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Crystallization and preliminary X-ray crystallographic analysis of enoyl-ACP reductase III (FabL) from *Bacillus subtilis*

Kook-Han Kim, Joon Kyu Park, Byung Hak Ha, Jin Ho Moon and Eunice EunKyeong Kim*

Life Sciences Division, Korea Institute of Science and Technology, Seoul 136-791, South Korea

Correspondence e-mail: eunice@kist.re.kr

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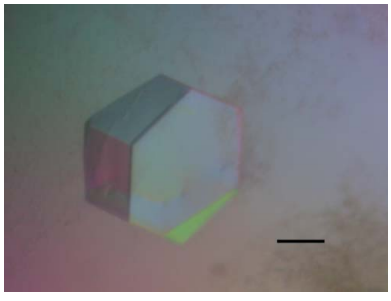
Enoyl-[acyl-carrier protein] reductase (enoyl-ACP reductase; ENR) is a key enzyme in type II fatty-acid synthase that catalyzes the last step in each elongation cycle. It has been considered as an antibiotic target since it is an essential enzyme in bacteria. However, recent studies indicate that some pathogens have more than one ENR. *Bacillus subtilis* is reported to have two ENRs, namely *Bs*FabI and *Bs*FabL. While *Bs*FabI is similar to other FabIs, *Bs*FabL shows very little sequence similarity and is NADPH-dependent instead of NADH-dependent as in the case of FabI. In order to understand these differences on a structural basis, *Bs*FabL has been cloned, expressed and and crystallized. The crystal belongs to space group *P*622, with unit-cell parameters $a = b = 139.56$, $c = 62.75$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and one molecule of FabL in the asymmetric unit. Data were collected using synchrotron radiation (beamline 4A at the Pohang Light Source, Korea). The crystal diffracted to 2.5 Å resolution.

1. Introduction

The increasing resistance of clinically important pathogens to antibiotic treatment is of worldwide concern and there have been continuous efforts to find new targets that will offer distinctively different mechanisms of action from current drug therapies. Among these is the fatty-acid biosynthesis pathway (Campbell & Cronan, 2001; Payne, 2004). The biosynthesis of fatty acids is a fundamental component of the cellular metabolic pathway, as fatty acids are the essential building blocks for membrane phospholipid formation (Wakil *et al.*, 1983). Most bacteria and plants synthesize fatty acids using a discrete and highly conserved group of enzymes called the type II fatty-acid synthase (FAS II) system, while yeast and animals utilize type I fatty-acid synthase (FAS I). FAS I consists of a polypeptide about 270 kDa in size with multiple active sites that perform all the catalytic reactions in the pathway, while FAS II is a dissociated system in which each component is encoded by a separate gene (see White *et al.*, 2005 and references therein). Since FAS II is quite distinct from FAS I, the enzymes in the type II FAS system have received enormous attention as possible antibiotic targets.

Trans-enoyl-[acyl-carrier-protein] reductase (enoyl-ACP reductase; ENR; EC 1.3.1.9) is one of the key components of FAS II. It completes each cycle of elongation by catalyzing the stereospecific reduction of the double bond at position 2 of a growing fatty-acid chain linked to the acyl-carrier protein in an NADH- or NADPH-dependent manner (Heath & Rock, 1995, 1996) and therefore plays a key role in the regulation of the pathway. As such, FabI, the NADH-dependent ENR, has been identified as a target for antimicrobial therapy. In fact, clinically important anti-infective agents such as triclosan (Levy *et al.*, 1999) and the antitubercular drug isoniazid (Banerjee *et al.*, 1994) target ENR and further novel classes of inhibitors have been reported (Ling *et al.*, 2004; Payne *et al.*, 2002; Sivaraman *et al.*, 2004). Genes encoding FabI are identified based on a high degree of overall sequence identity and the conserved spacing in the Tyr-$(Xaa)_6$-Lys dyad. The structure of FabI has been determined for *Escherichia coli* (Roujeinikova *et al.*, 1999; Stewart *et al.*, 1999; Baldock *et al.*, 1996, 1998), *Mycobacterium tuberculosis* (Rozwarski *et al.*, 1998, 1999) and *Brassica napus* (Rafferty *et al.*, 1995), including protein–inhibitor complexes.



Until recently, FabI was assumed to be the only enoyl-ACP reductase in the FAS II system and it was thus assumed that inhibitors effective against FabI would be effective against a broad spectrum of bacteria. However, an investigation of the molecular basis of the different sensitivities of Gram-positive and Gram-negative bacteria to triclosan led to a discovery that some bacteria such as *Streptococcus pneumoniae* and *Clostridium acetobutylicum* lack *fabI*. Instead, the corresponding catalysis is carried out by protein called FabK (also known as enoyl-ACP reductase II), a flavoprotein with very little sequence similarity encoded by *fabK*. Furthermore, pathogens such as *M. tuberculosis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* have been reported to have both FabI and FabK (Heath & Rock, 2000).

B. subtilis, one of the best-characterized members of the Gram-positive bacteria for which the complete genome sequence is known (Kunst *et al.*, 1997), is reported to have two ENRs (Heath, Li *et al.*, 2000; Heath, Su *et al.*, 2000). The first NADH-dependent FabI is homologous to *Escherichia coli* FabI (51% sequence identity to *Ec*FabI) and the second shows NADPH-dependent activity with very little sequence similarity to FabI (25% similarity to *Ec*FabI). The latter, named FabL (or YgaA or enoyl-ACP reductase III), is encoded by the *ygaA* gene and is comprised of 250 amino-acid residues (subunit MW = 27 178 Da) and contains the Tyr-$(Xaa)_6$-Lys dyad. It is inhibited by triclosan in a reversible manner, but does not form a stable NAD–triclosan ternary complex, indicating a clear difference from FabI. As the first step toward its structure elucidation, we have overexpressed *B. subtilis* FabL and crystallized it. Here, we report its purification and crystallization conditions as well as preliminary X-ray crystallographic data.

2. Experimental methods

2.1. Construct and expression

The *ygaA* gene was amplified by polymerase chain reaction (PCR) from *B. subtilis* strain 168 genomic DNA and the primers 5′-GGA ATT CCA TAT GGA ACA AAA TAA ATG TGC ACT CG-3′ and 5′-GGC CTC GAG AAC GAG CAG TGA GCG TCC-3′. The PCR product was purified, digested with *Nde*I and *Xho*I and ligated into pET22b expression vector (Novagen). This construction added a hexahistidine tag, with two intervening residues (Leu-Glu), at the C-terminus of the recombinant FabL. After confirmation of the DNA sequence, the resulting plasmid was transformed into *E. coli* BL21 (DE3) strain. Cells were grown in Luria–Bertani medium at 310 K with ampicillin (100 µg ml^{-1}) and expression was induced by 0.5 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) at an optical density of about 0.6 at 600 nm. Cells were allowed to grow at 291 K for 16 h after induction and were harvested and resuspended in ice-cold lysis buffer (50 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 2 m*M* 2-mercaptoethanol, 2.5% glycerol and 0.2 m*M* phenylmethanesulfonyl fluoride). The cells were disrupted by sonification and the crude lysate was centrifuged at 16 000*g* (Sorvall GSA rotor) for 30 min at 277 K; the cell debris was discarded.

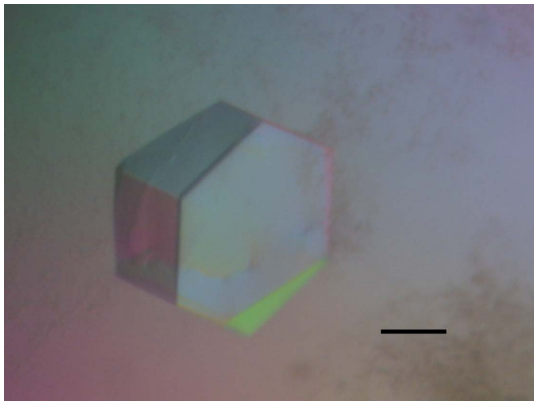


Figure 1
A crystal of FabL from *B. subtilis*. The scale bar is 0.1 mm in length.

2.2. Protein purification

The supernatant was loaded onto a nickel-chelated Hi-Trap chelating column (Amersham Biosciences) and eluted with a linear gradient of 20–500 m*M* imidazole in 50 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 2 m*M* 2-mercaptoethanol, 2.5% glycerol. The active fractions were pooled and applied onto a Pharmacia Hi-Trap Blue HP column, which was then eluted with a step salt gradient (0–2 *M* NaCl) in buffer. The active fraction was dialyzed and applied onto an anion-exchange column (Q-Sepharose, Amersham Biosciences), which was eluted at a rate of 3 ml min^{-1} with 50 m*M* Tris–HCl pH 8.0, 2.5% glycerol, 1 *M* NaCl, 1 m*M* dithiothreitol (DTT) plus a linear gradient of 0.1–1 *M* NaCl. The final purification step was gel filtration on a HiLoad 26/60 Superdex-75 prep-grade column (Amersham Biosciences), which was previously equilibrated with a buffer containing 50 m*M* Tris–HCl pH 8.0, 2.5% glycerol, 500 m*M* NaCl, 1 m*M* DTT. The protein was incubated with NADPH (Sigma) in a 1:2 molar ratio for 1 h prior to loading. An AKTA FPLC system (Amersham Pharmacia) was used throughout.

The protein was concentrated to 8.2 mg ml^{-1} using a YM10 membrane and Amicon concentrator (Amicon) and the concentration was determined using the Bio-Rad protein assay with BSA as a standard. FabL protein in 50 m*M* Tris–HCl pH 8.0, 2.5% glycerol, 500 m*M* NaCl, 1 m*M* DTT was used for crystallization.

2.3. Crystallization

Initial screening for crystallization conditions was carried out by the sitting-drop vapour-diffusion method using 96-well Intelli plates (Hampton Research) and a Hydra II Plus One (Matrix Technology) robotic system at 295 K. The protein concentration was 8.2 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0, 2.5% glycerol, 500 m*M* NaCl, 1 m*M* DTT. A sitting drop was prepared by mixing 200 nl each of the protein solution and the reservoir solution and was equilibrated against 70 µl reservoir solution. The initial search for crystallization conditions was performed using commercially available kits from Hampton Research (Aliso Viejo, CA, USA), Jena Bioscience (Jena, Germany) and Emerald BioSystems (Bainbridge Island, WA, USA). Out of the 1200 conditions screened, five gave microcrystals after 1–5 d: Grid Screen MPD No. 9 [0.1 *M* MES pH 6.0, 20%(*v*/*v*) MPD], Natrix I No. 15 [0.04 *M* $MgCl_2$, 0.05 *M* sodium cacodylate pH 6.0, 5%(*w*/*v*) MPD] and Crystal Screen I No. 13 [30%(*w*/*v*) PEG 400, 0.1 *M* Na HEPES pH 7.5, 0.2 *M* $MgCl_2$] from Hampton Research, Wizard I No. 23 [15%(*v*/*v*) ethanol, 0.1 *M* imidazole pH 8.0, 0.2 *M* $MgCl_2$, final pH 7.8] from Emerald Biostructures and JB Screen 7 No. 23 [47%(*w*/*v*) MPD, 2%(*w*/*v*) *t*-butanol] from Jena Bioscience.

These conditions were optimized using hanging-drop vapour-diffusion experiments. Each hanging drop was prepared by mixing 1.5 µl protein solution and 1.5 µl reservoir solution and was equilibrated over 0.5 ml reservoir solution. Optimization gave a few reasonably sized crystals, but they were generally not suitable for data collection. Diffraction-quality crystals were obtained with a reservoir solution consisting of 0.04 *M* $MgCl_2$, 0.05 *M* sodium cacodylate pH 6.0 and 11%(*v*/*v*) MPD at 277 K, an optimization of Natrix I No. 15.

Table 1
Data-collection statistics for BsFabL.

Values in parentheses are for the highest resolution shell.

X-ray source	Pohang Light Source, beamline 4A
Wavelength (Å)	0.97980
Space group	*P*622
Unit-cell parameters (Å, °)	$a = b = 139.56$, $c = 62.75$, $\alpha = \beta = 90$, $\gamma = 120$
Temperature (K)	100
Resolution range (Å)	50.0–2.5 (2.59–2.50)
Total/unique reflections	932524/12983
Completeness (%)	99.4 (98.9)
Mean $I/\sigma(I)$	24.3 (2.5)
$R_{\rm merge}$† (%)	6.4 (30.6)

† $R_{\rm merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements.

The single hexagonal plate-shaped crystals grew in 3–5 d (see Fig. 1). The crystals grew to approximate dimensions of 0.18 × 0.12 × 0.15 mm at 277 K.

2.4. Data collection

For X-ray data collection, a crystal was transferred to a solution consisting of 0.04 *M* $MgCl_2$, 0.05 *M* sodium cacodylate pH 6.0, 11%(*v*/*v*) MPD, 25%(*w*/*v*) glycerol before being flash-frozen. X-ray diffraction data were collected at 100 K using an ADSC Quantum CCD detector (Madison, WI, USA) at beamline 4A of Pohang Light Source (PLS), South Korea. The crystal-to-detector distance was set to 250 mm. A total of 250 images were collected with 1.0° oscillation and 10 s exposure per image. The wavelength used was 0.97980 Å.

Diffraction data were collected to 2.50 Å resolution and were processed and scaled with the programs *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997). The crystals belong to the hexagonal space group *P*622, with unit-cell parameters $a = b = 139.56$, $c = 62.75$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. A total of 932 524 reflections were measured, yielding 12 983 unique reflections with an $R_{\rm merge}$ (on intensity) of 6.4%. The merged data set is 99.4% complete to 2.50 Å resolution. The presence of one molecule of FabL in the asymmetric unit gives a crystal volume per protein weight ($V_{\rm M}$) of 3.3 $Å^3\,Da^{-1}$, with a corresponding solvent content of 62.7% (Matthews, 1968). The statistics of data collection are summarized in Table 1. Based on the assumption that FabL would have a conserved fold like that of FabI, we tried to solve the structure of BsFabL by molecular replacement using the programs *CNS* (Brünger *et al.*, 1998) and *AMoRe* (Navaza, 1994) using various structures of FabI as a search model (PDB codes 1i2z, 1d7o, 1d8a, 1jw7 and 1spx), but all attempts have been so far unsuccessful. There are eight methionine residues in BsFabL and efforts towards structure determination using MAD with selenomethione-substituted FabL crystals are currently in progress.

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