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Crystallization and preliminary crystallographic analysis of a novel plant type III polyketide synthase that produces pentaketide chromone

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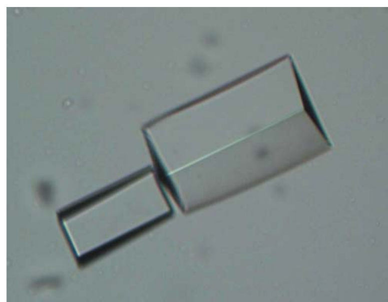

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Pentaketide chromone synthase (PCS) from *Aloe arborescens* is a novel plant-specific type III polyketide synthase that catalyzes the formation of 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA. Recombinant PCS expressed in *Escherichia coli* was crystallized by the hanging-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 73.2$, $b = 88.4$, $c = 70.0$ Å, $\alpha = \gamma = 90.0$, $\beta = 95.6°$. Diffraction data were collected to 1.6 Å resolution using synchrotron radiation at BL24XU of SPring-8.

1. Introduction

The chalcone synthase (CHS; EC 2.3.1.74) superfamily of type III polyketide synthases (PKSs) are structurally and mechanistically distinct from the modular type I and the dissociated type II PKSs of bacterial origin. The 40–45 kDa enzymes, which form simple homodimers, catalyze sequential decarboxylative condensations of malonyl-CoA with a CoA-linked starter molecule without the involvement of the acyl carrier protein to produce a variety of biologically active plant secondary metabolites (Schröder, 1999; Austin & Noel, 2003). For example, CHS, the pivotal enzyme in flavonoid biosynthesis, produces naringenin chalcone through the sequential condensation of 4-coumaroyl-CoA with three C_2 units from malonyl-CoA. On the other hand, 2-pyrone synthase (2PS) from *Gerbera hybrida* selects acetyl-CoA as a starter and carries out only two condensations with malonyl-CoA to produce triacetic acid lactone (TAL). Recent crystallographic and site-directed mutagenesis studies have begun to reveal the structural and functional details of the type III PKSs, which share a common three-dimensional overall fold and active-site architecture with a conserved Cys-His-Asn catalytic triad (Ferrer *et al.*, 1999; Jez *et al.*, 2000; Austin *et al.*, 2004). The polyketide-formation reaction is initiated by the starter molecule loading at the active-site Cys, which is followed by malonyl-CoA decarboxylation, polyketide chain elongation and cyclization of the polyketide intermediate. In principle, the remarkable functional diversity of the CHS-superfamily type III PKSs is derived from only a small modification of the active-site geometry (Jez *et al.*, 2000; Abe, Oguro *et al.*, 2005; Abe *et al.*, 2006).

Pentaketide chromone synthase (PCS) from *Aloe arborescens* is a novel plant-specific type III PKS that produces 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA (Fig. 1*a*; Abe, Utsumi *et al.*, 2005). The aromatic pentaketide is a biosynthetic precursor of the anti-asthmatic furochromones kehellin and visnagin. The amino-acid sequence of *A. arborescens* PCS shares 50–60% identity with those of the CHS-superfamily enzymes from other plants: 58% identity with *Medicago sativa* CHS and 57% identity with *G. hybrida* 2PS. Sequence comparisons revealed that the Cys-His-Asn catalytic triad and most of the CHS active-site residues are well conserved in *A. arborescens* PCS: however, the conserved Thr197, Gly256 and Ser338 in the CHSs (numbering according to *M. sativa* CHS; Ferrer *et al.*, 1999), which are uniquely altered in a number of divergent type III PKSs, are replaced with Met, Leu and Val, respectively, in *A. arborescens* PCS. The three residues lining the active-site cavity are also missing in *G. hybrida* 2PS (T197L/G256L/S338I; Jez *et al.*, 2000) and in *A. arborescens* octaketide synthase

(OKS; T197G/G256L/S338V; Abe, Oguro *et al.*, 2005). Remarkably, the replacement of Met207 of *A. arborescens* PCS (corresponding to Thr197 in the CHSs) with the less bulky Gly yielded a mutant enzyme that efficiently catalyzes the successive condensation of eight molecules of malonyl-CoA to produce SEK4 and SEK4b (Fig. 1*b*; Abe, Utsumi *et al.*, 2005). The octaketides are known to be the products of the minimal type II PKS for actinorhodin (*act* from *Streptomyces coelicolor*) and the longest polyketides ever generated by the structurally simple type III PKS. The pentaketide-forming PCS was thus functionally transformed into an octaketide-producing enzyme by the single amino-acid substitution. The functional diversity of the type III PKS was thus demonstrated to be evolved from the simple steric modulation of a single chemically inert residue lining the active-site cavity (Abe, Oguro *et al.*, 2005; Abe *et al.*, 2006). To further clarify the intimate three-dimensional structural details of the enzyme-catalyzed processes and to elucidate the structure–function relationship of the type III PKS enzymes, we now report the crystallization and preliminary crystallographic analysis of *A. arborescens* PCS.

2. Experimental

2.1. Expression and purification

A pET-41a(+) vector encoding full-length *A. arborescens* PCS (Abe, Utsumi *et al.*, 2005) was used as a template to amplify the PCS gene by PCR, using 5′-GCGCCCGGGAATGAGTTCACTCTCCAACG-3′ as a sense primer that introduces a *Sma*I restriction site and 5′-GCGCTCGAGTTACATGAGAGGCAGGCTGTGAA-3′ as an antisense primer that introduces a *Xho*I restriction site. The amplified DNA fragment was digested with *Sma*I/*Xho*I and was cloned into the *Sma*I/*Sal*I sites of a modified pET24a(+) expression vector (Novagen) for expression as a glutathione *S*-transferase (GST) fusion protein at the N-terminus. Between GST and PCS, a PreScission Protease (Amersham Biosciences) cleavage site (LEVLFQGP) was introduced. After confirmation of the sequence, the resultant expression plasmid was transformed into *Escherichia coli* BL21(DE3) pLysS.

Protein expression, extraction and initial purification by Glutathione Sepharose 4B affinity chromatography (Amersham Biosciences) were carried out as previously reported, except for the use of PreScission Protease digestion to remove the GST tag (Abe, Utsumi *et al.*, 2005). The resultant PCS protein thus contains three additional residues (GPG) at the N-terminal flanking region derived from the PreScission Protease recognition sequence. After affinity purification and GST-tag removal, the protein solution containing the recombinant PCS was diluted fivefold with 50 m*M* HEPES–NaOH buffer pH 7.0 containing 5% glycerol and 2 m*M* DTT and was then applied onto a Resource-Q column (Amersham Biosciences). The column was washed with HEPES–NaOH buffer containing 50 m*M* NaCl and the protein was subsequently eluted using a linear gradient of 50–200 m*M* NaCl. The protein solution was further purified to homogeneity by chromatography on Superdex 200HR (10/100 GL; Amersham Biosciences) and was concentrated to 10 mg ml^{-1} in 20 m*M* HEPES–NaOH pH 7.0 buffer containing 100 m*M* NaCl and 2 m*M* DTT. The typical yield of protein was about 0.5 mg per litre of culture.

2.2. Crystallization and X-ray data collection

Initial crystallization attempts were carried out at 293 K using an original 96-condition screening set (ZOEGENE Corporation) with the sitting-drop vapour-diffusion method. Crystals were observed under many crystallization conditions and one of the more promising crystallization conditions was further optimized. Finally, diffraction-quality crystals were obtained at 293 K in 100 m*M* Tris–HCl buffer pH 8.5 containing 14%(*w*/*v*) PEG 8000 and 350 m*M* KF using the hanging-drop vapour-diffusion method. The crystallization drops were prepared by mixing 0.5 µl protein solution and an equal volume of reservoir solution and were equilibrated against 500 µl reservoir solution. The crystals appeared reproducibly within 2 d and grew to average dimensions of approximately 0.2 × 0.1 × 0.1 mm (Fig. 2).

The crystals were transferred to the reservoir solution with 20%(*v*/*v*) glycerol as a cryoprotectant, picked up in a nylon loop and then flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data were collected from a single crystal at SPring-8 beamline BL24XU using a Rigaku R-AXIS V imaging-plate area detector with 180 frames. The 1° oscillation images were recorded with exposure times of 48 s. The wavelength of the synchrotron radiation was

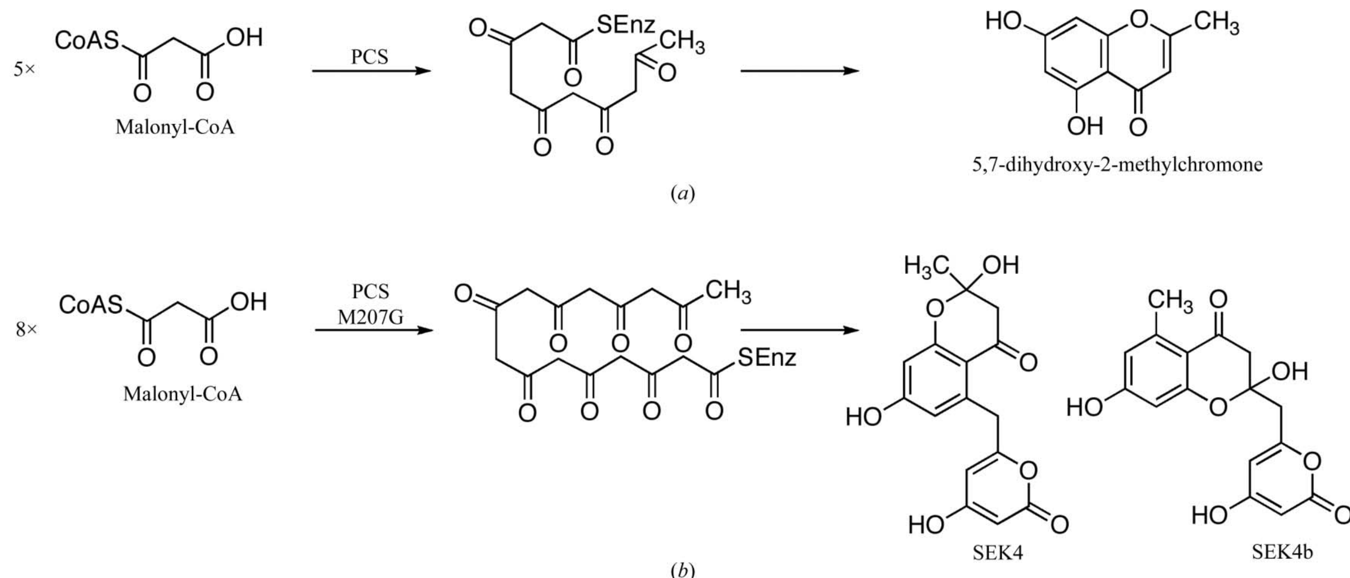

Figure 1
Proposed mechanism for the formation of (*a*) 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA by PCS and (*b*) SEK4 and SEK4b from eight molecules of malonyl-CoA by the PCS M207G mutant.

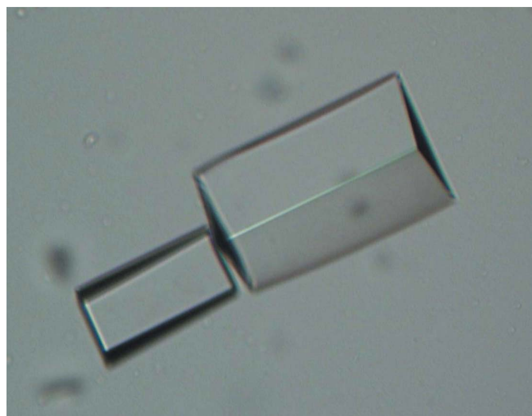

Figure 2
Crystals of *A. arborescens* PCS grown by the hanging-drop method. The dimensions of the largest crystal were approximately 0.2 × 0.1 × 0.1 mm.

0.82656 Å and the distance between the crystal and the detector was 340 mm. Data were indexed, integrated and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant PCS was heterologously expressed in *E. coli* as a fusion protein with GST at the N-terminus. After cleavage of the GST tag, the purified enzyme migrated as a single band with a molecular weight of 44 kDa on SDS–PAGE, which is in good agreement with the calculated value of 44 779 Da. A gel-filtration experiment gave a molecular weight of 88 kDa, suggesting that *A. arborescens* PCS is a homodimeric enzyme, as is the case for other known type III PKSs. A complete data set was collected to 1.6 Å resolution. Detailed data-processing statistics are shown in Table 1. From the diffraction data collection, the space group was determined to be monoclinic $P2_1$, with unit-cell parameters a = 73.2, b = 88.4, c = 70.0 Å, $\alpha = \gamma = 90.0$, $\beta = 95.6°$. With two monomers in the asymmetric unit, the Matthews coefficient (V_M; Matthews, 1968) was calculated to be 2.5 $Å^3$ Da^{-1}; the estimated solvent content is thus 49.0%, which is in the range normally observed for protein crystals.

The preliminary crystal structure of PCS was determined by the molecular-replacement method, with the reported crystal structure of *G. hybrida* 2PS (PDB code 1ee0; Jez *et al.*, 2000) as a search model, using the *EPMR* program (Kissinger *et al.*, 1999). The initial model had an *R* factor of 41% and showed an unknown compound within the traditional CoA-binding tunnel. Further refinements and analyses of the structure are in progress. Simultaneously, we are attempting to crystallize the PCS M207G mutant. Structural analyses of both proteins will provide valuable insights into the molecular mechanism of the functionally diverse type III PKSs.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	73.2
b (Å)	88.4
c (Å)	70.0
β (°)	95.6
Resolution (Å)	30.0–1.6 (1.66–1.60)
Unique reflections	116521
Redundancy	3.7 (3.8)
Completeness (%)	99.9 (100)
$\langle I/(\sigma I)\rangle$	33.5 (8.4)
R_{sym}† (%)	6.9 (23.0)

† $R_{sym} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

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