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Crystal structure and biochemical properties of *Re*H16_A1887, the 3-ketoacyl-CoA thiolase from *Ralstonia eutropha* H16

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ABSTRACT

*Re*H16_A1887 from *Ralstonia eutropha* is an enzyme annotated as a 3-ketoacyl-CoA thiolase, and it catalyzes the fourth step of β-oxidation degradative pathways by converting 3-ketoacyl-CoA to acyl-CoA. We determined the crystal structures of *Re*H16_A1887 in the apo-form and in complex with its CoA substrate. *Re*H16_A1887 functions as a dimer, and the monomer of *Re*H16_A1887 comprises three subdomains (I, II, and III). The structural comparison between the apo-form and the CoA-bound form revealed that *Re*H16_A1887 undergoes a structural change in the lid-subdomain (subdomain III) upon the binding of the CoA substrate. The CoA molecule was stabilized by hydrogen bonding with positively charged residues such as Lys18, Arg210, and Arg217, and residues Thr213 and Gln151 aid its binding as well. At the active site of *Re*H16_A1887, highly conserved residues such as Cys91, His348, and Cys378 were located near the thiol-group of CoA, indicating that *Re*H16_A1887 might catalyze the thiolase reaction in a way similar to other thiolases. Moreover, in the vicinity of the covalent nucleophile Cys91, a hydrophobic hole that might serve as a binding site for the acyl-group of 3-ketoacyl-CoA was observed. The residues involved in enzyme catalysis and substrate-binding were further confirmed by site-directed mutagenesis experiments.

1. Introduction

Ralstonia eutropha H16 first attracted biotechnological interest nearly 50 years ago with the realization that its ability to produce and store large amounts of polyhydroxyalkanoate (PHA) that could be harnessed to make biodegradable plastics. The strain can store PHA up to 80% of its cell dry weight as a result of nutrient limitation [1]. Many groups have explored production of PHAs from renewable carbon sources such as plant oils. Plant oils are a suitable carbon source for this endeavor as 3-hydroxyacyl coenzyme A (3-hydroxyacyl-CoA) PHA precursors can be produced from intermediates in the fatty acid degradation pathway [2,3]. Plant oils consist of triacylglycerols (TAGs), in which three fatty acids are joined to a glycerol backbone. Recently, plant oils have been explored as a possible feedstock alternative to petroleum for chemical production [4]. These oils can also be used as sources of carbon for bioplastic production by bacteria such as *R. eutropha*. *R. eutropha* must therefore employ a fatty acid degradation pathway to consume oils and fatty acids.

Fatty acids are fundamental biomolecules that are abundant in all life forms. With their enormous variation in chain length and degree of saturation, they are essential for energy storage, form structural entities in biomembranes, and serve as signaling molecules. Fatty acids are broken down in a cyclic manner, two carbons at a time, to generate a range of products by the process known as β-oxidation [5]. The shortened fatty acyl-CoA can then be subjected to further rounds of β-oxidation or directed to other pathways. The fatty acid β-oxidation spiral involves four enzymes, acyl-CoA dehydrogenase (ACD), 2-enoyl-CoA hydratase (ECH), L-3-hydroxyacyl-CoA dehydrogenase (HACD) and 3-ketoacyl-CoA thiolase (KACT) [6]. Among these enzymes, KACT catalyzes the degradative cleavage of a β-ketoacyl-CoA to acyl-CoA and a two-carbon shortened acyl-CoA [7,8].

There are two distinct forms of 3-ketoacyl-CoA thiolases. Type I is the 3-ketoacyl-CoA thiolase (EC 2.3.1.16), a catabolic enzyme performing the reverse Claisen condensation reaction involved in such as the β-oxidation cycle. Type II is the acetoacetyl-CoA thiolase (ACAT; EC 2.3.1.9), which is involved in the anabolic mevalonate pathway performing Claisen condensation. *R. eutropha* possesses both type I and II thiolases. The fatty acid β-oxidation pathway in

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R. eutropha is uncharacterized in the literature. Most studies of microbial fatty acid β-oxidation have been conducted in *Escherichia coli* and *Bacillus subtilis* [9,10], although some information is available regarding fatty acid degradation in *Pseudomonas* species [11,12]. A search of the *R. eutropha* H16 genome reveals many potential β-oxidation pathway gene homologs [13]. For example, 50 genes in the *R. eutropha* H16 genome are annotated as enoyl-CoA hydratases and 46 genes are annotated as acyl-CoA dehydrogenases. However, it is not known which of these homologs actually play a role in fatty acid breakdown.

In this study, we aimed to determine the crystal structure of *Ralstonia eutropha* 3-ketoacyl-CoA thiolase A1887 (*Re*H16_A1887), an enzyme that catalyzes the fourth step of β-oxidation degradative pathways and converts 3-ketoacyl-CoA to acyl-CoA. Biochemical and mutagenesis experiments were also performed.

2. Materials and methods

2.1. Preparation of H16_A1887

Cloning, expression, purification, and crystallization of *Re*H16_A1887 will be described elsewhere (Kim et al., in preparation). Briefly, the *Re*H16_A1887 coding gene (Met1-Leu392, M.W. 41.5 kDa) was amplified by polymerase chain reaction using *R. eutropha* chromosomal DNA as a template. The PCR product was then subcloned into pET30a (Invitrogen), and the resulting expression vector pET30a: *Re*H16_A1887 was transformed into an *E. coli* BL21(DE3)-T1^{R} strain, which was grown in 1 L of LB medium containing kanamycin (50 mg/ml) at 37 °C. After induction via the addition of 1.0 mM IPTG, the culture medium was further maintained for 20 h at 18 °C. The culture was harvested by centrifugation at 4000 g at 4 °C. The cell pellet was resuspended in ice-cold buffer A (40 mM Tris–HCl at pH 8.0 and 5 mM β-mercaptoethanol) and then disrupted by ultrasonication. The cell debris was removed by centrifugation at 13,500 g for 25 m, and lysate was bound to an Ni-NTA agarose (QIAGEN). After washing with buffer A containing 20 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, the trace amount of contamination was removed by applying Sephacryl S-300 HR prep grade (320 ml, GE Healthcare) size exclusion chromatography equilibrated with buffer A containing 5 mM β-mercaptoethanol (BME). All purification experiments were performed at 4 °C. The degree of protein purification was confirmed by SDS-PAGE. The purified protein showed ~95% purity on SDS-PAGE, was concentrated to 25 mg/ml in 40 mM Tris–HCl, pH 8.0, 5 mM BME.

2.2. Crystallization, data collection, and structure determination of ReH16_A1887

Crystallization of the purified protein was initially performed with commercially available sparse-matrix screens from RIGAKU and Molecular Dimensions using the hanging-drop vapor-diffusion method at 295 K. Each experiment consisted of mixing 1.2 μl protein solution (25 mg/ml in 40 mM Tris–HCl, pH 8.0) with 1.2 μl reservoir solution and then equilibrating it against 0.5 ml of the reservoir solution. *Re*H16_A1887 crystals were observed from several crystallization screening conditions. After several steps that improved the crystallization process using the hanging-drop vapor-diffusion method, crystals of the best quality appeared at 20 °C in 7 days and reached their maximal dimensions of approximately 0.2 × 0.2 × 0.5 mm using reservoir solution containing 17% PEG 8 K, 0.1 M HEPES, pH7.0.

The crystals were transferred to cryoprotectant solution containing 20% PEG 8 K, 0.1 M HEPES pH 7.0 and 30% (v/v) glycerol, fished out with a loop larger than the crystals and flash-frozen by immersion in liquid nitrogen at 100 K. The data were collected to a resolution of 1.4 Å at 7A beamline of the Pohang Accelerator Laboratory (PAL, Pohang, Korea) using a Quantum 270 CCD detector (ADSC, USA). The data were then indexed, integrated, and scaled using the HKL2000 suite [14]. Crystals of an apo-form belonged to space group $p4_32_12$, with unit cell parameters of $a = b = 129.52$ Å, $c = 114.13$ Å, $\alpha = \beta = \gamma = 90$. Assuming 2 molecules of *Re*H16_A1887 per asymmetric unit, the crystal volume per unit of protein mass was 2.99 Å^3 Da^{-1} [15], which corresponds to a solvent content of approximately 58.9%. *Re*H16_A1887 crystals in complex with CoA were crystallized with the same crystallization condition supplemented with 20 mM of CoA. Crystals in complex with CoA belonged to space group $p3_121$, with unit cell parameters of $a = b = 141.43$ Å, $c = 52.979$ Å, $\alpha = \beta = 90$ and $\gamma = 120$. Assuming 1 molecule of *Re*H16_A1887 per asymmetric unit, the crystal volume per unit of protein mass was 2.88 Å^3 Da^{-1} [15], which corresponds to a solvent content of approximately 57.36%.

The structure was determined by molecular replacement method with the CCP4 version of MOLREP using the structure of a thiolase from *Mycobacterium tuberculosis* (*Mt*FadA5, PDB code 4UBU) [16] as a search model. Model building was performed manually using the program WinCoot [17] and the refinement was performed with CCP4 refmac5 [18] and CNS [19]. The structures of *Re*H16_A1887 in complex with CoA were solved by molecular replacement method using the crystal structure of the apo-form of *Re*H16_A1887. Model building and structure refinement of the CoA-bound form were performed similarly to the apo-form of *Re*H16_A1887. The data statistics are summarized in Table 1. The refined *Re*H16_A1887 models will be deposited.

2.3. Activity measurement and site-directed mutagenesis

Site-specific mutations were created with the QuikChange kit (Stratagene), and sequencing was performed to confirm correct

Table 1
Data collection and refinement statistics.

	Apo	CoA-bound
Data collection		
Space group	$P4_32_12$	$P3_121$
Cell dimensions		
a, *b*, *c* (Å)	129.52,129.52, 114.13	141.43,141.43, 52.979
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 120.00
Resolution (Å)	50.0–1.4 (1.42–1.4)[a]	50.0–1.5 (1.53–1.5)
R_{sym} or R_{merge}	6.9 (30.9)	10.7 (34.0)
$I/\sigma(I)$	38.00 (3.78)	30.0 (4.8)
Completeness (%)	95.7 (87.2)	97.3 (93.2)
Redundancy	6.7 (4.2)	6.9 (3.9)
Refinement		
Resolution (Å)	50.0–1.4	50.0–1.5
No. reflections	172,263	89,331
R_{work}/R_{free}	15.6/18.1	15.20/17.61
No. atoms	6786	3396
Protein	5812	2906
Ligand/ion	–	48
Water	974	442
B-factors	17.531	12.564
Protein	15.869	11.041
Ligand/ion	–	15.206
Water	36.090	31.680
R.m.s. deviations		
Bond lengths (Å)	0.0291	0.0295
Bond angles (°)	2.6252	2.4719

AU: Equations defining various *R*-values are standard and hence are no longer defined in the footnotes.
AU: Ramachandran statistics should be in Methods section at the end of Refinement subsection.
AU: Wavelength of data collection, temperature and beamline should all be in Methods section.
[a] Number of xtals for each structure should be noted in footnote. Values in parentheses are for highest-resolution shell.