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Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of malonyl-CoA–acyl carrier protein transacylase (FabD) from *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (Xoo) causes bacterial blight in rice, which is one of the most devastating diseases in rice-cultivating countries. The *Xoo0880* (*fabD*) gene coding for a malonyl-CoA–acyl carrier protein transacylase (MCAT) from Xoo was cloned and expressed in *Escherichia coli*. MCAT is an essential enzyme that catalyzes a key reaction of fatty-acid synthesis in bacteria and plants: the conversion of malonyl-CoA to malonyl-acyl carrier protein. The FabD enzyme was purified and crystallized in order to elucidate its three-dimensional structure and to determine its enzymatic reaction mechanism and biological importance. The crystal obtained diffracted to 1.9 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 41.4$, $b = 74.6$, $c = 98.5$ Å. According to Matthews coefficient calculations, the crystallographic structure contains only one monomeric unit in the asymmetric unit with a V_M of $2.21 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 44.3%.

1. Introduction

Rice is an important staple for human consumption all over the world. Bacterial blight (BB) is a highly destructive bacterial disease of rice that is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo; Ezuka & Kaku, 2000). According to a report of the Agricultural Department, BB resulted in a rice-production loss worth more than 100 million dollars in South Korea in 2006 alone. To date, no effective drugs have been developed against this disease to halt rice-production losses. The whole genomic sequences of Xoo has been determined recently (Lee *et al.*, 2005) and provides valuable information for the selection of drug-target proteins from Xoo. As an initial step in drug development against Xoo, hundreds of genes coding essential enzymes have been selected as target proteins for drug candidates (Payne *et al.*, 2004, 2007) from the 4538 putative genes (Lee *et al.*, 2005). The selected target genes have been cloned and expressed in *Escherichia coli* systematically in order to obtain the essential drug-target enzymes and to determine their three-dimensional structures via X-ray crystallography.

Xoo0880 (FabD) is one of the most important drug-target enzymes of Xoo. The *fabD* gene expresses malonyl-CoA–acyl carrier protein transacylase (MCAT), which transfers the malonyl moiety to holo acyl carrier protein (ACP), forming a malonyl-ACP intermediate, in type II fatty-acid synthesis in bacteria (Ruch & Vagelos, 1973). The malonyl-ACP intermediate then acts as a two-carbon donor in the elongation step of fatty-acid synthesis. Fatty-acid synthesis (FAS) is essential for the survival of an organism (Magnuson *et al.*, 1993; White *et al.*, 2005) and MCAT takes part in this by extending the length of the growing acyl chain by two C atoms. MCAT has also been reported to be involved in polyketide biosynthesis, producing one of the largest classes of secondary metabolites, which includes tetracyclines and erythromycins (Keatinge-Clay *et al.*, 2003; Summers *et al.*, 1995). Therefore, MCAT is considered to be a vital enzyme in bacterial metabolic activity.

Here, we report the cloning and expression of the *fabD* (*Xoo0880*) gene and the purification, crystallization and preliminary X-ray crystallographic analysis of the coded enzyme. Crystallographic

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three-dimensional structural studies of FabD will elucidate the molecular basis of its enzymatic reaction mechanism as an MCAT and be helpful in the design of a potential drug against Xoo.

2. Methods and results

2.1. Cloning

The FabD-coding sequence of *Xoo0880* was amplified via the polymerase chain reaction (PCR) using bacterial cells (*Xoo* KACC10331 strain) as a template. The sequences of the forward and reverse oligonucleotide primers, designed based on the published genome sequence (Lee *et al.*, 2005), were as follows: 5'-GGG GGG **CAT ATG** ACC GAA TCC ACT CTC GCC TTC A-3' and 5'-GGG GGG **GGA T CC** TCA GTG GCC CCA CGC GTC GAG A-3'. The bases in bold designate *Nde*I and *Bam*HI restriction sites. The amplicon was double-digested with the two DNA-restriction enzymes and inserted into a modified pET11a vector (His-TEV-pET11a), which was engineered to contain additional residues for a 6×His tag and a tobacco etch virus (TEV) protease cleavage site before the *Nde*I site in a pET11a vector (Novagen) to facilitate purification of the expressed protein.

2.2. Overexpression and purification

The His-TEV-pET11a expression vector containing the coding sequence for *Xoo0880* was introduced into *Escherichia coli* BL21 (DE3) cells. Cells were grown at 310 K to an OD₆₀₀ of 0.5 in Luria-Bertani (LB) medium containing 50 µg ml⁻¹ ampicillin. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were cultured at the same temperature for an additional 16 h. Cultured cells were harvested by centrifugation for 20 min at 6000 rev min⁻¹ (Vision VS24-SMTi V5006A rotor) at 277 K. The cell pellet was then resuspended in ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM imidazole) and homogenized using ultrasonication (Sonomasher, S&T Science, Korea). The lysate was centrifuged for 30 min at 12 000 rev min⁻¹ (Vision VS24-SMTi V508A rotor) at 277 K. Only

10% of the total expressed *Xoo0880* was soluble (data not shown). The supernatant containing soluble FabD protein was applied onto Ni-NTA resin (Novagen) and affinity purification was performed according to the manufacturer's protocol at 277 K. The 6×His-tagged FabD protein was then eluted using lysis buffer containing 200 mM imidazole and treated with TEV protease overnight at 277 K to remove the 6×His tag. The resulting protein solution was dialyzed for 6 h at 277 K in buffer A (25 mM Tris-HCl pH 7.5, 15 mM NaCl) and

Figure 1
SDS-PAGE analysis of FabD (*Xoo0880*) during purification. Proteins were analyzed on 12% SDS-PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane *P*, purified FabD after UNO 6Q column chromatography.

(a)

(b)

(c)

Figure 2
Crystals of the FabD enzyme from *X. oryzae* pv. *oryzae* (*Xoo*) obtained from (a) a sitting-drop setup (Wizard G-5) using a Hydra automated high-throughput crystal screening machine, (b) a hanging-drop vapour-diffusion setup using 1.5 M ammonium sulfate, 0.1 M CHES pH 9.3, 0.2 M NaCl and (c) a well diffracting triangular shaped crystal grown at pH 9.0.

Table 1

Data-collection statistics.

Values in parentheses are for the last resolution shell.

Synchrotron	PF 17A
Wavelength (Å)	0.96418
Resolution range (Å)	50.0–1.9
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
<i>a</i>	41.4
<i>b</i>	74.6
<i>c</i>	98.5
Total No. of reflections	62374
No. of unique reflections	18355
Completeness (%)	74.9 (32.7)
Molecules per ASU	1
V_M (Å ³ Da ^{−1})	2.21
Solvent content (%)	44.3
Average $I/\sigma(I)$	30.9 (3.7)
$R_{\text{merge}}^{\dagger}$ (%)	6.0 (33.0)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl .

further purification was carried out on an UNO 6Q column (BioRad). The homogeneity of the purified protein was assessed *via* SDS–PAGE (Fig. 1). The purified protein was concentrated to 7 mg ml^{−1} and used in the crystallization process.

2.3. Crystallization and X-ray diffraction (data collection)

Crystallization of this enzyme was initiated by high-throughput crystal screening at 283 K using a Hydra II e-drop automated pipetting system (Matrix) with 96-well Intelli-Plates (Art Robbins) and various screening kits such as Crystal Screen HT, Index HT and Salt Rx HT (Hampton Research). Initially, very thin needle-like crystals (Fig. 2*a*) were obtained using Wizard condition G-5 (0.1 M CHES pH 9.5, 1.26 M ammonium sulfate and 0.2 M sodium chloride). Crystals were reproduced using the hanging-drop method, with a drop made up of 1 µl protein solution mixed with 1 µl reservoir solution and equilibrated against 1 ml reservoir solution; crystals grew over a period of a week. Since the initial crystals were not good enough for X-ray diffraction, the crystallization conditions were optimized by changing the pH and the salt concentration. Finally, a well diffracting crystal (Fig. 2*c*) was obtained in a week using 0.1 M CHES pH 9.0, 1.5 M ammonium sulfate and 0.2 M sodium chloride. The crystal was frozen in liquid nitrogen with 25% (v/v) glycerol as a cryoprotectant and used for data collection. The crystal data were collected using an ADSC Quantum 270 CCD detector on beamline 17A at the Photon Factory (KEK), Japan. X-ray diffraction data were collected to 1.9 Å resolution and each data set was integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski

& Minor, 1997). The crystallographic space group $P2_12_12_1$ was derived for the crystal by auto-indexing (Otwinowski & Minor, 1997), with unit-cell parameters $a = 41.4$, $b = 74.6$, $c = 98.5$ Å. Data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), only one crystallographic molecule was present in the asymmetric unit, with a solvent content of 44.3%. Structure determination was initiated by molecular replacement (MR) using *MOLREP* (Vagin & Teplyakov, 1997) with the structure of malonyl-CoA–acyl carrier protein transacylase (PDB code 1mla, Serre *et al.*, 1995) from *E. coli* as a template and the solution indicated that only one crystallographic molecule was present in the asymmetric unit. The template structure shared about 54.2% sequence identity with the Xoo FabD enzyme. The output model structure of this MR solution shows a well fitted electron-density map ($2F_o - F_c$) for all residues and the model will be improved by refinement. The structural details will be described in a separate paper. The structural data of the FabD (Xoo0880) protein will provide an insight into its enzymatic mechanism and be useful for developing antibacterial drugs against Xoo.

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