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Crystallization and preliminary crystallographic data of SnoaL, a polyketide cyclase in nogalamycin biosynthesis

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Nogalonic acid methyl ester cyclase (SnoaL) catalyzes the last ring-closure step in the biosynthesis of the polyketide antibiotic nogalamycin. Crystals of a complex of SnoaL with the substrate nogalonic acid methyl ester have been obtained using PEG 4000 as precipitant. The crystals are orthorhombic, space group $I222$, with unit-cell parameters a = 69.1, b = 72.0, c = 65.4 Å. They diffract to 1.35 Å resolution using synchrotron radiation. A Matthews coefficient of 2.0 Å^3 Da^{-1} suggests one subunit in the asymmetric unit. Diffraction data for an isomorphous uranium derivative were collected and a difference Patterson map showed strong peaks which allowed determination of the position of the uranium ions.

1. Introduction

Nogalamycin is a polyketide antibiotic produced by *Streptomyces nogalater* (Wiley *et al.*, 1977). This compound belongs to the group of glycosylated aromatic secondary metabolites denoted as anthracyclines, which provide several of the most common antitumour agents presently used in chemotherapy (Grein, 1987). Anthracycline antibiotics show severe side effects such as cardiotoxicity, which limit their clinical use (Myers *et al.*, 1988). Because of the structural complexity of these compounds, competitive chemical synthesis is not possible at present, but combinatorial biosynthesis appears to be a promising route towards novel anthracyclines with less severe side effects. Consequently, the genes and enzymes of the biosynthetic pathways of anthracyclines in *Streptomyces* have attracted considerable interest (for reviews, see Hutchinson, 1997; Hopwood, 1981, 1997). So far, a number of novel anthracyclines have been produced using gene transfer of biosynthetic enzymes between various *Streptomyces* strains (Hopwood *et al.*, 1985; Niemi *et al.*, 1994; Ylihonko, Hakala *et al.*, 1996; Metsä-Ketelä *et al.*, 2003).

The gene cluster for nogalamycin biosynthesis has been cloned and characterized (Ylihonko, Tuikkanen *et al.*, 1996; Torkkell *et al.*, 1997). The biosynthesis of this metabolite starts from acetate and elongation with nine additional acetate units *via* a polyketide synthase results in a linear polyketide chain of ten such building blocks (Wiley *et al.*, 1978). The chain undergoes subsequent cyclization steps leading to the formation of the metabolic intermediate nogalamycinone (Torkkell *et al.*, 2000). This polyketide aglycone is then further modified by tailoring enzymes through hydroxylation and glycosylation. Nogalamycin is structurally distinct from most other anthracyclines through (i) having a difference in its glycosylation profile, (ii) having a methyl rather than an ethyl substituent at the C-9 position and (iii) having opposite stereochemistry at C-9 of ring *A* of the polyketide aglycon (Wiley *et al.*, 1977; Arora, 1983) (Fig. 1).

The difference in stereochemistry at the C-9 position reflects the stereospecificity of the cyclase catalysing the last cyclization step that leads to the formation of the aromatic multicyclic ring system. Final ring closure in nogalamycin biosynthesis is catalyzed by a small enzyme, SnoaL, consisting of 144 amino acids per subunit (Torkkell *et al.*, 2000). This enzyme is homologous to other cyclases, which catalyze the last ring-closure step leading to the formation of the aklavinone skeleton; for instance, DrnD and DauD in *S. peucetius* and *Streptomyces* sp. strain C5, respectively (Dickens & Strohl, 1995; Madduri & Hutchinson, 1995; Kendrew *et al.*, 1999), RdmA in *S. purpurascens* (Niemi & Mäntsälä, 1995) and AknH in *S. galileus* (Räty *et al.*, 2002). SnoaL is 60–70% identical in amino-acid sequence to these other members of this enzyme family; however, it is unique in that the product of cyclization differs in stereochemistry at the C-9 atom from most other anthracyclines: it is 9*S* rather than 9*R* (Torkkell *et al.*, 2000) (Fig. 1).

The reaction catalysed by these enzymes is an aldol condensation (Fig. 1). However, these polyketide cyclases do not appear to use a Schiff-base mechanism as class I aldolases do, nor cofactors such as metal ions as class II aldolases do for stabilization of the enol(ate) intermediate (Kendrew *et al.*, 1999) and thus may represent a novel mechanistic solution to enzymatic carbon–carbon bond formation *via* aldol condensation. The three-dimensional

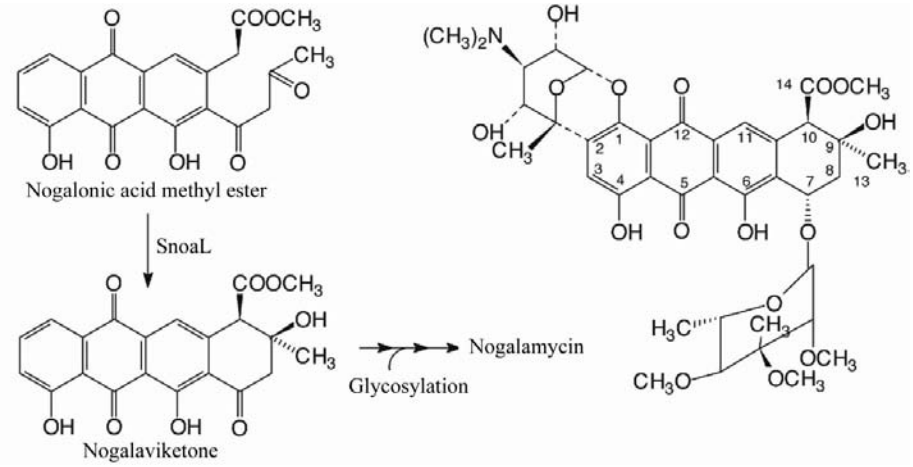

Figure 1
Reaction catalyzed by SnoaL, the formation of nogalaviketone from nogalonic acid methyl ester, in nogalamycin biosynthesis.

Table 1
Statistics of data collection for SnoaL.

Values in parentheses are for the highest resolution shell.

Parameters	SnoaL (low-pH)	SnoaL (high-pH)	SnoaL (U derivative)
Beamline	B711	ID14 EH-1	B711
Wavelength (Å)	1.099	0.934	1.099
Resolution (Å)	28.63–1.87	36.03–1.35	28.65–2.64
Space group	*I*222	*I*222	*I*222
Unit-cell parameters			
a (Å)	68.3	69.1	68.4
b (Å)	72.3	72.0	72.4
c (Å)	64.5	65.4	64.5
No. observations	48371	402458	18134
No. unique reflections	12194	35447	4771
R_{sym} (%)	6.6 (23.2)	8.5 (48.5)	5.3 (7.6)
Completeness (%)	99.6 (99.6)	98.5 (98.5)	97.0 (97.0)
Multiplicity (%)	3.8 (3.8)	11.3 (8.3)	3.8 (3.7)
Average $I/\sigma(I)$	20.2 (4.8)	23.3 (3.4)	23.7 (10.9)
Wilson *B* factor (Å^2)	24.3	16.6	54.1

structure of these cyclases or any sequence homologue is unknown. As part of our interest in the structural biology of anthracycline biosynthesis, we set out to crystallize and determine the structure of a representative of this polyketide cyclase family by protein crystallography. Here, we describe the crystallization and preliminary analysis of crystals of nogalonic acid methyl ester cyclase, SnoaL, from *S. nogalater* involved in nogalamycin biosynthesis.

2. Materials and methods

2.1. Protein production and crystallization

SnoaL was produced in *Escherichia coli* with an N-terminal 6×His tag attached to the SnoaL polypeptide chain *via* a 29-amino-acid linker. Attempts to obtain soluble active native enzyme, *i.e.* without any fusion peptide, were not successful. In short, the gene coding for SnoaL was inserted into the pBAD/hisB expression plasmid (Invitrogen) at the *Bgl*II/*Hin*dIII site located downstream from the coding region for an N-terminal His_6 tag. This construct results in a fusion polypeptide consisting of 178 amino-acid residues, 143 of which are of the cyclase (the N-terminal methionine was removed by the cloning procedure) and 35 of which are of the N-terminal fusion peptide, including the hexahistidine tag. The sequence of this N-terminal fusion peptide is

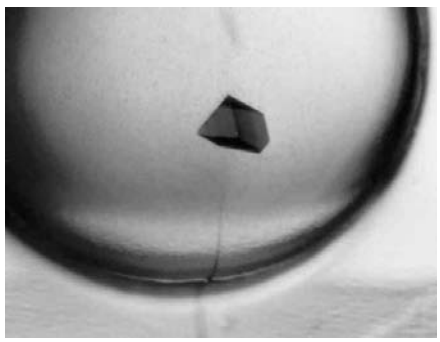

Figure 2
Low-pH crystal of SnoaL. The size of the crystal is approximately 0.2 × 0.1 × 0.1 mm.

GGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPSSR.

The construct was expressed in *E. coli* strain TOP 10 at 303 K. The protein was purified in three consecutive steps by Ni^{2+}-affinity chromatography, gel-filtration and anion-exchange chromatography. The purity of the samples was verified by native and SDS gel electrophoresis. Mass-spectrometric analysis of the purified protein gives a value of 20 472 Da, very close to the expected molecular weight of 20 471 Da for this construct. Samples of the substrate, nogalonic acid methyl ester, were kindly provided by Dr Tero Kunnari. Gel-filtration chromatography suggests a tetramer of SnoaL in solution (data not shown).

A solution of SnoaL in 25 m*M* Tris buffer pH 8.5 was incubated on ice for 1 h with the substrate nogalonic acid methyl ester in a 1:8 ratio. After incubation, the solution was concentrated to a protein concentration of 7.5 mg ml^{-1} and a final substrate concentration of 3 m*M*. Crystallization trials were performed with this solution at 293 K using the hanging-drop vapour-diffusion technique. Hampton Research Crystal Screen kits I and II were used for initial screening. 4 µl droplets consisting of equal volumes of protein solution and mother liquor were allowed to equilibrate with 1 ml of reservoir solution. Further optimizations were not required because high-quality crystals were obtained directly from some of the screening conditions.

2.2. Data collection and processing

Native SnoaL crystals were soaked in a cryoprotectant solution consisting of 25% PEG 400 (polyethylene glycol) for 30 s and then flash-frozen at 100 K by transfer into a gaseous nitrogen stream. The X-ray diffraction data (low-pH form) were collected at a wavelength of 1.099 Å at beamline B711, Max II Laboratory, Lund, Sweden. The crystal-to-detector distance was 120 mm and the oscillation angle was 0.8°. Data from the crystals obtained at high pH were collected at a wavelength of 0.934 Å at beamline ID14 EH-1, European Synchrotron Radiation Facility (ESRF), Grenoble, France using an oscillation angle of 1° and a crystal-to-detector distance of 120 mm. Prior to data collection, crystals were soaked in a solution of 25% PEG 400 for 30 s and flash-frozen at 100 K in the cryostream.

For the preparation of a heavy-atom derivative of SnoaL, the crystals were soaked in 1 m*M* uranium acetate solution for 30 min, quickly transferred into a solution of 25% PEG 400 as cryoprotectant and

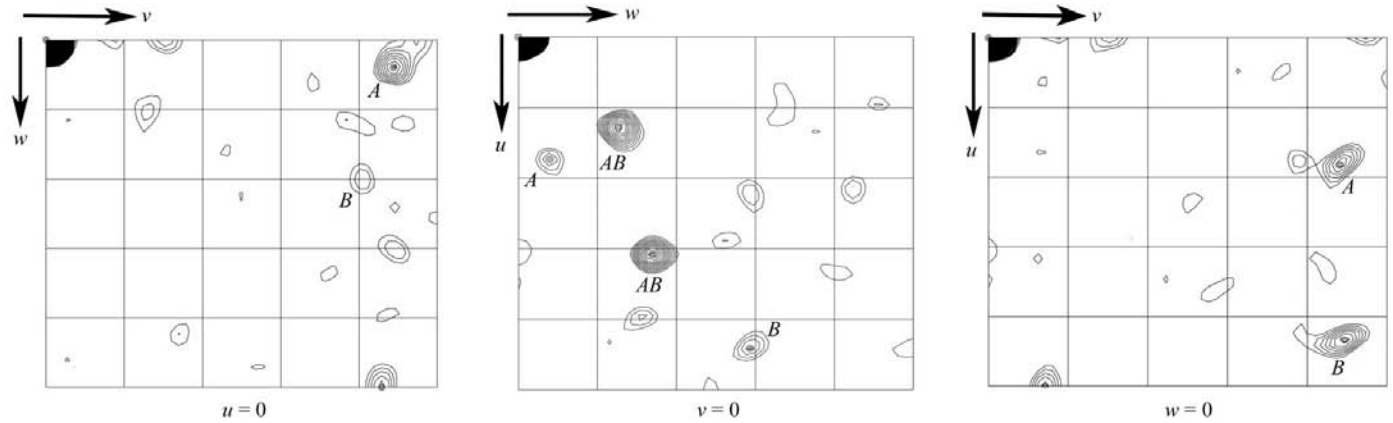

Figure 3
Harker sections of the difference Patterson map for the uranium derivative of SnoaL calculated at 2.6 Å resolution. The sections are contoured starting at 1.5σ and increasing in 0.5σ. Peaks *A* and *B* are U—U self-vectors corresponding to two independent uranium sites in the asymmetric unit and the peaks denoted *AB* are cross-vectors.

then flash-frozen at 100 K. Data were collected at beamline B711, Max II Laboratory, Lund, Sweden with a crystal-to-detector distance of 120 mm and an oscillation angle of 0.8°.

All images were processed using *MOSFLM* (Collaborative Computational Project, Number 4, 1994) and the data were scaled using the program *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The space group and unit-cell parameters were determined using the autoindexing option in *MOSFLM* and pseudo-precession images were generated with the program *PATTERN* (Lu, 1999).

3. Results

Square-shaped crystals of SnoaL with maximum dimensions of 0.2 × 0.1 × 0.1 mm (Fig. 2) were obtained using a crystallization solution consisting of 0.1 *M* sodium acetate pH 4.6 and 8%(*w*/*v*) PEG 4000. The crystals had a magenta/red colour (Fig. 2) indicating the binding of substrate and diffracted to 1.87 Å resolution using synchrotron radiation (Table 1). Analysis of the diffraction pattern suggested that the crystals belong to the orthorhombic space group *I*222, with unit-cell parameters a = 68.3, b = 72.3, c = 64.5 Å. The Matthews coefficient calculated for one molecule in the asymmetric unit was 2.0 Å^3 Da^{-1}, corresponding to a solvent content of 37.2%. This value strongly suggests that the asymmetric unit contains one subunit of SnoaL, since two or more subunits would result in an unreasonable packing density.

Crystals of SnoaL were also obtained at high pH. These crystals are more needle-shaped and were grown from a solution containing 0.2 *M* sodium acetate, 0.1 *M* Tris buffer pH 8.5 and 30%(*w*/*v*) PEG 4000. The magenta/red-coloured crystals diffracted to 1.35 Å resolution at beamline ID 14, ESRF. They also belong to space group *I*222, with unit-cell parameters a = 69.1, b = 72.0, c = 65.4 Å.

While production of selenomethionine-substituted proteins was in progress, we identified a heavy-metal derivative by soaking the low-pH crystals in a 1 m*M* solution of uranium acetate. A data set from these crystals was collected to 2.64 Å resolution and these crystals were isomorphous to the native crystals, with unit-cell parameters a = 68.4, b = 72.4, c = 64.5 Å (Table 1).

After data processing and scaling, difference Patterson maps were calculated using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The Harker sections for space group *I*222 show clear peaks (Fig. 3), indicating the presence of bound heavy-metal ions. The positions of the heavy-metal ions were determined and the parameters were refined using the program *SOLVE* (Terwilliger & Berendzen, 1999). The figure of merit after refinement was 0.63. Tracing of the polypeptide chain is now in progress.

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