Crystallization and preliminary crystallographic analysis of the first condensation domain of viomycin synthetase

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Nonribosomal peptide synthetases (NRPSs) are large multimodular enzymes that synthesize important secondary metabolites such as antibiotics. NRPSs follow a modular synthetic logic whereby each successive amino-acid monomer is added to the peptide chain by successive multi-domain modules. The condensation domain catalyzes the central chemical event in the synthetic cycle, peptide-bond formation, and is present in every elongation module of the NRPS. Viomycin is an antituberculosis nonribosomal peptide that is synthesized by a series of four NRPS proteins and then modified by tailoring proteins. In order to study the mechanisms of peptide-bond formation in viomycin and in NRPSs in general, a structural study of the first condensation domain of the viomycin synthetase protein VioA (VioA-C1) was initiated. The gene for VioA-C1 was cloned from genomic DNA of *Streptomyces vinaceus*, expressed as an octahistidine-tagged construct and purified by column chromatography. VioA-C1 was crystallized using the sitting-drop vapor-diffusion method. X-ray diffraction data were collected on a rotating-anode source to 2.9 Å resolution. The data could be indexed in the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 46.165$, $b = 68.335$, $c = 146.423$ Å. There is likely to be one monomer in the asymmetric unit, giving a solvent content of 49.2% and a Matthews coefficient (V_M) of $2.42 \text{ Å}^3 \text{ Da}^{-1}$. Structural determination is in progress.

1. Introduction

Nonribosomal peptide synthetases (NRPSs) are a family of large multimodular enzymes that synthesize peptide chemicals with interesting and useful biological properties which include antifungals, antibacterials, antivirals, antitumors, siderophores and immunosuppressants (Konz & Marahiel, 1999; Walsh, 2008). NRPSs are organized into modules of ~1100 residues, with each module responsible for the addition of one specific monomer to the peptide product (Strieker *et al.*, 2010). In general, a module consists of three domains that are essential to the elongation of the peptide: the adenylation (A) domain, the peptidyl carrier protein (PCP) domain and the condensation (C) domain. In an elongation cycle, the A domain binds specifically to one monomer, activates it by adenylation and then transfers it onto the 4'-phosphopantetheinyl arm of the PCP domain. The PCP domains of the current and previous modules transport their covalently bound substrates into the active site of the C domain, which catalyzes the formation of a peptide bond between the two substrates (Fig. 1). The elongated molecule is then carried to the C domain in the next module by the current module's PCP domain, to continue this assembly-line elongation.

Although the C domain plays the central chemical role in nonribosomal peptide synthesis and structures of C domains in their unliganded forms have been determined (Keating *et al.*, 2002; Samel *et al.*, 2007; Tanovic *et al.*, 2008), key questions remain unanswered. The C domains possess a highly conserved histidine motif (HHXXXDG) (Marahiel *et al.*, 1997) involved in catalysis, but there is disagreement about the precise enzymatic mechanism. It has been proposed that the second histidine of the motif acts as a general base in the reaction (Bergendahl *et al.*, 2002), but a computational analysis

of the structure of a C domain from tyrocidine synthetase suggests that the pK_a of this residue makes it incapable of acting as a general base, and an electrostatic role was put forward for it instead (Samel *et al.*, 2007). In addition, although A domains are primarily responsible for substrate selection, C domains are also known to display some substrate specificity, the basis of which is unknown (Stachelhaus & Marahiel, 1995; Belshaw *et al.*, 1999; Ehmann *et al.*, 2000; Linne & Marahiel, 2000; Clugston *et al.*, 2003; Kraas *et al.*, 2012). Clarification of the catalytic mechanism and substrate selectivity of C domains would lead to a better fundamental understanding of NRPSs and could potentiate future bioengineering efforts to produce designer peptides from modified NRPS systems.

Viomycin is a cyclic nonribosomal peptide antibiotic in the tuberactinomycin family. These drugs are an important class of antibiotics used specifically against multidrug-resistant tuberculosis (Caminero *et al.*, 2010), some of which are included on the World Health Organization's *Model List of Essential Medicines* (World Health Organization, 2011). Viomycin acts by binding the small subunit of the bacterial ribosome and strongly stabilizes tRNA in the ribosomal A site, which blocks tRNA translocation to the P site, and thus inhibits protein synthesis (Modolell & Vázquez, 1977; Stanley *et al.*, 2010). To study viomycin synthesis and NRPS condensation in general, we initiated structural studies of the first C domain of viomycin synthetase from *Streptomyces vinaceus*, VioA-C1 (Yin *et al.*, 2003).

We cloned, expressed, purified and crystallized VioA-C1. We obtained a diffraction data set which extends to 2.9 Å resolution. Future structure determination of apo and co-complex forms of VioA-C1 should help clarify mechanisms of the key catalytic step for one of nature's most elegant macromolecular machines.

Figure 1
The peptidyl transferase reaction catalyzed by VioA-C1 (Helmetag *et al.*, 2009). The wavy lines represent the 4'-phosphopantetheinyl arms of the PCP domains.

2. Materials and methods

2.1. Cloning and expression

VioA-C1 was cloned from the gene *vioA* (European Nucleotide Archive accession number AAP92491.1) in *S. vinaceus* genomic DNA obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA; ATCC number 11861) using primers VioAC1-F (5'-AAAAAATCATGAGAACCGCGGAAGGCG-AGCTGTC-3') and VioAC1-R (5'-AAAAAATCGAGTTAGGACAGCAGAGGCAGCCGGG-3'). These primers contain *Bsp*HI and *Xho*I restriction sites, respectively, and allow placement into a pET28-derived vector featuring an N-terminal octahistidine tag and tobacco etch virus (TEV) protease cleavage site of sequence MHHHHHHHPDLGTGSENLYFQGAMR to give the expression vector pHTVioAC1. Protein production was performed in Rosetta2 (DE3) *Escherichia coli* (EMD Millipore, Billerica, Massachusetts, USA) cells grown in 6 l of LB medium supplemented by 300 µg ml⁻¹ kanamycin and 340 µg ml⁻¹ chloramphenicol. VioA-C1 expression was induced at an OD₆₀₀ of ~0.4–0.6 with the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and expression continued for 5 h at 295 K. Cells were then harvested and stored at 193 K.

2.2. Protein purification

VioA-C1 cell pellets were resuspended in a buffer consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol, 1 mM imidazole pH 8.0, 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were lysed using an EmulsiFlex-C3 homogenizer (Avestin Inc., Canada) and centrifuged for 30 min at 40 000g and 277 K. The resulting supernatant was pooled and applied onto a 5 ml HiTrap IMAC FF column (GE Healthcare) charged with Ni²⁺. VioA-C1 was eluted using a buffer consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol, 250 mM imidazole pH 8.0, 1 mM PMSF; the purity was assessed using SDS-PAGE.

The fractions containing VioA-C1 were pooled and digested overnight with TEV protease, using a ratio of 1 mg TEV protease per 40 mg VioA-C1, while dialysed against a buffer consisting of 50 mM

Figure 2
12% SDS-PAGE used to assess the purity of VioA-C1. Molecular-weight markers are present in the left lane, labelled in kDa.

Tris–HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol (dialysis buffer). Cleavage with TEV protease results in a remnant sequence of GAMR on the N-terminus of VioA-C1. The sample was reapplied onto the 5 ml HiTrap IMAC FF column charged with Ni^{2+} and the flowthrough was collected and pooled. The sample was concentrated and applied onto a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare) equilibrated in dialysis buffer. VioA-C1-containing fractions were pooled and diluted 1:1 with 50 mM Tris–HCl pH 7.5 and 2 mM β -mercaptoethanol for anion-exchange chromatography with a MonoQ 5/50 GL column (GE Healthcare). VioA-C1 was eluted from the MonoQ column by a gradient of 0–1 M NaCl. High- and low-salt MonoQ buffers also contained 50 mM Tris–HCl pH 7.5 and 2 mM β -mercaptoethanol. Finally, pure VioA-C1 (Fig. 2) was placed into a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol, by serial dilution and concentration using an Amicon Ultra 10 K centrifugal filter device (EMD Millipore, Billerica, Massachusetts, USA). At a concentration of 10 mg ml^{−1}, VioA-C1 was flash-frozen in liquid nitrogen and stored at 193 K.

2.3. Crystallization

Sparse-matrix crystallization trials of VioA-C1 were performed using the sitting-drop vapor-diffusion method with the aid of a Phoenix crystallization robot (Art Robbins, Sunnyvale, California, USA) in Intelli-Plates 96 (Art Robbins, Sunnyvale, California, USA). Crystallization drops with 0.2 μ l of 5 or 10 mg ml^{−1} protein sample and 0.2 μ l crystallization solution were equilibrated against 75 μ l reservoir volume. Crystallization solutions from the PACT, Classics, Classics II, PEGs and JCSG+ crystallization suites (Qiagen, Germantown, Maryland, USA) were assayed for promotion of crystal growth at temperatures of 277 and 295 K.

Initial crystallization screens yielded small, fragile needles growing in heavy precipitate from an experiment using 0.2 M magnesium chloride, 0.1 M HEPES pH 7.5, 25% (w/v) PEG 3350 as the crystallization solution. Subsequent optimization was performed in larger format 24-well sitting-drop Cryschem Plates (Hampton Research, Aliso Viejo, California, USA) by manual two-dimensional grid screens around the initial conditions. In these screens, the drop contained 2 μ l of 10 mg ml^{−1} protein sample and 2 μ l of crystallization solution, and was equilibrated against a 400 μ l reservoir of crystallization solution. Further optimization was done with multiple

Figure 3

A drop containing heavy precipitate and crystals of VioA-C1. The largest crystals are approximately 400 × 30 × 10 μ m. Arrows indicate the positions of crystals.

screenings of the Hampton Additive Screen (Hampton Research, Aliso Viejo, California). Crystals suitable for data collection were grown using a crystallization solution of 11–16% PEG 3350, 0.2 M ammonium acetate, 0.1 M HEPES pH 7.5, 0.01–0.025 mM MnCl_2 at 277 K. VioA-C1 crystals grew to a maximum size of approximately 400 × 30 × 10 μ m and grew only out of drops containing heavy precipitate (Fig. 3). Crystals were cryoprotected by adding 25% PEG 400 to the equilibrated crystal drop, mounting them in cryoloops and flash-cooling in liquid nitrogen.

2.4. Data collection and processing

Diffraction data were collected using a Rigaku rotating copper-anode generator fitted with Osmic confocal optics and an R-Axis IV⁺⁺ image-plate detector (Rigaku, Tokyo, Japan) at the Biochemistry Department of McGill University, Montreal, Canada. A 98° data set was collected with an image oscillation width of 1°, an exposure time of 10 min per image and a crystal-to-detector distance of 230 mm (Fig. 4).

The raw data were processed using the program *HKL-2000* (Otwinowski & Minor, 1997). The data were indexed in the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 46.165$, $b = 68.335$, $c = 146.423$ Å. The statistics for data collection and processing are listed in Table 1. Calculations reveal that there is likely to be one monomer in the asymmetric unit, which would give a solvent content of 49.2% and a Matthews coefficient (V_M ; Matthews, 1968) of 2.42 Å³ Da^{−1}.

3. Results and discussion

VioA-C1 from *S. vinaceus* was heterologously expressed in Rosetta2 (DE3) *E. coli* as an octahistidine-tagged construct and purified to homogeneity by a four-step purification consisting of immobilized metal-affinity chromatography, reverse immobilized metal-affinity

Figure 4

An X-ray diffraction image from a crystal of VioA-C1. The crystal-to-detector distance was 230 mm; the exposure time was 10 min per image.

Table 1

Data-collection and processing statistics.

Values in parentheses are for the highest-resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 46.165$, $b = 68.335$, $c = 146.423$
Wavelength (Å)	1.5418
Resolution (Å)	50.00–2.86 (2.91–2.86)
No. of unique reflections	11209 (527)
No. of observed reflections	39463
Completeness (%)	99.3 (98.7)
Multiplicity	3.5 (3.5)
$R_{\text{merge}}^{\dagger}$ (%)	16.0 (67.1)
$\langle I/\sigma(I) \rangle$	7.0 (1.6)
Temperature (K)	100
Matthews coefficient (Å ³ Da ^{−1})	2.42
Solvent content (%)	49.2
No. of molecules in the asymmetric unit	1

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ represents the intensity of the i th measurement and $\langle I(hkl) \rangle$ represents the average intensity of reflection hkl .

chromatography, size-exclusion chromatography and anion-exchange chromatography. Each litre of bacterial culture yielded approximately 2.1 mg of pure VioA-C1. Crystallization conditions obtained from initial screens were optimized using sitting-drop vapor-diffusion experiments: 2 µl of 10 mg ml^{−1} VioA-C1 in a buffer of 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol were mixed with 2 µl reservoir solution and equilibrated against 400 µl reservoir solution. The best diffracting crystals grew in 11–16% PEG 3350, 0.2 M ammonium acetate, 0.1 M HEPES pH 7.5, 0.01–0.025 mM MnCl₂, at a temperature of 277 K. This condition produced drops with both crystals and heavy precipitate, but attempts to grow crystals in conditions without precipitate using micro- or macroseeding did not succeed. Diffraction data were collected with the in-house X-ray source and the crystal diffracted to a resolution of 2.86 Å with an outer shell $\langle I/\sigma(I) \rangle$ of 1.6 [or 2.96 Å with an $\langle I/\sigma(I) \rangle$ of 2]. Indexing of the data indicated that the crystals belonged to the orthorhombic space group $P2_12_12_1$ with unit-cell parameters $a = 46.165$, $b = 68.335$, $c = 146.423$ Å. The Matthews coefficient calculation for one monomer in the asymmetric unit is 2.42 Å³ Da^{−1}, making it likely that there is one monomer in the asymmetric unit and a solvent content of 49.2%.

There are structures available for C domains from tyrocidine synthetase III (PDB entry 2jgp, sequence identity with VioA-C1 of 25%; Samel *et al.*, 2007), vibriobactin synthase (PDB entry 115a, 17% sequence identity; Keating *et al.*, 2002) and surfactin A synthetase C (PDB entry 2vsq, 20% sequence identity; Tanovic *et al.*, 2008). Thus far we have been unable to determine the structure of VioA-C1 using molecular-replacement phasing, despite trials with all three full C

domain structures and multiple subdomain models of them as search models. The lack of success is likely due to conformational changes between and within the N- and C-terminal subdomains of the C domains, which can be observed in the published C domain structures, as well as fairly low sequence conservation between C domains. We have grown crystals of selenomethionine-derivatized VioA-C1 for structure determination with multiple- or single-wavelength anomalous dispersion phasing techniques.

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References

- Belshaw, P. J., Walsh, C. T. & Stachelhaus, T. (1999). *Science*, **284**, 486–489.
- Bergendahl, V., Linne, U. & Marahiel, M. A. (2002). *Eur. J. Biochem.* **269**, 620–629.
- Caminero, J. A., Sotgiu, G., Zumla, A. & Migliori, G. B. (2010). *Lancet Infect. Dis.* **10**, 621–629.
- Clugston, S. L., Sieber, S. A., Marahiel, M. A. & Walsh, C. T. (2003). *Biochemistry*, **42**, 12095–12104.
- Ehmann, D. E., Shaw-Reid, C. A., Losey, H. C. & Walsh, C. T. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 2509–2514.
- Helmetag, V., Samel, S. A., Thomas, M. G., Marahiel, M. A. & Essen, L. O. (2009). *FEBS J.* **276**, 3669–3682.
- Keating, T. A., Marshall, C. G., Walsh, C. T. & Keating, A. E. (2002). *Nature Struct. Biol.* **9**, 522–526.
- Konz, D. & Marahiel, M. A. (1999). *Chem. Biol.* **6**, R39–R48.
- Kraas, F. I., Giessen, T. W. & Marahiel, M. A. (2012). *FEBS Lett.* **586**, 283–288.
- Linne, U. & Marahiel, M. A. (2000). *Biochemistry*, **39**, 10439–10447.
- Marahiel, M. A., Stachelhaus, T. & Mootz, H. D. (1997). *Chem. Rev.* **97**, 2651–2674.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Modolell, J. & Vázquez, D. (1977). *Eur. J. Biochem.* **81**, 491–497.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Samel, S. A., Schoenafinger, G., Knappe, T. A., Marahiel, M. A. & Essen, L. O. (2007). *Structure*, **15**, 781–792.
- Stachelhaus, T. & Marahiel, M. A. (1995). *J. Biol. Chem.* **270**, 6163–6169.
- Stanley, R. E., Blaha, G., Grodzicki, R. L., Strickler, M. D. & Steitz, T. A. (2010). *Nature Struct. Mol. Biol.* **17**, 289–293.
- Strieker, M., Tanović, A. & Marahiel, M. A. (2010). *Curr. Opin. Struct. Biol.* **20**, 234–240.
- Tanovic, A., Samel, S. A., Essen, L. O. & Marahiel, M. A. (2008). *Science*, **321**, 659–663.
- Walsh, C. T. (2008). *Acc. Chem. Res.* **41**, 4–10.
- World Health Organization (2011). *WHO Model List of Essential Medicines*, 17th list. Geneva: WHO.
- Yin, X., O'Hare, T., Gould, S. J. & Zabriskie, T. M. (2003). *Gene*, **312**, 215–224.