Crystallization and preliminary X-ray diffraction analysis of cyclolavandulyl diphosphate synthase, a new member of the *cis*-isoprenyl diphosphate synthase superfamily

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Cyclolavandulyl diphosphate synthase (CLDS; estimated molecular weight 23.1 kDa) from the soil bacterium *Streptomyces* sp. CL190 is an enzyme that catalyzes both the condensation of two molecules of C₅ dimethylallyl diphosphate (DMAPP) and the subsequent cyclization. CLDS was crystallized in the absence and the presence of the substrate DMAPP. Diffraction data were collected at a synchrotron source and the crystals diffracted to 2.00 and 1.73 Å resolution, respectively. The crystal obtained in the absence of DMAPP belonged to space group *P*₂₁₂₁₂₁, with unit-cell parameters *a* = 39.0, *b* = 87.5, *c* = 113.6 Å. The crystal obtained in the presence of DMAPP belonged to space group *P*1, with unit-cell parameters *a* = 46.9, *b* = 61.7, *c* = 82.2 Å, α = 74.0, β = 84.5, γ = 86.0°.

1. Introduction

Terpenoids represent an important class of biologically active compounds owing to their structural diversity (Maimone & Baran, 2007; Sacchettini & Poulter, 1997). Over the past several decades, various studies have sought to identify the biosynthetic mechanisms responsible for terpenoid complexity (Christianson, 2006). The complexity of the terpenoid skeleton arises from the condensation of C₅ isoprene units and a subsequent cyclization. Typically, these condensation and cyclization reactions are independently catalyzed by isoprenyl diphosphate synthase (IDS) and terpene cyclase, respectively.

Cyclolavandulyl diphosphate (CLDP) is a C₁₀ monoterpene with a branched and cyclized carbon skeleton (Fig. 1). The skeleton is found in some natural products derived from plants and bacteria. Although the cyclolavandulyl skeleton was discovered in nature approximately half a century ago (Logani *et al.*, 1967), the enzyme responsible for synthesizing this skeleton remained largely unknown. Recently, we identified and characterized CLDP synthase (CLDS) from the soil bacterium *Streptomyces* sp. CL190 (Ozaki *et al.*, 2014); this bacterium produces lavanducyanin, which is a phenazine with an *N*-linked cyclolavandulyl structure (Imai *et al.*, 1989). CLDS is a single-domain enzyme consisting of only 217 residues and catalyzes both the condensation of two molecules of C₅ dimethylallyl diphosphate (DMAPP) and the subsequent cyclization to form CLDP (Fig. 1).

CLDS belongs to the *cis*-IDS superfamily. Undecaprenyl diphosphate synthase (UPPS), which is also a member of this family, catalyzes the sequential *cis* condensations of eight C₅ isopentenyl diphosphates with a C₁₅ *trans,trans*-farnesyl diphosphate to produce the acyclic C₅₅ undecaprenyl diphosphate, which is indispensable for peptidoglycan biosynthesis in the bacterial cell wall. The crystal structures of UPPS from *Micrococcus luteus* and *Escherichia coli* have previously been determined, and the structural features necessary for the condensation reaction have been clarified (PDB entries 1f75, 1ueh and 1x09; Fujihashi *et al.*, 2001; Chang *et al.*, 2003; Guo *et al.*, 2005). The structures enabled the identification of the amino-acid residues that recognize the phosphate moiety of the isoprenyl diphosphate substrates. A comparison of the amino-acid sequences of CLDS and UPPS revealed that these residues are highly conserved between CLDS and UPPS (Ozaki *et al.*, 2014). However, because the dual nature of the CLDS activity is unique within the *cis*-IDS superfamily, the determination of the crystal structure of CLDS is

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Table 1
Overexpression information.

	CLDS-NH	CLDS-CH
Source organism	<i>Streptomyces</i> sp. CL190	<i>Streptomyces</i> sp. CL190
DNA source	gDNA (accession No. AB872045)	pH8CLDS (Ozaki <i>et al.</i> , 2014)
Forward primer	5'-GGGGATCCACACGTTGATGCTGCTGCG-3'	5'-GGGGAATCCATATGACCAGTTGATGCTGCTG-3'
Reverse primer	5'-GGAAGCTTTACGCCGGTAGCCGCCGAAG-3'	5'-CCCCAAGCTTTAGTGATGGTATGGTATGCGCCGGTAGCCGCCGAAG-3'
Cloning vector	pt7blue t-vector	pBluescript II SK(+)
Expression vector	pHIS8 (Jez <i>et al.</i> , 2000)	pET-26b(+)
Expression plasmid	pH8CLDS (Ozaki <i>et al.</i> , 2014)	pET-CLDS-CH
Expression host	<i>E. coli</i> BL21-CodonPlus(DE3)-RIL	<i>E. coli</i> BL21-CodonPlus(DE3)-RIL
Complete amino-acid sequence of the construct produced	MKHHHHHHHGGGLVPRGSHGGSTLMLLPDGMRRWSEKNGVSLDDGYAAMGDKIIE-FMGWAKEEGVKTLYITASSAANHGRPEAAVNTFMEAFTEVIRCHSQKFDFSG-SLDLVSEDTLTELALRDKSDSESDFTLHYILGMSLSHEVVGFINKLNGKIPEM-TEEILAEAYVPTQVDYIIRTGGAIRMSSFFPLMSPYAEHFSPVLPDTPTRADFDAALKDLRDRRFGGYPA	MTTLMMLPDGMRRWSEKNGVSLDDGYAAMGDKIIEFMGWAKEEGVKTLYITASSAANHGRPEAAVNTFMEAFTEVIRCHSQKFDFSGSLDLVSEDTLTELALRDKSDSESDFTLHYILGMSLSHEVVGFINKLNGKIPEMTEEILAEAYVPTQVDYIIRTGGAIRMSSFFPLMSPYAEHFSPVLPDTPTRADFDAALKDLRDRRFGGYPAHHHHH

Table 2
Crystallization.

	CLDS-NH	CLDS-CH
Method	Hanging-drop vapour diffusion	Hanging-drop vapour diffusion
Plate type	24-well VDX plate (Hampton Research)	24-well VDX plate (Hampton Research)
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	10	10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DMAPP, 2 mM MgSO ₄	20 mM Tris-HCl pH 8.0, 150 mM NaCl
Composition of reservoir solution	100 mM HEPES-NaOH pH 7.0, 24% PEG 2000	100 mM Tris-HCl pH 7.0, 1 M sodium citrate tribasic, 200 mM NaCl
Volume and ratio of drop	2 µl, 1:1 protein:reservoir solution	2 µl, 1:1 protein:reservoir solution
Volume of reservoir (µl)	500	500

necessary to elucidate the structural basis for this unique CLDS-catalyzed reaction.

To understand the structural basis for this unusual two-step CLDS-catalyzed reaction, we crystallized CLDS and collected and analyzed X-ray diffraction data from the CLDS crystals.

2. Materials and methods

2.1. Overexpression and purification

The previously described expression plasmid pH8CLDS (Ozaki *et al.*, 2014) was used to express CLDS with an N-terminal eight-histidine tag (CLDS-NH) in *E. coli* BL21-CodonPlus(DE3)-RIL cells. To express CLDS with a C-terminal six-histidine tag (CLDS-CH), we constructed the expression plasmid as described below. The *clds* gene encoding CLDS (accession No. AB872045) from *Streptomyces* sp. CL190 was amplified by polymerase chain reaction using two oligonucleotides (Table 1). The amplified DNA fragment encoding CLDS with a C-terminal (His)₆ tag was cloned into the *Eco*RI/*Hind*III sites of the pBluescript II SK(+) vector. After verifying the nucleotide sequence, the DNA fragment was cloned into the *Nde*I/*Hind*III sites of the pET-26b(+) vector to construct the plasmid pET-CLDS-CH, which was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells.

The cells harbouring pH8CLDS or pET-CLDS-CH were grown in 2×YT broth in the presence of kanamycin (50 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹) at 303 K. When the optical density at 600 nm of the culture reached approximately 0.6, protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside and the culture was incubated at 303 K for an additional 12 h. The cells were harvested, washed in buffer A (20 mM Tris-HCl pH 8.0) and resuspended in this buffer. The resuspended cells were lysed by sonication and centrifuged at 40 000g for 20 min. The supernatant was applied onto a Ni²⁺-affinity column (Novagen) equilibrated with buffer B (20 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 20 mM imidazole, followed by successive washing with this buffer. Proteins bound to the resin were eluted with buffer B containing 500 mM imidazole. The fractions containing the histidine-tagged CLDS were concentrated by ultrafiltration using Vivaspinn 20 concentrators (10 kDa molecular-weight cutoff; Sartorius Stedim Biotech). The concentrated sample was applied onto a HiLoad 26/60 Superdex 75 (GE Healthcare) size-exclusion column that was equilibrated with buffer B, and fractions were collected every 2.5 ml at a flow rate of 2.5 ml min⁻¹. The oligomeric state of the protein was estimated to be a dimer based on the elution volume from size-exclusion chromatography. The purity of the purified CLDS was greater than 95%, as verified by SDS-PAGE (Supplementary Figs. S1

Figure 1
Proposed reaction mechanism for CLDS. LDP, lavandulyl diphosphate.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	CLDS-NH-DMAPP-MgSO ₄	CLDS-CH
Beamline	BL-5A, PF, KEK	AR-NE3A, PF, KEK
Wavelength (Å)	1.0	1.0
Temperature (K)	95	95
Detector	ADSC Quantum 315r	ADSC Quantum 270
	CCD	CCD
Crystal-to-detector distance (mm)	214	229
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	180	180
Exposure time per image (s)	0.9	1.0
Space group	<i>P</i> 1	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	46.9, 61.7, 82.2	39.0, 87.5, 113.6
α , β , γ (°)	74.0, 84.5, 86.0	90, 90, 90
Resolution range (Å)	50–1.73 (1.76–1.73)	50–2.00 (2.03–2.00)
Total No. of reflections	154893	189589
No. of unique reflections	92115	27009
Completeness (%)	85.9 (95.5) [†]	99.7 (100.0)
Multiplicity	2.0 (2.0)	7.0 (7.3)
$\langle I/\sigma(I) \rangle$	18.1 (1.8)	21.8 (3.2)
<i>R</i> _{int}	0.083 (0.434)	0.117 (0.521)

[†] Owing to the ice rings observed in the diffraction image (Supplementary Fig. S1).

and S2¹). Greater than 40 mg of CLDS-NH and CLDS-CH was purified from 1.6 l of culture, and the proteins were used for crystallization without removal of the tags.

2.2. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method using Crystal Screen (Hampton Research) and the Wizard crystallization screen series (Emerald Bio). The screens were set up using 2 μ l drops consisting of 1 μ l reservoir solution and 1 μ l 10 mg ml^{−1} CLDS solution with and without 5 mM DMAPP and 2 mM MgSO₄ (Table 2).

2.3. Data collection and processing

Prior to data collection from the CLDS-NH-DMAPP-MgSO₄ crystal, the crystal was directly flash-cooled in a nitrogen-gas stream at 95 K because it was unstable in cryoprotectant buffer containing PEG 400. Prior to data collection of CLDS-CH, the crystal was briefly soaked in a cryoprotectant solution consisting of 100 mM Tris-HCl pH 7.0, 1 M sodium citrate tribasic, 200 mM sodium chloride, 30% (v/v) glycerol and then flash-cooled in a nitrogen-gas stream at 95 K. Diffraction data ($\lambda = 1.0$ Å) for CLDS-NH-DMAPP-MgSO₄ and CLDS-CH were collected using charge-coupled device detectors (ADSC Quantum 315r and ADSC Quantum 270) at the BL-5A and AR-NE3A stations of the Photon Factory, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan, respectively (Table 3). Diffraction images (Supplementary Figs. S1 and S2) were indexed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). Molecular replacement was performed with *Phaser* (McCoy *et al.*, 2007) in the *CCP4* program suite (Winn *et al.*, 2011).

3. Results and discussion

Crystals of CLDS-NH appeared in a droplet containing 100 mM HEPES-NaOH pH 7.0, 24% PEG 2000 in the presence of 5 mM DMAPP and 2 mM MgSO₄ (Fig. 2). Crystals of CLDS-CH appeared

Figure 2

Crystal of CLDS-NH in the presence of DMAPP and MgSO₄.

in a droplet containing 100 mM Tris-HCl pH 7.0, 1 M sodium citrate tribasic, 200 mM sodium chloride (Fig. 3). X-ray diffraction data from these CLDS crystals were collected and analyzed. Details of the data-collection statistics are summarized in Table 3. The crystal obtained in the presence of DMAPP belonged to space group *P*1, with unit-cell parameters *a* = 46.9, *b* = 61.7, *c* = 82.2 Å, α = 74.0, β = 84.5, γ = 86.0°. The crystal obtained in the absence of DMAPP belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 39.0, *b* = 87.5, *c* = 113.6 Å. We attempted molecular replacement with UPPS from *Campylobacter jejuni* (26% identity; PDB entry 3ugs; Center for Structural Genomics of Infectious Diseases, unpublished work), UPPS from *E. coli* (27% identity; PDB entry 1ueh; Chang *et al.*, 2003) and UPPS from *Helicobacter pylori* (27% identity; PDB entry 2d2r; Kuo *et al.*, 2008) as search models. However, this attempt did not provide a possible solution. Therefore, we are now trying to determine the three-dimensional structure of CLDS by the multi-wavelength anomalous diffraction method. These preliminary results will enable the elucidation of the structural basis of the unique CLDS activity.

Figure 3

Crystal of CLDS-CH.

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: US5062).

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References

- Chang, S.-Y., Ko, T.-P., Liang, P.-H. & Wang, A. H.-J. (2003). *J. Biol. Chem.* **278**, 29298–29307.
- Christianson, D. W. (2006). *Chem. Rev.* **106**, 3412–3442.
- Fujihashi, M., Zhang, Y.-W., Higuchi, Y., Li, X.-Y., Koyama, T. & Miki, K. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 4337–4342.
- Guo, R.-T., Ko, T.-P., Chen, A. P.-C., Kuo, C.-J., Wang, A. H.-J. & Liang, P.-H. (2005). *J. Biol. Chem.* **280**, 20762–20774.
- Imai, S., Furihata, K., Hayakawa, Y., Noguchi, T. & Seto, H. (1989). *J. Antibiot.* **42**, 1196–1198.
- Jez, J. M., Ferrer, J.-L., Bowman, M. E., Dixon, R. A. & Noel, J. P. (2000). *Biochemistry*, **39**, 890–902.
- Kuo, C.-J., Guo, R.-T., Lu, I.-L., Liu, H.-G., Wu, S.-Y., Ko, T.-P., Wang, A. H.-J. & Liang, P.-H. (2008). *J. Biomed. Biotechnol.* **2008**, 841312.
- Logani, M. K., Varshney, I. P., Pandey, R. C. & Dev, S. (1967). *Tetrahedron Lett.* **28**, 2645–2648.
- Maimone, T. J. & Baran, P. S. (2007). *Nature Chem. Biol.* **3**, 396–407.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Ozaki, T., Zhao, P., Shinada, T., Nishiyama, M. & Kuzuyama, T. (2014). *J. Am. Chem. Soc.* **136**, 4837–4840.
- Sacchettini, J. C. & Poulter, C. D. (1997). *Science*, **277**, 1788–1789.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.