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Expression, purification and crystallization of a fungal type III polyketide synthase that produces the csypyrone

CsyB from *Aspergillus oryzae* is a novel type III polyketide synthase that catalyzes the formation of csypyrone B1 [4-(3-acetyl-4-hydroxy-2-oxo-2H-pyran-6-yl)butyric acid] from fatty acyl-CoA, malonyl-CoA and acetoacetyl-CoA. Recombinant CsyB expressed in *Escherichia coli* was crystallized by the sitting-drop vapour-diffusion method. The crystals belonged to space $P2_1$, with unit-cell parameters $a = 70.0$, $b = 104.8$, $c = 73.5$ Å, $\beta = 114.4^\circ$.

1. Introduction

The chalcone synthase (CHS; EC 2.3.1.74) superfamily of type III polyketide synthases (PKSs) is structurally and mechanistically distinct from the modular type I and the dissociated type II PKSs. These 40–45 kDa enzymes, which form simple homodimers, catalyze sequential decarboxylative condensations of malonyl-CoA with an aromatic or aliphatic CoA-linked starter molecule without using the acyl carrier protein to produce a variety of biologically active secondary metabolites (Schröder, 1999; Austin & Noel, 2003). Although type III PKSs are mainly distributed in plants, they are also found in microbes, not only in bacteria but also in eukaryotic microbes, including fungi.

CsyB from *Aspergillus oryzae* is a unique type III PKS that is involved in the biosynthesis of csypyrone B1. The enzyme shares 37% identity with *Neurospora crassa* 2'-oxoalkylresorcylic acid synthase (NcORAS) and 45% identity with *A. oryzae* CsyA, which produce pentaketide alkylresorcylic acid (Funa *et al.*, 2007) and dihydroxybenzoic acid (Seshime, Juvvadi, Kitamoto, Ebizuka & Fujii, 2010), respectively. A feeding experiment with [1,2-¹³C₂]-labelled acetate revealed that csypyrone B1 consists of two independent polyketide chains (Seshime, Juvvadi, Kitamoto, Ebizuka & Fujii, 2010). Thus, two distinct catalytic mechanisms of CsyB including a unique coupling reaction of the two independent polyketide chains have been proposed (Fig. 1; Seshime, Juvvadi, Kitamoto, Ebizuka & Fujii, 2010; Hashimoto *et al.*, 2013). One is that CsyB catalyzes the formation of 3-oxohexanoyl-CoA and acetoacetyl-CoA from decarboxylative condensation of malonyl-CoA with butyryl-CoA and acetyl-CoA, respectively, and then accepts them as the substrates to produce a precursor of csypyrone B1 through the formation of a branched linear intermediate and a pyrone-ring cyclization reaction. After this, the precursor would likely be oxidized by the host fungus to form csypyrone B1. Another proposed mechanism is the one-pot formation of csypyrone B1 from condensation of 3-oxoadipyl-CoA and acetoacetyl-CoA derived from decarboxylative condensation of malonyl-CoA with succinyl-CoA and acetyl-CoA, respectively, through the formation of a branched linear intermediate and a pyrone-ring cyclization reaction.

To clarify the CsyB-catalyzed processes from the point of view of the three-dimensional structure and to elucidate the structure–function relationship of the functionally diverse type III PKS enzymes, we expressed hexahistidine (His₆)-fused recombinant *A. oryzae* CsyB in *Escherichia coli* and obtained good-quality crystals from the His₆-tag-fused recombinant CsyB.

Figure 1
Proposed mechanism for the formation of csypyrone B1 by CsyB.

2. Experimental

2.1. Expression and purification

The cDNA encoding full-length CsyB from *A. oryzae* was purchased from Operon Biotechnologies Inc. and was used as the template to amplify the CsyB gene by PCR using 5'-CCTCATATG-ATCGAACCGTTACCGAC-3' as the sense primer, which introduces an *NdeI* restriction site, and 5'-AATGCGGCCGCTTATGCGTG-CAGATACGAGC-3' as the antisense primer, which introduces a *NotI* restriction site. The amplified DNA fragment was digested with *NdeI/NotI* and ligated into the *NdeI/NotI* sites of the pET-28a vector (Merck Millipore) for expression as a fusion protein with a His₆ tag at the N-terminus.

After confirmation of the sequence, the pET-28a vector encoding full-length CsyB was transformed into *Escherichia coli* BLR (DE3). The cells harbouring the plasmid were cultured to an OD₆₀₀ of 0.6 in LB medium containing 50 µg ml⁻¹ kanamycin at 310 K. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 0.5 mM to induce gene expression and the culture was incubated for a further 16 h at 293 K.

All of the following procedures were performed at 277 K. The *E. coli* cells were harvested by centrifugation at 5000g and resuspended in 50 mM Tris-HCl buffer pH 7.5 containing 250 mM NaCl, 5% (v/v) glycerol, 5 mM imidazole (buffer A). The cells were disrupted by sonication and the lysate was centrifuged at 12 000g for 30 min. The supernatant was loaded onto COSMOGEL His-Accept (Nacalai Tesque) equilibrated with buffer A. After washing the resin with buffer A containing 10 mM imidazole, the recombinant CsyB protein was subsequently eluted with buffer A containing 300 mM imidazole. The protein solution was concentrated to 5 ml, further purified to homogeneity by gel-filtration chromatography on HiLoad 16/60 Superdex 200 pg (GE Healthcare) and concentrated to 13 mg ml⁻¹ in 20 mM Tris-HCl buffer pH 7.5 containing 150 mM NaCl, 2 mM DTT.

A dynamic light-scattering (DLS) analysis was performed using a DynaPro-MSXTC molecular-sizing instrument (Protein Solutions). After centrifugation through a 0.22 µm Ultrafree-MC filter (Millipore) to remove particulate material from the protein solution, the solution properties of the purified protein were monitored. Data were acquired as 50 scattering measurements at 293 K and data from four sets of measurements were analyzed using the DYNAMICS software package (Protein Solutions) and averaged.

2.2. Crystallization and X-ray data collection

Initial crystallization attempts were carried out at 293 K using the sitting-drop vapour-diffusion method with a 96-condition crystallization screen originally designed by Mitsubishi Chemical Corporation. Single crystals were observed in a crystallization condition consisting of 100 mM PIPES pH 6.5, 4% (w/v) PEG 4000, 200 mM LiCl. Thus, cocrystallization attempts with CoA-SH, a common byproduct released from the substrates during the iterative condensation reactions of type III PKSs, were then carried out. Finally, diffraction-quality crystals were obtained at 293 K using 100 mM PIPES pH 6.5, 4% (w/v) PEG 4000, 200 mM LiCl, 2 mM CoA-SH by the sitting-drop vapour-diffusion method. The optimized crystallization drops were prepared by mixing 0.5 µl protein solution and an equal volume of reservoir solution and were equilibrated against 50 µl reservoir solution.

Figure 2
Purification of CsyB by gel-filtration chromatography. Peak 1 represents CsyB. Inset, SDS-PAGE gel (10%) for peak 1. The approximate molecular weight of CsyB is 45 kDa.

Figure 3
Crystals of CsyB. The dimensions of the crystals were approximately $0.1 \times 0.1 \times 0.02$ mm.

The crystals were transferred into a cryoprotectant consisting of 100 mM PIPES pH 6.5, 4%(w/v) PEG 4000, 200 mM LiCl, 2 mM CoA-SH, 20%(v/v) glycerol. After a few seconds, the crystals were picked up in a nylon loop and then flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data sets were collected on beamline BL-17A of the Photon Factory (PF; wavelength 0.9800 Å) using an ADSC Quantum 270 detector with a distance of 188 mm between the crystal and the detector. A total 360 frames were recorded with a 0.5° oscillation angle and 1 s exposure time. The data were indexed, integrated and scaled with the *XDS* program package (Kabsch, 2010).

3. Results and discussion

The recombinant CsyB protein was expressed in *E. coli*. The purified His₆-tag-fused CsyB protein migrated as a single band with a molecular mass of 45 kDa on SDS-PAGE, which agrees well with the calculated value of 45.2 kDa (Fig. 2). In contrast, the recombinant

Figure 4
Self-rotation function calculated from diffraction data of CsyB at $\kappa = 180^\circ$. The horizontal and vertical polar coordinates of the rotation axis are ω (0 – 90° from the centre to the edge of the circle) and ϕ (plotted around the circumference as shown in the figure). The peaks at $\phi = 90$ and -90° on the circumference arise from the crystallographic twofold axis of the space group.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	BL17-A, PF
Wavelength (Å)	0.9800
Space group	$P2_1$
Unit-cell parameters	
a (Å)	70.0
b (Å)	104.8
c (Å)	73.5
β ($^\circ$)	114.4
Resolution (Å)	50.00–1.71 (1.81–1.71)
Total reflections	388694 (62153)
Unique reflections	101115 (16084)
Multiplicity	3.8 (3.9)
Completeness (%)	96.3 (95.3)
$\langle I/\sigma(I) \rangle$	17.3 (5.3)
$R_{\text{merge}}^\dagger$ (%)	5.2 (26.3)
Molecules per asymmetric unit	2
V_M (Å ³ Da ^{−1})	2.71
V_{solv} (%)	54.69

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

CsyB protein yielded a molecular mass of 105 kDa in a gel-filtration experiment. Furthermore, DLS analysis after gel filtration revealed a monomodal distribution, with a polydispersity value of 10.2% and an estimated molecular mass of 89 kDa, suggesting that the recombinant CsyB is a homodimeric enzyme, as in the cases of other known type III PKSs (Austin & Noel, 2003; Abe & Morita, 2010).

Crystals appeared reproducibly within a day and the largest crystal grew to dimensions of approximately $0.1 \times 0.1 \times 0.02$ mm (Fig. 3). A complete data set was collected to 1.71 Å resolution. Detailed data-processing statistics are shown in Table 1. It should be noted that the data collection was processed only to the corners of the square detector. Thus, higher resolution data may be obtained in the future. Based on the diffraction data, the space group was determined to be $P2_1$, with unit-cell parameters $a = 70.0$, $b = 104.8$, $c = 73.5$ Å, $\beta = 114.4^\circ$. With two monomers in the asymmetric unit, the Matthews volume (V_M ; Matthews, 1968) was calculated to be $2.71 \text{ Å}^3 \text{ Da}^{-1}$ and the estimated solvent content was 54.69%, which is in the range normally observed for protein crystals. The self-rotation function was calculated between 15.0 and 8 Å resolution applying an integration radius of 18 Å using *POLARRFN* in the *CCP4* suite (Winn *et al.*, 2011). The peaks represent twofold noncrystallographic symmetry axes in the $\kappa = 180^\circ$ section (Fig. 4). Structure determination by the molecular-replacement method is now under way using *MOLREP* (Vagin & Teplyakov, 2010) with the CsyB structure model as a search model generated by the *SWISS-MODEL* package (<http://swissmodel.expasy.org/>) based on the crystal structure of NcORAS (PDB entry 3e1h; Goyal *et al.*, 2008), which shares 37% identity with CsyB. Simultaneously, we are also attempting to crystallize CsyB complexed with substrate and product analogues. These structural analyses will provide valuable insights into the novel polyketide-formation reaction of CsyB.

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