, 1H), 7.25–7.20 (m, 1H), 7.12 (d, \( J = 2.6, 1H \)), 6.95–6.88 (m, 1H), 3.58 (s, 2H), 2.28 (s, 2H). \(^{13}\)C NMR (ppm) (100 MHz, CDCl₃): 157.6 (d, \( J = 135.8, 1H \)), 127.2 (Cq), 126.0 (Cq), 125.1 (Cq), 113.2 (Cq), 79.8 (Cq), 79.6 (Cq), 75.2 (Cq), 65.2 (Cq), 53.9 (Cq), 53.7 (Cq).
(CH\textsubscript{arom}, 128.1 (CH\textsubscript{arom}), 127.5 (CH\textsubscript{arom}, 127.4 (CH\textsubscript{arom}, 123.3 (CH\textsubscript{arom}, 120.2 (C\textsubscript{q}, 115.7 (CH\textsubscript{arom}, 110.8 (C\textsubscript{q}, 66.1 (-CH\textsubscript{\textsuperscript{-}}, 61.0 (-CH\textsubscript{\textsuperscript{2}}, 29.3 (-CH\textsubscript{\textsuperscript{2}}, 14.2 (-CH\textsubscript{\textsuperscript{3}}).

**Deprotection of the Amino Group**

As described by Wartmann et al. (39), the intermediate esters 9A and 9F were dissolved in THF (0.1 m), treated with 2 m HCl (3.5 eq), and stirred for 1 h at room temperature. The reaction mixture was diluted with ethyl acetate and neutralized with saturated aqueous NaHCO\textsubscript{3}. The aqueous phase was extracted three times with ethyl acetate, and the combined organic phases were dried with MgSO\textsubscript{4}. The solvent was evaporated under reduced pressure.

**Ethyl 2-amino-3-(5-fluoro-1H-indol-3-yl)propanoate (10F)**—Following the above described procedure, protected 5-fluorooamino ester 9F (520 mg, 1.26 mmol) gave the desired product after flash column chromatography (DCM/MeOH: 7:1) as a yellow viscous oil (260 mg, 0.62 mmol, 83%). 1H NMR (ppm) (400 MHz, CDCl\textsubscript{3}): 8.19 (s, 1H, 1H), 7.27–7.23 (m, 2H), 7.11 (d, J = 2.4, 1H), 6.93 (dd, J = 9.0, J = 2.4), 4.16 (dd, J = 7.2, J = 2.5, 2H), 3.78 (dd, J = 7.5, J = 5.0, 1H), 3.21 (dd, J = 14.45, J = 4.98, 1H), 3.03 (dd, J = 14.49, J = 7.47, 1H), 1.25 (t, J = 7.15). 13C NMR (ppm) (100 MHz, CDCl\textsubscript{3}): 175.1 (C\textsubscript{q}, 150.2 (C\textsubscript{arom}), 148.7 (C\textsubscript{q}, 142.8 (CH\textsubscript{arom}), 127.4 (CH\textsubscript{arom}), 123.6 (CH\textsubscript{arom}, 120.2 (C\textsubscript{q}, 115.5 (CH\textsubscript{arom}, 109.7 (C\textsubscript{q}, 61.0 (-CH\textsubscript{\textsuperscript{-}}, 55.1 (-CH\textsubscript{\textsuperscript{2}}, 30.7 (-CH\textsubscript{\textsuperscript{2}}, 14.2 (-CH\textsubscript{\textsuperscript{3}}).

**Saponification to Amino Acids**

To a solution of KOH (3 eq) in MeOH/H\textsubscript{2}O (5:3) was added a solution of the respective ester in MeOH (0.6 m), and the resulting mixture was stirred overnight. MeOH was evaporated under reduced pressure, and the aqueous residue was washed with diethyl ether. The aqueous phase was acidified with AcOH to pH 4–5. Reversed phase column chromatography on RP-18 (100 g, MeOH/H\textsubscript{2}O) gave the desired amino acid.

**2-Amino-3-(5-fluoro-1H-indol-3-yl)propanoic Acid (11F)**—Reaction performed with amino ester 10F (156 mg, 0.623 mmol) yielded the desired product after flash column chromatography on RP-18 (MeOH/H\textsubscript{2}O 60:40 → 50:50) as a slightly yellow solid (84%). 1H NMR (ppm) (400 MHz, D\textsubscript{2}O): 7.51 (dd, J = 8.8, J = 4.6, 1H), 7.44 (dd, J = 10.0, J = 2.2, 1H), 7.13–7.03 (m, 1H), 4.07 (dd, J = 7.6, J = 4.8, 1H), 3.46 (dd, J = 15.2, J = 4.84, 1H), 3.32 (dd, J = 15.4, J = 7.9, 1H). No 13C NMR spectra were recorded due to low solubility.

**VioA Activity Assays**

VioA enzyme kinetics were determined in a coupled peroxidase assay mainly as described previously (7, 9). A reaction mixture of 600 \(\mu\)l containing 50 mM Tris–HCl, pH 9.0, 0.2 \(\mu\)M VioA, 1 mM 4-aminooantipyrine, 1 mM phenol, and 15 units/mL horseradish peroxidase was pre-incubated at 30 °C. Then, VioA catalysis was initiated by the addition of 1 mM L-Trp, and the formation of the red quinoneimine dye was monitored at a wavelength of 505 nm in the linear range of the activity assay (~3 min). The specific activity of VioA was determined by calculation of the slope in the linear range. An extinction coefficient of \(\varepsilon_{505} = 6400 \text{ M}^{-1} \text{cm}^{-1}\) was used to determine the specific activity of VioA.

Alternatively, the activity of VioA was directly measured with an HPLC-based substrate depletion assay as described elsewhere (7). Samples of 100 \(\mu\)l were taken from a reaction mixture (1 ml) containing 50 mM Tris–HCl, pH 9.0, 1 \(\mu\)M VioA, and 500 \(\mu\)M L-Trp at various time points (every 20 s for 3 min). Samples were quenched by the addition of 20 \(\mu\)l of 50% (v/v) trichloroacetic acid and centrifuged for 10 min at 12,100 \(\times\) g, and the supernatant was analyzed on a L-Trp and the VioA reaction product were removed by a buffer exchanged step (Illustra\textsuperscript{TM} NAP\textsuperscript{TM}-5 column Sephadex\textsuperscript{TM}, GE Healthcare, using buffer 1). The residual FAD content of the VioA sample was determined by UV-visible absorption spectroscopy.
Crystal Structure of VioA

Analytic Gel Permeation Chromatography

The native molecular mass of VioA was determined on an Äkta purifier system equipped with a Superdex™ 200 Increase 5/150 GL column (GE Healthcare). The system was equilibrated with buffer 1 or alternatively with buffer 1 containing 500 μM Trp derivative IEA at a flow rate of 0.45 ml min⁻¹. The column was calibrated using protein standards (gel filtration markers kit for protein molecular mass 12,000–200,000 Da, Sigma) according to the manufacturer’s instructions. Samples of VioA (20 μl) at a concentration of 215 μM were injected. Alternatively VioA was pre-incubated in the presence of 500 μM IEA for 3 h. Elution was monitored at 280 and 450 nm, respectively.

Small-angle X-ray Scattering

SAXS experiments were carried out on beamline BM29 (51) of the European Synchrotron Radiation Facility (Grenoble, France) to elucidate the oligomeric architecture of VioA. Samples of VioA (or of VioA in the presence of 1 mM IEA) at concentrations ranging from 10 to 265 μM in buffer 1 were loaded by an automated sample changer to a quartz capillary mounted in vacuum and exposed to an x-ray energy of 12.5 keV. Scattering images covering a momentum transfer range of 0.0025–5 nm⁻¹ were recorded with a Dectris Pilatus 1M hybrid photon counting detector. A total of 10 images were recorded per sample with an exposure time of 1 s per frame. To reduce radiation damage, the protein solution was kept under steady flow while being irradiated. The scattering functions derived from the images were normalized for exposure time and sample transmission and checked for effects of radiation damage in PRIMUS (52). Data unaffected by radiation damage were averaged, corrected for buffer scattering, normalized, and merged from the different concentrations. The comparison of the determined scattering data with the theoretical scattering curves of a VioA monomer or two potential dimers (33) was performed with CRYSOL (53). The radius of gyration (Rg) and the pair distance distribution P(r) were derived with GNOM (54). Twenty models were computed from SAXS data with DAMMIF (55) and used for the construction of a representative ab initio model using DAMAVER and SUPCOMP (56, 57). The models had a mean normalized spatial discrepancy (NSD) of 1.244 and a standard deviation of NSD (σNSD) of 0.189. One of the 20 models showed an NSD of greater than 1.622 (= NSD + 2 × σNSD) and was thus omitted for the calculation of the final ab initio model.

Micro Scale Thermophoresis

VioA-ligand/inhibitor interaction was measured on a MonoLith NT.115 instrument (NanoTemper Technologies, GmbH). VioA (20 μM) was labeled using the MonoLith NT™ protein labeling kit (NT-647-Red-NHS amine reactive fluorescent dye, NanoTemper Technologies, GmbH). The labeled protein was supplemented with 0.05% (w/v) Tween 20 and different concentrations of IAA, IEA, and citrate (30 nM to 20 mM) were titrated, respectively. Thermophoresis was measured in hydrophilic capillaries (NanoTemper Technologies, GmbH) using a laser intensity of 20% for 35 s.

Protein Crystallization

Purified VioA was concentrated to 150–200 μM using a Vivaspin™ 4 centrifugal concentrator (30,000 molecular weight cutoff PES, Sartorius). Crystals were prepared as sitting drops by mixing 1 μl of protein solution with 1 μl of reservoir solution. Native VioA crystals grew at 4 °C from 80 mM Tris-HCl, pH 8.5, 24% (w/v) PEG 4000, 20% (v/v) glycerol after 5 weeks. A heavy metal derivative was produced by soaking VioA crystals with 10 mM Sm(NO₃)₃ for 2 weeks. Co-crystals of VioA and IEA grew after 5 days at 17 °C in sitting drops in the presence of 100 mM HEPES, pH 7.0, 10% PEG 6000, 100 mM lithium chloride, and 3.75 mM IEA. Crystals were cryo-protected in the presence of 20% glycerol (v/v) and flash-cooled in liquid nitrogen.

Data Collection, Structure Determination, and Refinement

Diffraction experiments were performed on the beamline X06DA, operated by the Paul Scherrer Institute at the Swiss Light Source (Villigen, Switzerland), on beamline P11, operated by DESY at PETRA III synchrotron (Hamburg, Germany) (58), and on beamline 14.1, operated by the Helmholtz-Zentrum Berlin at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (59). Data processing was carried out with XDS (60), and the upper resolution limits were assessed through careful observation of I/σI and CC₁/₂ (61). The phase problem was solved by a single-wavelength anomalous diffraction experiment at λ = 1.77 Å using the anomalous signal arising from the proximity of samarium’s L-III absorption edge. ShelxC/D/E (62) was employed to locate heavy metal sites, to compute a first electron density map, and to build an initial structural model. The other crystal forms were phased by molecular replacement, with Phaser (63), using the initial model obtained from the Sm derivative. With subsequent refinement in Phenix.refine (64) and manual rebuilding in Coot (65), the models were finalized. Data collection and refinement statistics are shown in Table 1.

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