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Crystal structure and biochemical characterization of beta-keto thiolase B from polyhydroxyalkanoate-producing bacterium *Ralstonia eutropha* H16

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ABSTRACT

*Re*BktB is a β-keto thiolase from *Ralstonia eutropha* H16 that catalyzes condensation reactions between acetyl-CoA with acyl-CoA molecules that contains different numbers of carbon atoms, such as acetyl-CoA, propionyl-CoA, and butyryl-CoA, to produce valuable bioproducts, such as polyhydroxybutyrate, polyhydroxybutyrate-hydroxyvalerate, and hexanoate. We solved a crystal structure of *Re*BktB at 2.3 Å, and the overall structure has a similar fold to that of type II biosynthetic thiolases, such as PhbA from *Zoogloea ramigera* (*Zr*PhbA). The superposition of this structure with that of *Zr*PhbA complexed with CoA revealed the residues that comprise the catalytic and substrate binding sites of *Re*BktB. The catalytic site of *Re*BktB contains three conserved residues, Cys90, His350, and Cys380, which may function as a covalent nucleophile, a general base, and second nucleophile, respectively. For substrate binding, *Re*BktB stabilized the ADP moiety of CoA in a distinct way compared to *Zr*PhbA with His219, Arg221, and Asp228 residues, whereas the stabilization of β-mercaptoethyamine and pantothenic acid moieties of CoA was quite similar between these two enzymes. Kinetic study of *Re*BktB revealed that K_m, V_{max}, and K_{cat} values of 11.58 μM, 1.5 μmol/min, and 102.18 s^{-1}, respectively, and the catalytic and substrate binding sites of *Re*BktB were further confirmed by site-directed mutagenesis experiments.

1. Introduction

Ralstonia eutropha H16 is a gram-negative lithoautotrophic bacterium that inhabits soil and freshwater [1]. *R. eutropha* has received a significant amount of attention from the biotechnology community because it can utilize both organic compounds and molecular hydrogen (H_2) as energy sources. Furthermore, *R. eutropha* can synthesize polyhydroxyalkanoates (PHA) polymers while storing surplus organic compounds [2,3]. Recently, the analysis of the *R. eutropha* genome revealed genes involved in the biosynthesis of PHA [2,3], and the granule shaped carbon polymer synthesized by *R. eutropha* has been extensively used to make biodegradable thermoplastics [4–6].

Among many different types of PHAs, *R. eutropha* mainly biosynthesizes the polyhydroxybutyrate (PHB) monopolymer [7] by utilizing three enzymes, β-ketothiolase (PhbA), NADPH-dependent acetoacetyl-CoA reductase (PhbB), and PHB synthase (PhbC), whose coding genes are located on the same operon [8–11]. β-ketothiolase is an enzyme that catalyzes the first step of PHA synthesis, and is also involved in many other important biosynthetic pathways [12,13]. Thiolases can be divided two categories, type I degradative (EC 2.3.1.16) and type II biosynthetic (EC 2.3.1.9) thiolases. Among the 37 β-ketothiolase homologues that are present in the *R. eutropha* genome, two β-ketothiolases, PhbA and β-ketothiolase B (BktB), are known to play a role in the biosynthesis of PHA by catalyzing Claisen condensation reactions of 2 molecules of acetyl-CoA to form acetoacetyl-CoA [14].

Although the functions of *Re*PhbA and *Re*BktB are similar as β-ketothiolase enzymes, *Re*BktB is also involved in the biosynthesis of longer chain polymers in *R. eutropha*. *Re*BktB catalyzes not only a condensation reaction between 2 acetyl-CoA molecules to produce acetoacetyl-CoA, but it also catalyzes a condensation reaction between acetyl-CoA and propionyl-CoA to produce valeryl-CoA. On the other hand, *Re*PhbA utilizes acetyl-CoA as its sole substrate and produces acetoacetyl-CoA [7]. Due to the function of *Re*BktB, this enzyme has been used in the synthesis of poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBHV) or longer chain copolymers [7]. Furthermore, *Re*BktB has been shown to catalyze a condensation reaction between acetyl-CoA and butyryl-CoA to form 3-ketohexanoyl-CoA, which can be used to produce hexanoate or *n*-hexanol [15].

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In the present study, we report a crystal structure of β-ketothiolase B from *R. eutropha* H16 (*Re*BktB) and reveal its residues involved in substrate binding. Biochemical properties of *Re*BktB were also elucidated by kinetic analysis and site-directed mutagenesis experiments. Importantly, our results provide useful information for engineering *Re*BktB to have an increased rate of producing valuable bio-products such as bio-plastics and bio-fuels.

2. Materials and methods

2.1. Cloning, expression, and purification

Cloning, expression, purification, and crystallization of *Re*BktB will be described elsewhere (Kim et al., in preparation). Briefly, the recombinant *Re*BktB protein was expressed using the pPROEX Hta (Invitrogen) bacterial expression system and purified through sequential chromatographic steps including Ni–NTA, ion-exchange, and size-exclusion chromatography. All purification experiments were performed at 4 °C. The degree of protein purification was confirmed by SDS–PAGE. The purified protein was concentrated to 20 mg/ml in 40 mM Tris–HCl, pH 8.0 and 1 mM Dithiothreitol.

2.2. Crystallization and data collection

Crystallization of the purified protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald BioSystems using the hanging-drop vapor-diffusion method at 295 K. Crystals of the best quality appeared in 2 days and reached their maximum dimensions of $0.05 \times 0.2 \times 0.2$ mm using reservoir solution containing 25% polyethylene glycol 3350, 0.1 M Bis-Tris, pH 6.5 and 0.2 M lithium sulfate. The crystals were transferred to a cryoprotectant solution containing 25% polyethylene glycol, 0.1 M Bis-Tris, pH 6.5, 0.2 M lithium sulfate 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 2.3 Å at 7A beamline of the Pohang Accelerator Laboratory (PAL, Pohang, Korea) using a Quantum 270 CCD detector (ADSC, USA). All data were indexed, integrated and scaled together using the HKL2000 software package.[16] The crystals of *Re*BktB belonged to the space group $C222_1$. Assuming that an asymmetric unit contains four molecules of *Re*BktB, the crystal volume per unit of protein mass is 2.54 $Å^3$ Da^{-1}, which means the solvent content is approximately 51.5%.

2.3. Crystallization and data collection

The structure was determined by molecular replacement with the CCP4 version of MOLREP [17] using the structure of PhbA thiolase from *Zoogloea ramigera* (*Zr*PhbA) (PDB code 1DM3) as a search model. Model building was performed manually using the program WinCoot [18] and the refinement was performed with CCP4 refmac5 [19] and CNS [20]. The data statistics are summarized in Table 1. The refined *Re*BktB models and structure factors were deposited in the Protein Data Bank as the PDB code 4NZS.

2.4. Site-directed mutagenesis and activity assay

Site-specific mutations were created with the QuikChange kit (stratagene), and sequencing was performed to confirm correct incorporation of the mutations. The mutant proteins were purified in the same manner as the wild type. Enzyme activities of wild type and mutant proteins were measured by monitoring the change of acetoacetyl-CoA absorbance at 303 nm. The reaction mixture (1 ml) contained 100 mM Tris, pH 8.0, 10 mM Magnesium chloride, 1 mM Dithiothreitol, 0.05 mM CoA. For each reaction 0.15 μg of wild-type or mutant *Re*BktB protein was added to start the reaction, and the decrease of absorbance at 303 nm was monitored at room temperature for 5 min.

Table 1
Data collection and refinement statistics.

	*Re*BktB
Data collection	
Space group	$C222_1$
Cell dimensions	
a, *b*, *c* (Å)	106.95, 107.24, 144.14
Resolution (Å)	50.00–2.3 (2.34–2.3)*
R_{sym} or R_{merge}	7.0 (32.0)
$I/\sigma I$	25.57 (2.64)
Completeness (%)	85.9 (79.9)
Redundancy	5.6 (2.6)
Refinement	
Resolution (Å)	35.77–2.3
No. reflections	30408
R_{work}/R_{free}	25.3/31.6
No. atoms	5708
Protein	
Ligand/ion	
Water	
B-factors	64.617
Protein	
Ligand/ion	
Water	
R.m.s. deviations	
Bond lengths (Å)	0.0110
Bond angles (°)	1.548

[AU: Equations defining various *R*-values are standard and hence are no longer defined in the footnotes.]
[AU: Ramachandran statistics should be in Section 2 at the end of Refinement subsection.]
[AU: Wavelength of data collection, temperature and beamline should all be in Section 2.]
* Number of xtals for each structure should be noted in footnote. Values in parentheses are for highest-resolution shell.

3. Results and discussion

3.1. Overall structure of ReBktB

*Re*BktB is an enzyme that catalyzes a condensation reaction between acetyl-CoA with acyl-CoA molecules with a different number of carbon atoms, such as acetyl-CoA, propionyl-CoA, and butyryl-CoA, to produce acetoacetyl-CoA, valeryl-CoA, and 3-ketohexanoyl-CoA, respectively. The enzymatic products are further converted to valuable bioproducts, such as PHB, PHBHV, and hexanoate. To investigate the structural basis for the catalytic mechanism of β-ketothiolase from *R. eutropha* H16 (*Re*BktB), we solved a crystal structure of the *Re*BktB protein at 2.3 Å. The asymmetric unit contained 2 *Re*BktB molecules, and the tetrameric structure of the protein was easily generated by applying $C222_1$ symmetry (Fig. 1), which is consistent with our size-exclusion chromatography data (data not shown). The overall structure of *Re*BktB was similar to that of the PhbA thiolase from *Z. ramigera* (*Zr*PhbA) [21] with 52% amino acid sequence identity (Fig 2A). The structure of *Re*BktB was divided into three distinctive domains, two core domains and a loop domain. The two core domains consisted of N-terminal (residues 2–119 and 253–273) and C-terminal (residues 274–394) core domains, and the loop domain was located between the two core domains (L-domain, residues 120–252) (Fig. 2B). The N- and C-domains consisted of a mixture of α-helices and β-sheet strands (α–β–α–β–α structure), which is a typical topology for