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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

enzymes belonging to the thiolase family. In the N-domain, six β-sheets were packed and covered by four α-helixes. The C-domain was also composed of four β-sheets packed evenly, and four α-helices covered the β-sheets. The L-domain was composed of four α-helices and three short β-sheets, forming a triangular shape. This domain also contained a sequence motif (residues 127–144) involved in the tetramerization of the protein (Fig. 2B).

3.2. Catalytic site

Two cysteine residues (Cys89 and Cys378) have been reported to be catalytic residues in *Zr*PhbA, in which they each function as a second nucleophile and a covalent catalyst, respectively. When the structure of *Re*BktB was superposed with that of *Zr*PhbA (pdb code 1DLV) [21], 2 cysteine residues, Cys90 and Cys380, of *Re*BktB were observed to be located at similar positions that corresponded to cys89 and Cys378 residues in *Zr*PhbA. In *Re*BktB, the Cys90 residue was located at the connecting loop between β3 and α3 of the N-domain, while the Cys380 residue was located at the β12 of the C-domain (Fig. 3A). Furthermore, the His350 residue was located near the Cys90 residue, and appeared to function as a general base. The superposition of structures of *Re*BktB and *Zr*PhbA also revealed that the His348 residue in *Zr*PhbA was located at a position similar to the His350 residue in *Re*BktB (Fig. 3A). These observations indicated that *Re*BktB has a similar catalytic mechanism to that of *Zr*PhbA.

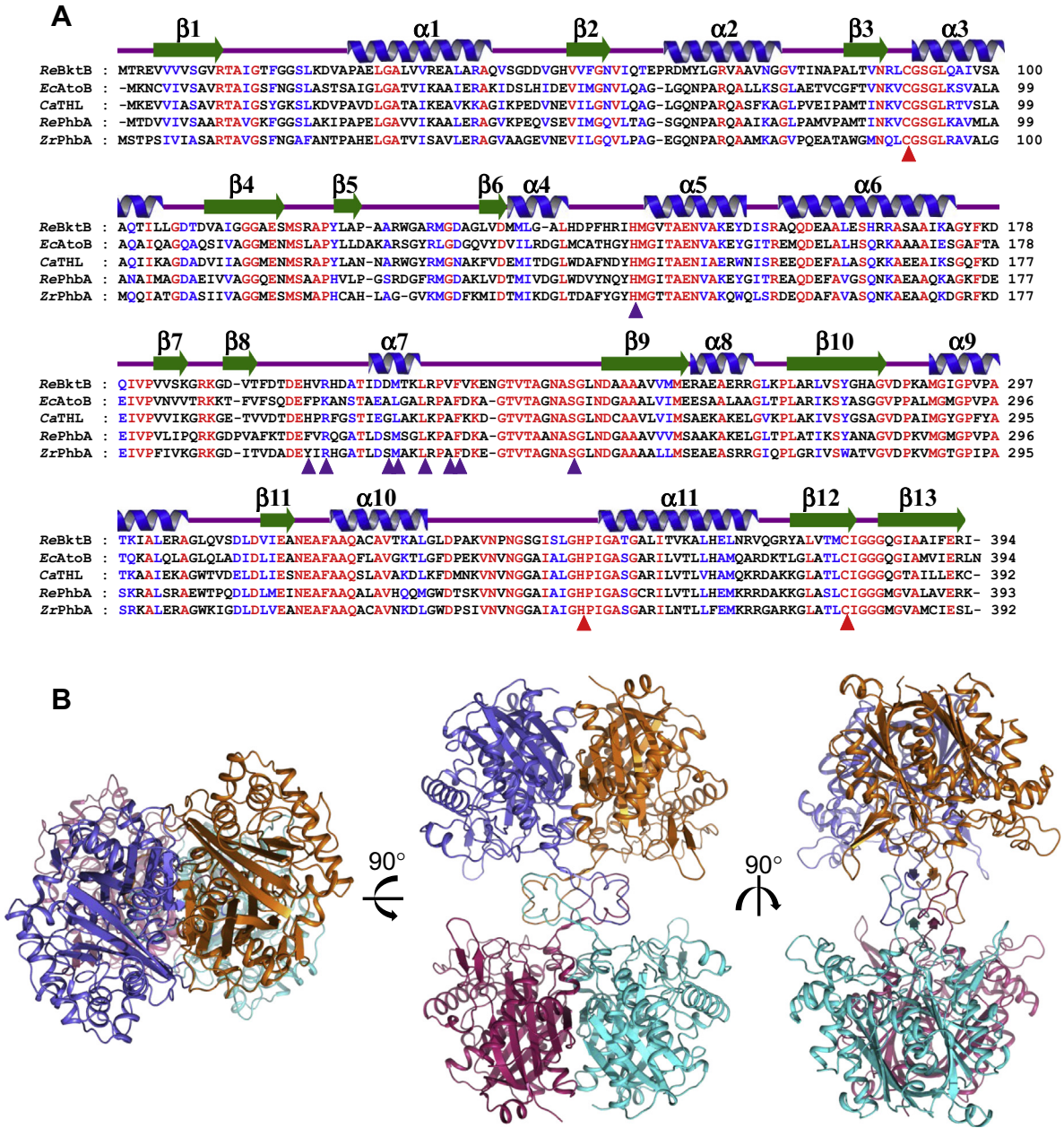

Fig. 1. Overall shape of *Re*BktB. (A) Amino acid sequence alignment of thiolases. Secondary structure elements are shown based on the *Re*BktB structure. Identical and highly conserved residues are presented in red and blue colored characters, respectively. Residues involved in the enzyme catalysis and substrate binding are marked with red- and purple-colored rectangles, respectively. *Re*, *Ec*, *Ca*, and *Zr* are abbreviations for *Ralstonia eutropha*, *Escherichia coli*, *Clostridium acetobutyricum*, and *Zoogloea ramigera*, respectively. (B) Overall shape of *Re*BktB tetramer. Four polypeptides are differentiated by colors of orange, light blue, hot pick, and cyan. Left and right side of figures are 90° rotations of central figure in vertical and horizon directions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

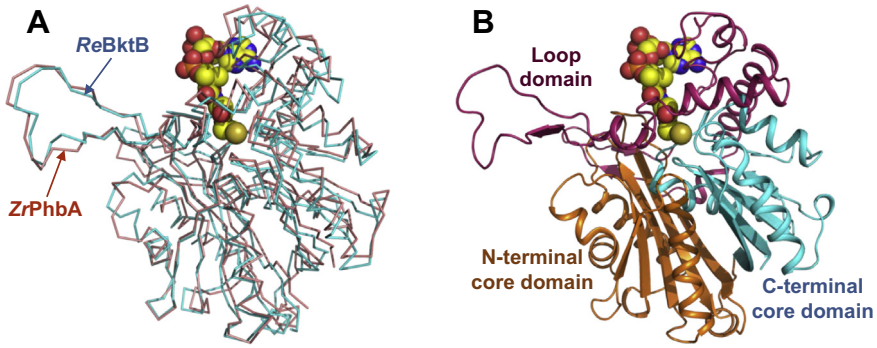

Fig. 2. Monomeric structure of *Re*BktB. (A) Superposition of monomeric structure of *Re*BktB with that of ZrPhbA complexed with CoA. Structures of *Re*BktB and ZrPhbA are shown as ribbon diagram with cyan and salmon colors, respectively. Bound CoA is shown as a sphere diagram. (B) Monomeric structure of *Re*BktB. Monomeric structure of *Re*BktB is shown as a cartoon diagram. N- and C-terminal core domains, and loop domain are distinguished with orange, cyan, and hot pink colors, respectively, and labeled appropriately. Bound CoA is shown as a sphere diagram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Substrate binding mode

Although determination of the structure of *Re*BktB in complex with its substrate was not successful, the substrate binding mode of the protein could be identified by the superposition of the protein with the *Zr*PhbA structure in complex with CoA. Stabilization of the β-mercaptoethylamine and pantothenic acid moieties of CoA in *Re*BktB were quite similar to those in *Zr*PhbA. In *Zr*PhbA, the side-chain hydroxyl group and main-chain carbonyl group of Ser247 and His156 were involved in the stabilization of the β-mercaptoethylamine and pantothenic acid moieties of CoA. Residues of Ser249 and His157 were also found in *Re*BktB at the similar positions to the corresponding residues in *Zr*PhbA. In *Zr*PhbA, the ADP moiety of CoA was bound in the hydrophobic pocket constituted by residues of Met228, Leu231, Ala234, and Phe235. The hydrophobic ADP binding pocket was similarly constituted in *Re*BktB by residues of Met229, Leu232, Val235, and Phe236. One exceptional residue was Val235, whose corresponding residue was Ala234 in *Zr*PhbA, and this somewhat bulky residue might aid in the tight binding of the ADP moiety in *Re*BktB. The binding of the ADP moiety is also mediated by hydrogen bonding interactions. However, the residues involved in the interactions are quite different between these two proteins. Unlike in *Zr*PhbA, where residues of Tyr218, Arg220, and Ser227 form direct and water-mediated hydrogen bonds with the ADP moiety, His219, Arg221, and Asp228 residues were located in *Re*BktB at the positions of corresponding residues in *Zr*PhbA. These observations indicated that *Re*BktB recognizes its substrate in a unique mode.

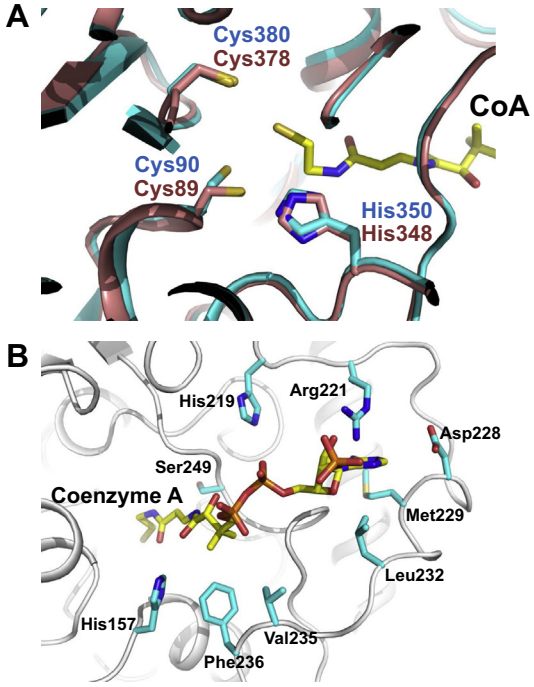

Fig. 3. Active site of *Re*BktB. (A) Catalytic site of *Re*BktB. Monomeric structure of *Re*BktB was superposed with that of *Zr*PhbA complexed with CoA. Structures of *Re*BktB and *Zr*PhbA are shown as cartoon diagram with cyan and salmon colors, respectively. Three catalytic residues of *Re*BktB and *Zr*PhbA are shown as stick models, and labeled appropriately. Bound CoA is shown as a stick model with yellow color. (B) Substrate binding site of *Re*BktB. Structure of *Re*BktB is shown as a cartoon diagram. Residues involved in the CoA binding are shown as stick models with cyan color, and labeled appropriately. CoA is shown as a stick model with yellow color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Kinetic and mutagenesis studies

To characterize the properties of *Re*BktB, kinetic analysis was performed by measuring thiolysis activity with the acetoacetyl-CoA substrate. Reaction rates corresponding to various concentrations of acetoacetyl-CoA were plotted, and determined to obey Michaelis–Menten kinetics (Fig. 4A and B). Based on this kinetic analysis, the K_m, V_{max}, and K_{cat} values of *Re*BktB with acetoacetyl-CoA were determined to be 11.58 μM, 1.5 μmol/min, and 102.18 s^{-1}, respectively.

In order to confirm the residues involved in *Re*BktB catalysis and substrate binding, we performed site-directed mutagenesis experiments based on structural observations of the protein and compared the enzymatic activity of the mutants with that of wild-type protein. To confirm the catalytic residues, two cysteine residues (Cys90 and Cys380) were mutated to serine, and the both mutants (C90S and C380S) showed almost complete loss of activity, indicating that *Re*BktB uses two conserved cysteine residues for enzyme catalysis (Fig. 4C). As described above, *Re*BktB uses unique hydrophilic residues (His219, Arg221, and Asp228) to stabilize the ADP moiety of CoA, which were confirmed by generating various mutants and measuring their enzyme activity. His219, a residue suspected to form a water-mediated hydrogen bond with the pyrophosphate moiety of ADP, was mutated to either phenylalanine or alanine. The H219F and H219A mutants exhibited 74% and 24% activity, respectively, compared to that of the wild-type protein, suggesting that the His219 residue is involved in making a complementary substrate binding pocket rather than stabilizing

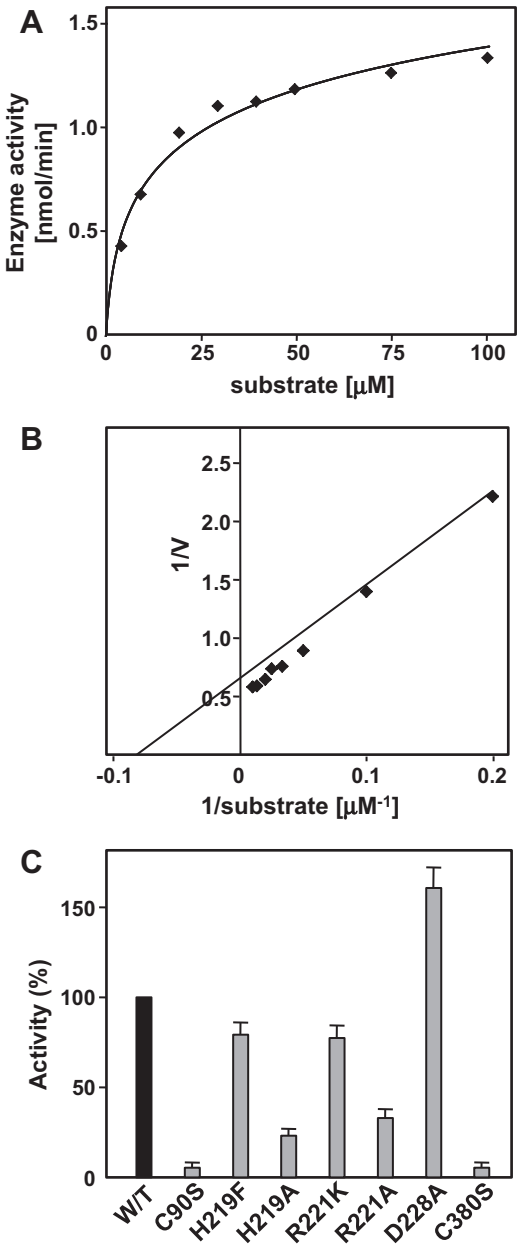

Fig. 4. Enzyme kinetics of *Re*BktB. (A) Michaelis–Menten equation based plot of reaction velocity versus substrate concentrations. For the various substrate concentrations, 5, 10, 20, 30, 40, 50, 75, and 100 mM acetoacetyl-CoA were used. (B) Lineweaver–Burk plot of (A). K_m, V_{max}, and K_{cat} of *Re*BktB were calculated with 11.58 μM, 1.5 μmol/min, and 102.18 s^{-1}, respectively. (C) Site-directed mutagenesis of *Re*BktB. Residues involved in enzyme catalysis and substrate binding were replaced by appropriate residues. The relative activities of recombinant mutant proteins were measured and compared with that of wild-type *Re*BktB.

a substrate via water-mediated hydrogen bond formation (Fig. 4C). The Arg221 residue was mutated to either lysine or alanine, and the resulting mutants, R221K and R221A, exhibited 67% and 38% activity, respectively, compared to that of the wild-type protein. Considering that arginine has a higher capability of forming hydrogen bonds than lysine, these results indicated that Arg221 aid in the stabilization of substrate through hydrogen bond formation with an adenine moiety of CoA. Interestingly, the D228A mutant showed a 1.5-fold increase in enzyme activity (Fig. 4C). Although the Asp228 residue may be involved in stabilizing an adenine moiety of CoA through hydrogen bonding, we speculate that creating a more hydrophobic environment with alanine may facilitate a preferable substrate binding pocket.

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