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Crystallization and preliminary X-ray crystallographic studies of a new class of enoyl-(acyl-carrier protein) reductase, FabV, from *Vibrio fischeri*

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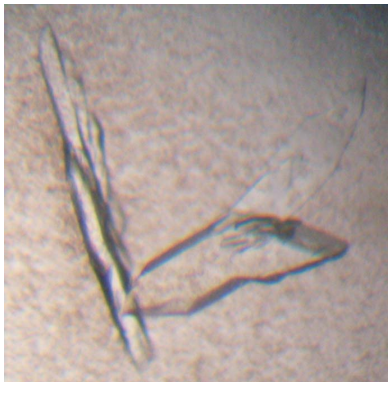
Enoyl-(acyl-carrier protein) reductase (ENR) catalyzes the last step of the fatty-acid elongation cycle of the bacterial fatty-acid biosynthesis (FAS II) pathway. Recently, a new class of ENR has been identified from *Vibrio cholerae* and was named FabV. In order to understand the molecular mechanism of the new class of ENR at the structural level, FabV from *V. fischeri* was overexpressed, purified and crystallized. Diffraction data were collected to 2.7 Å resolution from a native crystal. The crystal belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 123.53, b = 164.14, c = 97.07 Å. The presence of four molecules of FabV in the asymmetric unit gave a V_M value of 2.81 Å^3 Da^{-1}, with a corresponding solvent content of 54.5%.

1. Introduction

Fatty-acid biosynthesis is essential for survival in mammals, plants, fungi and bacteria, with the exception being the archaea. The bacterial fatty-acid synthesis system (FAS II) is carried out by a series of separate enzymes and differs significantly from the mammalian and fungal system (FAS I), which utilizes a large complex multi-functional enzyme (Lu *et al.*, 2004; White *et al.*, 2005). The differences between the FAS I and FAS II systems make the FAS II enzymes potential targets for antibacterial inhibitors (Campbell & Cronan, 2001; Heath & Rock, 2004; Payne *et al.*, 2001; Zhang *et al.*, 2006). In particular, the enoyl-(acyl-carrier protein) reductase (ENR), which catalyzes the last reaction in each elongation cycle, has been the most widely targeted enzyme of this pathway and antibacterial compounds that target this enzyme such as triclosan and isoniazid have been discovered (Heath *et al.*, 1998; McMurry *et al.*, 1998; Musser *et al.*, 1996; Tonge *et al.*, 2007).

In bacteria, four ENR isoenzymes have been identified: FabI (Bergler *et al.*, 1994; Thomas & Cronan, 2005), FabK (Heath & Rock, 2000; Marrakchi *et al.*, 2003), FabL (Heath *et al.*, 2000) and FabV (Massengo-Tiassé & Cronan, 2008). FabI, FabL and FabV belong to the short-chain dehydrogenase/reductase (SDR) superfamily, whereas FabK is a TIM-barrel flavoprotein. FabV was recently identified in the Gram-negative bacterium *Vibrio cholerae*, which causes cholera in humans. FabVs, the third SDR superfamily of ENRs, have very low sequence identity (below 20%) and have distinct features compared with the other SDR ENRs (FabI and FabL). Firstly, they are essentially refractory to inhibition by triclosan, whereas FabI is very sensitive to triclosan and FabL is very poorly inhibited by triclosan. Secondly, they have the typical Tyr-X_n-Lys motif found in the SDR superfamily, although it is considerably larger than in the other SDR superfamilies of ENRs. Instead of the Tyr-X_6-Lys active-site motif which is found in FabI and FabL, FabV contains a Tyr-X_8-Lys eight-residue gap between the tyrosine and lysine residues in the active site (Massengo-Tiassé & Cronan, 2008); this is the largest known spacing in the SDR superfamily (Persson *et al.*, 2003). Comparative analysis using a *BLAST* sequence-similarity search found homologues in a number of bacterial genomes, including the clinically important pathogens *Vibrio* spp., *Yersinia pestis* and *Pseudomonas aeruginosa*.



To date, no three-dimensional structure of FabV has been reported. Therefore, in this study we overexpressed, crystallized and conducted preliminary X-ray crystallographic analysis of FabV from *V. fischeri*, which has 87% sequence identity to that from *V. cholerae*, with the goal of better understanding the molecular mechanism of FabV.

2. Materials and methods

2.1. Cloning, overexpression and purification

The full-length *fabV* gene (EC 1.3.1.9; UniProtKB accession No. Q5E6G3) was amplified from *V. fischeri* genomic DNA by polymerase chain reaction (PCR) using the forward primer 5′-GGAATTC**CATATG**ATGATCATCAAACCTAGAAT-3′ and the reverse primer 3′-CCGCCG**CTCGAG**TTAGATATCAGCAACATCAA-5′ (*Nde*I and *Xho*I restriction sites are indicated in bold and the stop codon is underlined). PCR reactions were performed using 30 PCR cycles (denaturation at 368 K for 30 s, annealing at 328 K for 30 s and 345 K for 120 s). The PCR product was digested with *Nde*I and *Xho*I and ligated into the pET-22b vector (Novagen, USA), which was used to express the native protein without extra amino acids or tag sequences. The resulting plasmid was transformed into *Escherichia coli* BL21 (DE3) strain (Novagen, USA) and the cells were grown at 310 K in Luria–Bertani medium supplemented with ampicillin (50 µg ml^{-1}). Protein expression was induced by adding 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) once the cells reached an optical density at 600 nm of about 0.45. The cells were then grown for an additional 12 h at 291 K. The cells were harvested by centrifugation at 5000*g* for 30 min at 277 K. The pelleted cells were suspended in buffer *A* (20 m*M* Tris–HCl pH 7.9, 50 m*M* NaCl) and lysed by sonication. The crude lysate was centrifuged at 20 000*g* for 1 h at 277 K. The supernatant was subjected to ion-exchange chromatography on a HiTrap DEAE FF column (GE Healthcare, USA). The protein was stepwise eluted with 20 m*M* Tris–HCl pH 7.9 buffer containing 100, 200, 300 and 400 m*M* NaCl. Most of the protein was eluted with 100 and 200 m*M* NaCl. The fractions that eluted at 100 and 200 m*M* NaCl were pooled and applied onto a HiTrap Phenyl HP column (GE Healthcare, USA), which was previously equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.9, 2 *M* NaCl). After the protein sample had been loaded, the column was washed with two column volumes of buffer *B* followed by a linear gradient of buffer *C* (20 m*M* Tris–HCl pH 7.9) using ten column volumes. FabV was eluted with 20 m*M* NaCl. The protein was purified to its final state by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Healthcare, USA) that had previously been equilibrated with buffer *D* (20 m*M* Tris–HCl pH 7.9, 200 m*M* NaCl, 2 m*M* DTT). All chromatographic steps were carried out at 277 K. Prior to crystallization experiments, the purified protein was concentrated to 10 mg ml^{-1} using an Amicon Ultra-15 ultrafiltration device (Millipore, USA). This procedure yielded approximately 10 mg of FabV protein per litre of culture. The protein concentration was determined using the Bradford assay and protein purity was examined by 12% SDS–PAGE.

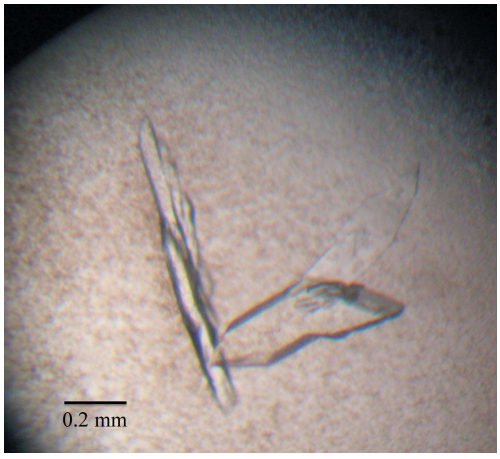


Figure 1
Crystals of the FabV protein from *V. fischeri*. The crystal dimensions were 0.3 × 0.6 × 0.1 mm.

2.2. Crystallization and X-ray analysis

Initial crystallization screens were performed using the sitting-drop vapour-diffusion method (0.5 µl protein solution and 0.5 µl reservoir solution equilibrated against 50 µl reservoir solution) to set up 96-well Intelli-Plates (Art Robbins Instruments, USA) at 295 K. The initial screening was set up by hand using an eLINE electronic pipette (Biohit, Finland). Commercial screening kits such as Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, PEGRx, PEGRx 2, Index (Hampton Research, USA) and Morpheus (Molecular Dimensions, UK) were used. Crystals were obtained under the following conditions: 0.1 *M* Morpheus buffer system 3 pH 8.5 (mixed together according to the buffer-composition table to achieve the appropriate pH; 30 ml 1 *M* Bicine and 46.8 ml 1 *M* Tris), 0.09 *M* NPS (stock solution consisting of 0.3 *M* sodium nitrate, 0.3 *M* sodium phosphate and 0.3 *M* ammonium sulfate) and 37.5%(*v*/*v*) MPD_P1K_P3350 [stock solution consisting of 25%(*v*/*v*) MPD, 25%(*v*/*v*) PEG 1K and 25%(*v*/*v*) PEG 3350]. To improve the quality of the crystals, further optimization was performed using the Additive Screen kit (Hampton Research, USA). Crystal optimization experiments were carried out using the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research, USA). The best crystals were grown at 295 K in drops consisting of a mixture of 1 µl protein solution and 1 µl reservoir solution, which consisted of 0.1 *M* Morpheus buffer system 3 pH 9.0 (a mixture of 30 ml 1 *M* Bicine with 160 ml 1 *M* Tris), 0.09 *M* NPS, 38%(*v*/*v*) MPD_P1K_P3350 and 0.2 µl 0.5 *M* sodium fluoride as an additive solution. Single crystals were obtained within 5 d. Crystals were flash-cooled directly in a liquid N_2 stream at 100 K as the mother liquor is an adequate cryoprotectant. Data sets were collected on beamline 6C at Pohang Light Source (Pohang, Republic of Korea) using an ADSC Quantum 210 CCD detector. The X-ray beam size was 80 × 300 µm. A total range of 240° was covered with 1.0° oscillation and 30 s exposure per frame. The wavelength of the synchrotron X-rays was 1.23986 Å. The crystal-to-detector distance was set to 150 mm. X-ray diffraction data were collected to 2.7 Å resolution and all data sets were indexed, processed and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

The gene encoding the FabV protein from *V. fischeri* was cloned into pET-22b vector. The protein, which contains 400 amino acids with a calculated molecular weight of 43.8 kDa, was overexpressed in *E. coli* BL21 (DE3). Crystals suitable for X-ray analysis were obtained under the optimized crystallization conditions using 0.1 *M* Morpheus buffer system 3 pH 9.0, 0.09 *M* NPS, 38%(*v*/*v*) MPD_P1K_P3350 and 50 m*M* sodium fluoride (Fig. 1). The crystal belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 123.53, b = 164.14, c = 97.07 Å , $\alpha = \beta = \gamma = 90°$. X-ray diffraction data were collected to 2.7 Å resolution. Although the FabV protein was shown to be a monomer by gel-filtration chromatography, as reported previously

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 123.53, b = 164.14, c = 97.07
Resolution range (Å)	50–2.7 (2.80–2.70)
Total reflections	309297
Unique reflections	52370
Multiplicity	5.9 (3.1)
Completeness (%)	96.1 (89.4)
$R_{\rm merge}$ (%)†	9.3 (40.9)
$\langle I/\sigma(I)\rangle$	15.0 (1.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ represents the observed intensity, $\langle I(hkl)\rangle$ represents the average intensity and i counts through all symmetry-related reflections.

(Massengo-Tiassé & Cronan, 2008), each asymmetric unit contained four molecules of protein, yielding a crystal volume per protein mass ($V_{\rm M}$) of 2.81 Å^3 Da^{-1} and a solvent content of 54.5% (Matthews, 1968). A self-rotation function calculation using the program *MOLREP* (Vagin & Teplyakov, 2010) only showed strong peaks in the $\kappa = 180^\circ$ section, revealing the existence of three mutually perpendicular twofold rotation axes (data not shown) and supporting the presence of a tetramer in the asymmetric unit with 222 point symmetry, as in other known crystal structures of the FabI isozyme. The data-collection statistics are summarized in Table 1. For MAD phasing, selenomethionine-substituted protein was expressed and purified and crystallization experiments are currently in progress.

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