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Purification, crystallization and preliminary X-ray diffraction analysis of 3-ketoacyl-CoA thiolase A1887 from *Ralstonia eutropha* H16

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The gene product of A1887 from *Ralstonia eutropha* (ReH16_A1887) has been annotated as a 3-ketoacyl-CoA thiolase, an enzyme that catalyzes the fourth step of β -oxidation degradative pathways by converting 3-ketoacyl-CoA to acyl-CoA. ReH16_A1887 was overexpressed and purified to homogeneity by affinity and size-exclusion chromatography. The degradative thiolase activity of the purified ReH16_A1887 was measured and enzyme-kinetic parameters for the protein were obtained, with K_m , V_{max} and k_{cat} values of 158 μM , 32 $mM \min^{-1}$ and $5 \times 10^6 s^{-1}$, respectively. The ReH16_A1887 protein was crystallized in 17% PEG 8K, 0.1 M HEPES pH 7.0 at 293 K and a complete data set was collected to 1.4 Å resolution. The crystal belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 129.52$, $c = 114.13$ Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contained two molecules, with a solvent content of 58.9%.

1. Introduction

Ralstonia eutropha H16 is a representative bacterial strain which has been used in polyhydroxyalkanoate (PHA) biosynthesis for nearly 50 years owing to its ability to produce and store large amounts of poly[*R*-(−)-3-hydroxybutyrate] and other polyesters that can be harnessed to make biodegradable plastics. The production of PHAs from renewable carbon sources such as plant oils has been explored. Plant oils could be used for a suitable carbon source for this procedure as 3-hydroxyacyl coenzyme A (3-hydroxyacyl-CoA) PHA precursors can be produced from intermediates in the fatty-acid degradation pathway (Lageveen *et al.*, 1988; Vo *et al.*, 2008). Recently, plant oils have been investigated as an alternative to petroleum for the production of chemicals (Carlsson, 2009). Plant oils are composed of triacylglycerols (TAGs), in which three fatty acids are joined to a glycerol backbone. *R. eutropha* must employ a fatty-acid degradation pathway to consume oils and fatty acids as carbon sources for the production of bioplastics.

Fatty acids are abundant in all life forms in diverse forms in the degree of saturation and chain length, and are essential elements for energy storage or as structural components of biomembranes. Fatty acids are broken down in a cyclic manner to generate a range of products by the process known as β -oxidation (Knoop, 1905). Fatty acyl-CoAs of short chain length can then be subjected to further rounds of β -oxidation or directed to other pathways. β -Oxidation is employed by channelling reactions, which involve four enzymatic activities. Four different enzymes, acyl-CoA dehydrogenase (ACD), 2-enoyl-CoA hydratase (ECH), L-3-hydroxyacyl-CoA dehydrogenase (HACD) and 3-ketoacyl-CoA thiolase (KACT) are

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involved in each reaction (Kim & Battaile, 2002). The bacterial and mitochondrial fatty-acid β -oxidation multi-enzyme complexes predominantly exhibit the last three of these four enzymic activities. The sequential β -oxidation reactions shorten the saturated acyl-CoA by two C atoms in each step to finally produce acetyl-CoA (Ac-CoA). The most obvious case of channelling is found in bacteria such as *Escherichia coli* and *Pseudomonas fragi* (Kunau *et al.*, 1995). The β -ketothiolase inhibitor acrylic acid has been shown to be a powerful tool for the diversion of intermediates of fatty-acid β -oxidation towards PHA biosynthesis in the non-PHA-accumulating *E. coli* and *P. fragi* when a PHA synthase gene was expressed (Antonio *et al.*, 2000; Qi *et al.*, 1998; Fiedler *et al.*, 2000).

3-Ketoacyl-CoA thiolase (KACT) catalyzes the last reaction of β -oxidation: degradative cleavage of a β -ketoacyl-CoA to acyl-CoA and a two-carbon shortened acyl-CoA (Gilbert, 1981; Gilbert *et al.*, 1981). There are two classes of 3-ketoacyl-CoA thiolases. Type I thiolases (3-ketoacyl-CoA thiolases; EC 2.3.1.16) are enzymes involved in the reverse Claisen condensation reaction in, for example, the β -oxidation cycle. Type II thiolases (acetoacetyl-CoA thiolases; ACATs; EC 2.3.1.9) are enzymes involved the anabolic mevalonate pathway that perform Claisen condensation. *R. eutropha* possess both kinds of thiolase, types I and II. Most studies of microbial fatty-acid β -oxidation have been conducted in *E. coli* and *Bacillus subtilis* (Fujita *et al.*, 2007; Matsuoka *et al.*,

2007). The fatty-acid β -oxidation pathway in *R. eutropha* is uncharacterized in the literature. A search of the *R. eutropha* H16 genome revealed 50 genes annotated as enoyl-CoA hydratases and 46 genes annotated as acyl-CoA dehydrogenases as potential β -oxidation pathway gene homologues (Pohlmann *et al.*, 2006). However, it has not been revealed which of these homologues actually plays a role in fatty-acid β -oxidation. In a previous study, we reported on PhaA derived from *Ralstonia eutropha* H16. This PhaA belongs to the type I thiolases, is involved in a biosynthetic pathway and exists as a tetramer (Kim & Kim, 2014). In this report, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of ReH16_A1887 (Uniprot ID Q0KAI3), a protein annotated as a 3-ketoacyl-CoA thiolase (type II) from *R. eutropha* (Fig. 1a). We also report the enzyme-kinetic properties of ReH16_A1887.

2. Materials and methods

2.1. Production and kinetic measurements of ReH16_A1887

The forward and reverse primers were designed as 5'-GCGCGCATATGCAACAAGCCGTCATCGTCGACGC-3' and 5'-GCGCGCTCGAGCAGGCGCTCGATGATGGTGGCG-3' to introduce NdeI and XhoI restriction sites, respectively (underlined). The ReH16_A1887 coding gene was amplified from *R. eutropha* H16 chromosomal DNA by

Figure 1

Purification and enzyme kinetics of ReH16_A1887. (a) Diagram of the enzyme reaction of ReH16_A1887. (b) SDS-PAGE of purification of recombinant ReH16_A1887 protein. Lane 1 shows molecular-weight markers (labelled in kDa). Lanes 2–10 show the purification procedure of ReH16_A1887 using Ni-NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, supernatant and pellet fraction after centrifugation of whole cell extract, respectively; lane 5, flowthrough from Ni-NTA column; lanes 6, 7, 8 and 9, wash with 0, 3, 6 and 9 mM imidazole, respectively; lane 10, elution with 300 mM imidazole. Purified ReH16_A1887 is indicated with an arrow. (c) Enzyme kinetics of ReH16_A1887. Reaction velocity was plotted *versus* substrate concentration based on the Michaelis–Menten equation. For the various substrate concentrations, 100, 200, 400, 600, 800 and 1000 μM acetoacetyl-CoA were used.

Table 1

Macromolecule-production information.

The underlined characters in the primers indicate restriction-enzyme sites.

Source organism	<i>R. eutropha</i> H16
DNA source	Chromosomal DNA
Forward primer	GCGCGCATATGCAACAAGCGTCATCGTCGACGC
Reverse primer	GCGCGCTCGAGCAGGCGCTCGATGATGGTGGCG
Cloning vector	pET-30a
Expression vector	pET-30a
Expression host	<i>E. coli</i> BL21(DE3)-T1 ^R
Complete amino-acid sequence of the construct produced	MQQAVIVDAIRSPMGRSKPGSAFTHELHATELLAQ-VIKGLVERNKLDPGLVDDVITGCVTQAGEQSA-GPGRVAWLAAGFPDHPATTIDRKCGSSQAV-HFAAQGIMAGAYDIVIACGIESMSRVPMGSAR-IQNPYGPSMEARYAPGLVSQGVAAELVAAKY-ELSRHMDSYSARSHELAATARESGAFREIL-GISTPNGLVEQDETIRPGTSVEKLGTLQASFR-NDELSARFPQIGWNVTAGNASQISDGASAMLL-MSEMAQRLGLKPRARFVAFDVCDDPVMMLT-APIPASQRAIKKSGLKLDQIDHYEINEAFACV-PLAWQALGADPARLNPRGGAIALGHPLASGV-RLMTTMLHALED SGQRYGLQSMCEAGGMANAT-IIERLLEHHHHH

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	SPL plate (24-well)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	25
Buffer composition of protein solution	40 mM Tris-HCl pH 8.0
Composition of reservoir solution	17% (w/v) polyethylene glycol 8K, 0.1 M HEPES pH 7.0
Volume and ratio of drop	2 µl; 1:1 ratio of protein and reservoir solutions
Volume of reservoir (ml)	0.5

polymerase chain reaction (PCR). The PCR product was then subcloned into pET-30a (Invitrogen) with a six-His tag at the C-terminus. The resulting expression vector pET-30a: *ReH16_A1887* was transformed into *E. coli* BL21(DE3)-T1^R strain, which was grown in 1 l LB medium containing kanamycin (50 mg ml⁻¹) at 310 K. At an OD₆₀₀ of 0.6, *ReH16_A1887* protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h at 291 K, the cells were harvested by centrifugation at 4000g for 15 min at 277 K. The cell pellet was resuspended in buffer A (40 mM Tris-HCl pH 8.0) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13 500g for 25 min and the lysate was applied onto an Ni-NTA agarose column (Qiagen). After washing with buffer A containing 30 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, the trace amount of contaminants was removed by size-exclusion chromatography using a Superdex 200 prep-grade column (320 ml, GE Healthcare) equilibrated with buffer A containing 5 mM β-mercaptoethanol (BME). The protein eluted at a molecular weight of ~80 kDa, indicating that the *ReH16_A1887* protein formed a dimeric structure. All purification experiments were performed at 277 K. The purity of the final protein was assessed by SDS-PAGE. The purified protein was concentrated to 25 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 5 mM BME.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 7A, PAL
Wavelength (Å)	0.979340
Temperature (K)	100
Detector	Quantum 270 CCD
Crystal-to-detector distance (mm)	160
Rotation range per image (°)	1.00
Total rotation range (°)	120
Exposure time per image (s)	1
Space group	<i>P</i> 4 ₃ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	129.52, 129.52, 114.13
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90.00, 90.00, 90.00
Mosaicity (°)	0.217
Resolution range (Å)	50.00–1.40 (1.42–1.40)
Total No. of reflections	1214750
No. of unique reflections	181268
Completeness (%)	95.7 (87.2)
Multiplicity	6.7 (4.2)
<i>I</i> / <i>σ</i> (<i>I</i>)	38.00 (3.78)
<i>R</i> _{merge} † (%)	6.9 (30.9)
Overall <i>B</i> factor from Wilson plot (Å ²)	14.584

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections.

Macromolecule-production information is summarized in Table 1.

The activity assays were performed with a reaction mixture of 1 ml in total volume. The reaction mixture consisted of 0.1 M Tris-HCl pH 8.3, 25 mM MgCl₂, 3-acetoacetyl-CoA, 1000 µM CoA and 410 nM *ReH16_A1887* enzyme. After pre-incubation, the reaction was initiated by addition of enzyme. The decrease in 3-ketoacyl-CoA was then measured at 303 nm using an extinction coefficient of $8.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. All experiments were performed at 303 K. The enzyme-kinetics experiments were performed by the addition of various concentrations of 3-acetoacetyl-CoA substrate: 100, 200, 400, 600, 800 and 1000 µM.

2.2. Crystallization of *ReH16_A1887*

Crystallization of the purified *ReH16_A1887* protein was initially performed with commercially available sparse-matrix screens including Index, PEG/Ion, PEG/Ion 2 (Hampton Research), Wizard Classic 1 and 2, Wizard Cryo 1 and 2 (Rigaku) and Structure Screen 1 and 2 (Molecular Dimensions) using the sitting-drop vapour-diffusion method at 293 K. Each experiment consisted of mixing 1.0 µl protein solution (140 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0) with 1.0 µl reservoir solution and then equilibrating against 0.5 ml reservoir solution. *ReH16_A1887* crystals were observed from three crystallization screen conditions: (i) 25% polyethylene glycol 3350, 0.1 M HEPES pH 7.5, (ii) 20% polyethylene glycol 8K, 0.1 M HEPES pH 7.5, (iii) 20% polyethylene glycol 8K, 0.1 M MES pH 8.5, 0.2 M calcium acetate. After several rounds of crystal improvement, suitable crystals for diffraction experiments were obtained from a precipitant consisting of 17% PEG 8K, 0.1 M HEPES pH 7.0 and reached maximum dimensions of 0.2 × 0.2 × 0.5 mm within 7 d. Crystallization information is summarized in Table 2.

2.3. X-ray diffraction analysis of *ReH16_A1887*

Diffraction data were collected from the *ReH16_A1887* crystals on beamline 7A of the Pohang Accelerator Laboratory (PAL) using a Quantum 270 CCD detector (ADSC, USA). The data were indexed, integrated and scaled using the *HKL-2000* software package (Otwinowski & Minor, 1997). The data statistics are summarized in Table 3.

3. Results and discussion

ReH16_A1887 was purified to apparent homogeneity by metal-affinity chromatography followed by size-exclusion chromatography. The elution volume of the major peak corresponds to a dimeric form, with an apparent molecular weight of ~ 80 kDa from a gel-filtration column (data not shown) calibrated with standard molecular-weight proteins.

Figure 2
Tetragonal crystals of *ReH16_A1887*. Crystals of the best quality were produced from the condition 17% PEG 8K, 0.1 M HEPES pH 7.0 and grew to maximum dimensions of $0.2 \times 0.2 \times 0.5$ mm within 7 d.

SDS-PAGE of the purified enzyme showed a single band with a monomeric molecular weight of 40 kDa (Fig. 1*b*). To characterize the properties of *ReH16_A1887*, kinetic analysis was performed by measuring the degradation reaction of acetoacetyl-CoA to acetyl-CoA. Reaction rates corresponding to various concentrations of substrate were plotted and determined to obey Michaelis–Menten kinetics. Based on this kinetic analysis, the K_m , V_{max} and k_{cat} values of *ReH16_A1887* with acetoacetyl-CoA were determined to be $158 \mu\text{M}$, 32 mM min^{-1} and $5 \times 10^6 \text{ s}^{-1}$, respectively (Fig. 1*c*).

The *ReH16_A1887* protein was crystallized in 17% PEG 8K, 0.1 M HEPES pH 7.0 at 293 K (Fig. 2). The crystals were transferred into a cryoprotectant solution consisting of 17% PEG 8K, 0.1 M HEPES pH 7.0, 30% glycerol, fished out with a loop larger than the crystals and flash-cooled by immersion in liquid nitrogen at 100 K. X-ray diffraction data were collected to a resolution of $1.4 \text{ \AA}$ on beamline 7A at the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using a Quantum 270 CCD detector (ADSC, USA) (Fig. 3). The crystals of *ReH16_A1887* belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 129.52$, $c = 114.13 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$. Calculation of a self-Patterson function revealed that a non-origin peak is present and calculation of the self-rotation function was consistent with packing in space group $P4_32_12$ (data not shown). Moreover, a twinning test revealed that there was no indication of twinning (data not shown). Assuming the presence of two molecules of *ReH16_A1887* per asymmetric unit, the crystal volume per unit of protein mass was $2.99 \text{ \AA}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 58.9%.

We attempted phase determination using the molecular-replacement method and a solution was found using the thiolase from *Mycobacterium tuberculosis* (MtFadA5; PDB

Figure 3
Diffraction pattern of the *ReH16_A1887* crystal. The crystal diffracted to a maximum resolution of $1.4 \text{ \AA}$.

entry 4ubu; Stillman *et al.*, 1993) with side chains converted to alanines as a search model. *ReH16_A1887* has 39% amino-acid sequence identity to *MtFadA5*. *MOLREP* (Vagin & Teplyakov, 2010) located two polyalanine-model molecules in the asymmetric unit. The resulting solution had a correlation coefficient and *R* factor of 0.532 and 62.1%, respectively. After rigid-body refinement using *REFMAC5* (Murshudov *et al.*, 2011) from the *CCP4* suite (Winn *et al.*, 2011) in the resolution range 50–1.4 Å, *R* and *R*_{free} were 32.52 and 42.23%, respectively, with a correlation coefficient of 0.652. The initial electron-density map, which was of good quality with backbone atoms well defined by electron density, allowed us to build a three-dimensional *ReH16_A1887* model. Crystallographic model building and refinement of the structure to 1.4 Å resolution are in progress.

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