Crystallization and preliminary X-ray diffraction analysis of AntE, a crotonyl-CoA carboxylase/reductase from *Streptomyces* sp. NRRL 2288

Lihan Zhang,^a Jing Chen,^{a,b}
Takahiro Mori,^{a,c} Yan Yan,^b Wen
Liu^b and Ikuro Abe^{a,*}

^aGraduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, ^bState Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, People's Republic of China, and ^cJST, CREST, 5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan

Correspondence e-mail:
abei@mol.f.u-tokyo.ac.jp

Received 20 March 2014
Accepted 14 April 2014

AntE from *Streptomyces* sp. NRRL 2288 is a crotonyl-CoA carboxylase/reductase that catalyzes the reductive carboxylation of various α,β -unsaturated acyl-CoAs to provide the building block at the C7 position for antimycin A biosynthesis. Recombinant AntE expressed in *Escherichia coli* was crystallized by the sitting-drop vapour-diffusion method. The crystals belonged to space group $I222$ or $I2_12_12_1$, with unit-cell parameters $a = 76.4$, $b = 96.7$, $c = 129.6$ Å, $\alpha = \beta = \gamma = 90.0^\circ$. A diffraction data set was collected at the KEK Photon Factory to 2.29 Å resolution.

1. Introduction

Crotonyl-CoA carboxylase/reductases (CCRs) belong to the medium-chain reductase/dehydrogenase (MDR) superfamily and catalyze the reductive carboxylation of α,β -unsaturated acyl-CoAs in the presence of CO₂ and NADPH (Erb *et al.*, 2007; Wilson & Moore, 2012). The carboxylation reaction mechanism begins with the transfer of the hydride from NADPH onto the β -carbon of the acyl-CoA to yield a thioester enolate, followed by the electrophilic attack of CO₂ at the α -carbon. In the carboxylation reaction of (*E*)-crotonyl-CoA, for instance, the pro-(4*R*) H atom of NADPH is transferred to the *re* face of the C3 position of crotonyl-CoA, followed by CO₂ incorporation at the C2 position in an *anti* fashion; hence, the reaction is stereospecific. In addition to the carboxylation reaction, CCRs also catalyze a reduction reaction with a solvent proton in the absence of CO₂ (Erb *et al.*, 2007).

The reactions catalyzed by CCRs are employed in both primary and secondary metabolism. The CCR enzymes were initially studied in fatty-acid biosynthesis, where CCRs are employed in the crotonyl-CoA reduction to form butyryl-CoA, a starter unit for fatty-acid biosynthesis (Langdon, 1955). It was subsequently revealed that some CCRs catalyze a carboxylation reaction to form ethylmalonyl-CoA, which is further processed to glyoxylate to enter the central metabolism pathway (Alber *et al.*, 2006; Erb *et al.*, 2007). Ethylmalonyl-CoA can be further incorporated in the secondary metabolism pathway, specifically in the polyketide synthase (PKS) pathway. Since ethylmalonyl-CoA and other C2-substituted alkylmalonyl-CoAs serve as PKS extender units *via* decarboxylative condensation, the alkylmalonyl-CoAs produced by CCRs allow the diversification of PKS products from ordinary malonyl-CoA or methylmalonyl-CoA units. Many polyketide compounds bearing unusual side chains are currently thought to be involved with CCRs (Wilson & Moore, 2012), and some of them have been characterized, such as CinF in cinnabaramide A biosynthesis to produce hexylmalonyl-CoA (Quade *et al.*, 2012), TscC in FK506 biosynthesis to produce allylmalonyl-CoA (Mo *et al.*, 2011) and SalG in salinosporamide biosynthesis to produce chloroethylmalonyl-CoA (Eustáquio *et al.*, 2009). The recent crystallographic analysis of CinF, the first crystallographic study of a CCR, has revealed the structural details of the reaction mechanism and the substrate recognition (PDB entry 4a10; Quade *et al.*, 2012). It was reported that CinF accepts 2-octenoyl-CoA as well as crotonyl-CoA as a substrate because of its large active-site cavity size; however, the substrate specificity of CinF for other substrates is as yet unknown (Quade *et al.*, 2012).

AntE (GenBank accession No. AGG37751.1), which catalyzes the reductive carboxylation of various α,β -unsaturated acyl-CoAs to

© 2014 International Union of Crystallography
All rights reserved

provide the building block at the C7 position of antimycin A biosynthesis, was identified in *Streptomyces* sp. NRRL 2288 (Fig. 1*a*; Yan *et al.*, 2012, 2013). The enzyme is composed of 406 amino acids, sharing 38% amino-acid sequence identity with CinF and 37% identity with a putative CCR from *S. coelicolor* A3 (PDB entry 3krt; New York SGX Research Center for Structural Genomics, unpublished work). As antimycin A is widespread in *Streptomyces* species (Seipke *et al.*, 2011; Yan *et al.*, 2012), AntE homologues are found in several species with nearly 100% homology (*S. albus* J1074 and *Streptomyces* sp. S4) or with ~85% homology (*S. blastomyceticus* and *S. ambofaciens* ATCC 23877), and the AntE homologue from *S. albus* J1074 has been characterized and found to have carboxylative activity, producing butylmalonyl-CoA from 2*E*-hexenoyl-CoA (Sandy *et al.*, 2012). Our previous *in vivo* and *in vitro* analyses of AntE demonstrated the extraordinary promiscuity in its substrate recognition, resulting in the production of a number of unnatural extender units such as heptynoylmalonyl-CoA, cyclohexanepropanoylmalonyl-CoA and chloropentanoylmalonyl-CoA, as well as various natural alkylmalonyl-CoA extender units, which enabled us to produce a series of unnatural antimycin derivatives (Fig. 1*b*; Yan *et al.*, 2013). These observations unambiguously demonstrated the promise of AntE as an enzyme for supplying non-natural extender units in PKS engineering. Moreover, the stereospecificity of the reactions by CCRs highlights AntE as a potent biocatalyst. By comparing the structures of AntE and other CCR enzymes, we can shed light on the currently less understood molecular mechanisms for the broad substrate specificities of the CCR enzymes. Thus, not only to clarify the three-dimensional structural details of the exceptionally promiscuous substrate recognition of AntE, but also to elucidate the structure–function relationships of the CCR enzymes in general, we expressed hexahistidine (His₆)-fused recombinant *Streptomyces* sp. NRRL 2288 AntE in *Escherichia coli* and obtained good-quality crystals from the recombinant enzyme.

2. Experimental

2.1. Construction of expression plasmids

The gene encoding full-length AntE was amplified from the genome of *Streptomyces* sp. NRRL 2288 by PCR using the forward primer 5'-AAAGAATTCCATATGACCAAGGACCTCTAC-3' and the reverse primer 5'-AAAAAGCTTCTACTCGGTGGCGTT-CACC-3' (the *Nde*I and *Hind*III restriction sites, respectively, are

represented by bold letters). The amplified DNA fragment was digested with *Nde*I/*Hind*III and was ligated into the *Nde*I/*Hind*III sites of the pET-28a(+) vector (TaKaRa) using a DNA ligation kit (TaKaRa) for expression as a fusion protein with a His₆ tag at the N-terminus.

2.2. Expression and purification of His₆-tag-fused AntE

After confirmation of the sequence, the pET-28a(+) vector encoding full-length AntE was transformed into *E. coli* BLR(DE3) cells. The cells harbouring the plasmid were cultured at 303 K to an OD₆₀₀ of 0.6 in LB medium containing 100 µg ml⁻¹ kanamycin. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 1 mM to induce gene expression, and the culture was incubated at 293 K for a further 16 h.

All of the following procedures were performed at 277 K. The *E. coli* cells were harvested by centrifugation at 5000*g* and resuspended in 50 mM Tris–HCl buffer pH 8.0 containing 100 mM NaCl, 5% (v/v) glycerol (buffer *A*). The cells were disrupted by sonication ten times for 15 s each with 20 s intervals between each sonication, and the lysate was centrifuged at 12 000*g* for 15 min. The supernatant was loaded onto a COSMOGEL His-Accept (Nacalai Tesque) Ni-NTA column equilibrated with buffer *A*. After washing the resin with 20 mM Tris–HCl buffer pH 7.5 containing 100 mM NaCl, 5% (v/v) glycerol (buffer *B*), the His₆-tag-fused AntE was subsequently eluted with buffer *B* containing 500 mM imidazole. The protein solution was purified to homogeneity by gel-filtration chromatography on a Superdex 200HR column (10/300 GL; GE Healthcare) and was concentrated to 12 mg ml⁻¹ in 20 mM Tris–HCl pH 7.5 buffer containing 100 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. The data were acquired from 50 scattering measurements at 278 K and the data from three sets were analyzed using the DYNAMICS software package (Protein Solutions) and were averaged.

2.3. Crystallization and X-ray data collection

Initial crystallization attempts and optimizations of the crystallization conditions were performed at 293 K using the sitting-drop vapour-diffusion method by mixing 0.5 µl purified His₆-tag-fused

Figure 1

(*a*) Reductive carboxylation reaction catalyzed by AntE in antimycin biosynthesis. (*b*) The structure of the substrates accepted by AntE.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	BL-17A, PF
Wavelength (Å)	0.9800
Space group	<i>I</i> 222 or <i>I</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters	
<i>a</i> (Å)	76.4
<i>b</i> (Å)	96.7
<i>c</i> (Å)	129.6
$\alpha = \beta = \gamma$ (°)	90.0
Resolution (Å)	50.0–2.29 (6.81–2.29)
Total reflections	160702 (25132)
Unique reflections	21854 (3433)
Multiplicity	7.35 (7.32)
Completeness (%)	99.8 (99.1)
$\langle I/\sigma(I) \rangle$	19.8 (3.96)
$R_{\text{merge}}^{\dagger}$ (%)	9.1 (53.8)
Molecules per asymmetric unit	1
V_M (Å ³ Da ^{−1})	2.56
V_{solv} (%)	52.0

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ 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 .

AntE and an equal volume of reservoir solution and equilibrating the mixture against 50 μ l reservoir solution using the Wizard I and II (Emerald Bio) and Crystal Screen and Crystal Screen 2 (Hampton Research) kits. As crystals were observed in a crystallization solution containing 100 mM CAPS–NaOH pH 10.5, 30%(v/v) PEG 400, further crystallization was attempted at various conditions with pH values ranging from pH 9.5 to 11.5 and concentrations of PEG 400 ranging from 25 to 40% in nearly 200 droplet conditions. Diffraction-quality crystals were finally obtained at 293 K in 100 mM CAPS–NaOH pH 10.0, 38%(v/v) PEG 400 using the sitting-drop vapour-diffusion method.

The crystals were transferred to a cryoprotectant consisting of 100 mM CAPS–NaOH pH 10.0, 38%(v/v) PEG 400, 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 from a single crystal on beamline BL-17A of the Photon Factory (PF; wavelength 0.9800 Å). A total of 240 frames were recorded with a 0.75° oscillation angle and a 2 s exposure time. The data were indexed, integrated and scaled with the XDS program package (Kabsch, 2010).

Figure 2

Crystal of AntE. The dimensions of the crystal were approximately 0.1 $\times$ 0.05 $\times$ 0.05 mm.

3. Results and discussion

The His₆-tag-fused recombinant AntE protein was expressed in *E. coli*. The recombinant enzyme exhibited AntE activity (Yan *et al.*, 2013) and the typical yield was about 5 mg per litre of culture. The purified His₆-tag-fused AntE migrated as a single band with a molecular mass of 47 kDa on SDS–PAGE, which agrees well with the calculated value of 46.7 kDa for His₆-tag-fused AntE. On the other hand, the gel-filtration analysis of the recombinant AntE yielded a molecular mass of 143 kDa, suggesting that the recombinant AntE exists as a trimeric or tetrameric enzyme. Furthermore, DLS analysis of the recombinant AntE after gel filtration revealed a tetrameric distribution, with a polydispersity value of 12.6% and an estimated molecular mass of 193 kDa, suggesting that this recombinant protein is a tetrameric enzyme in solution, as in the case of the other CCR enzyme CinF (Quade *et al.*, 2012).

The crystals appeared reproducibly within a week, and the largest crystal grew to dimensions of approximately 0.1 $\times$ 0.05 $\times$ 0.05 mm (Fig. 2). A complete data set was collected to 2.29 Å resolution. Details of the data-processing statistics are provided in Table 1. The Matthews coefficient (V_M ; Matthews, 1968) of 2.56 Å³ Da^{−1} and the estimated solvent content of 52.0% are in the range normally observed for protein crystals. No significant twin fraction was found by the *L*-test using *phenix.xtriage* (Adams *et al.*, 2010); the standard $\langle I^2 \rangle / \langle I \rangle^2$ test gave $\langle I^2 \rangle / \langle I \rangle^2$ of 1.935 for *I*222 and $\langle I^2 \rangle / \langle I \rangle^2$ of 1.939 for *I*2₁2₁2₁, and both *I*222 and *I*2₁2₁2₁ had $\langle |L| \rangle$ of 0.48 and $\langle L^2 \rangle$ of 0.31. The native Patterson map calculated with *phenix.xtriage* revealed that the largest off-origin peaks were 4.9% (*I*222) and 4.8% (*I*2₁2₁2₁) of the height of the origin peak, thus suggesting no significant pseudo-translation. Structure determination by the molecular-replacement method with *MOLREP* (Vagin & Teplyakov, 2010) is now in progress using the AntE structure model generated by the *SWISS-MODEL* package (<http://expasy.ch/spdpv/>) based on the crystal structure of CinF (PDB entry 4a10; Quade *et al.*, 2012) as a search model, because it possesses a well conserved Rossmann fold and highest homology to AntE among enzymes with available structures. Simultaneously, we are also attempting to crystallize the His₆-tag-fused AntE complexed with substrate and product analogues. These structural analyses will provide valuable insights into not only the mechanism underlying the exceptionally promiscuous substrate recognition by AntE but also the structure–function relationships of the CCR enzymes.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and CREST, Japan Science and Technology Agency.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Alber, B. E., Spanheimer, R., Ebenau-Jehle, C. & Fuchs, G. (2006). *Mol. Microbiol.* **61**, 297–309.
- Erb, T. J., Berg, I. A., Brecht, V., Müller, M., Fuchs, G. & Alber, B. E. (2007). *Proc. Natl Acad. Sci. USA*, **104**, 10631–10636.
- Eustáquio, A. S., McGlinchey, R. P., Liu, Y., Hazzard, C., Beer, L. L., Florova, G., Alhamadsheh, M. M., Lechner, A., Kale, A. J., Kobayashi, Y., Reynolds, K. A. & Moore, B. S. (2009). *Proc. Natl Acad. Sci. USA*, **106**, 12295–12300.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Langdon, R. G. (1955). *J. Am. Chem. Soc.* **77**, 5190–5192.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Mo, S. *et al.* (2011). *J. Am. Chem. Soc.* **133**, 976–985.
- Quade, N., Huo, L., Rachid, S., Heinz, D. W. & Müller, R. (2012). *Nat. Chem. Biol.* **8**, 117–124.
- Sandy, M., Rui, Z., Gallagher, J. & Zhang, W. (2012). *ACS Chem. Biol.* **7**, 1956–1961.

- Seipke, R. F., Barke, J., Brearley, C., Hill, L., Yu, D. W., Goss, R. J. M. & Hutchings, M. I. (2011). *PLoS One*, **6**, e22028.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Wilson, M. C. & Moore, B. S. (2012). *Nat. Prod. Rep.* **29**, 72–86.
- Yan, Y., Chen, J., Zhang, L., Zheng, Q., Han, Y., Zhang, H., Zhang, D., Awakawa, T., Abe, I. & Liu, W. (2013). *Angew. Chem. Int. Ed.* **52**, 12308–12312.
- Yan, Y., Zhang, L., Ito, T., Qu, X., Asakawa, Y., Awakawa, T., Abe, I. & Liu, W. (2012). *Org. Lett.* **14**, 4142–4145.