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Directed evolution and structural analysis of NADPH-dependent acetoacetyl-CoA reductase from *Ralstonia eutropha* reveals two mutations responsible for enhanced kinetics

Ken’ichiro Matsumoto‡†, Yoshikazu Tanaka§††, Tsuyoshi Watanabe‡††, Ren Motohashi‡‡, Koji Ikeda§, Kota Tobitani‡, Min Yao§††, Isao Tanaka§††, and Seiichi Taguchi‡¶#

from the ‡Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Japan Science and Technology Agency, CREST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, and §Graduate School of Life Sciences, and +Faculty of Advanced Life Sciences, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan

*Running title: Evolved NADPH-dependent acetoacetyl-CoA reductase

1 The first four authors equally contributed to the present study.

# To whom correspondence should be addressed: Div. of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan. Tel.: +81-11-706-6610; Fax: +81-11-706-6610; E-mail: staguchi@eng.hokudai.ac.jp

**Keywords:** in vitro evolution; polyhydroxyalkanoate; biobased plastic; 3-ketoacyl-ACP reductase
ABSTRACT

Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA reductase (PhaB) is a key enzyme in the synthesis of poly(3-hydroxybutyrate) [P(3HB)], along with \( \beta \)-ketothiolase (PhaA) and polyhydroxyalkanoate synthase (PhaC). In this study, PhaB from *Ralstonia eutropha* was engineered by means of directed evolution consisting of an error-prone PCR-mediated mutagenesis and a P(3HB) accumulation-based *in vivo* screening system using *Escherichia coli*. Out of approximately twenty thousand mutants, we obtained two mutant candidates bearing Gln47Leu (Q47L) and Thr173Ser (T173S) substitutions. The mutants respectively exhibited a 2.4 and 3.5-fold higher \( k_{\text{cat}} \) value compared to that of wild-type enzyme. In fact, the PhaB mutants did exhibit enhanced activity and P(3HB) accumulation when expressed in recombinant *Corynebacterium glutamicum*. Comparative three-dimensional structural analysis between the wild-type and highly active PhaB mutants revealed that the beneficial mutations affected the flexibility around the active site, which in turn play an important role in substrate recognition. Furthermore, both the kinetic analysis and crystal structure data supported the conclusion that PhaB forms a ternary complex with NADPH and acetoacetyl-CoA. These results suggest that the mutations affected the interaction with substrates, resulting in the acquirement of enhanced activity.

INTRODUCTION
Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA reductase (PhaB) stereoselectively reduces the 3-ketone group of acetoacetyl-CoA so as to synthesize \((R)-3\)-hydroxybutyryl(3HB)-CoA, which is known to be a monomer precursor of microbial polyester polyhydroxyalkanoate (PHA) (1-3). The PhaB-encoding gene is found in many bacteria, including *Ralstonia eutropha* (also known as *Cupriavidus necator*), and typically located in the *phb* operon together with \(\beta\)-ketothiolase (PhaA) and PHA synthase (PhaC). The three enzymes catalyze the successive reactions synthesizing P(3HB) from acetyl-CoA (4-5). This pathway has been extensively utilized for the microbial production of P(3HB) and 3HB-based copolymers (6), both of which can be used as biobased plastics. For efficient production of these polymers, the enhancement in the activities of these enzymes is effective (7-8). In several cases, it has been known that the enhanced expression of either PhaB by itself or both PhaA and PhaB increased P(3HB) production by as the result of an increase in gene dosage (9) and codon optimization (10).

There are two major strategies to engineer enzymes for acquiring enhanced activity; structural and non-structural approaches. The three-dimensional structure of PhaB has not been determined, despite its important role in PHA biosynthesis. PhaB possesses a primary structure that is similar to NADPH-dependent 3-ketoacyl-acyl-carrier-protein(ACP) reductase (FabG), which generates the \((R)-3\)-hydroxyacyl-ACP involved in fatty acid biosynthesis. However, although PhaB and FabG presumably have similar protein folding and reaction mechanisms to some extent, the regions contributing to the activity of PhaB and FabG have not been identified. Thus, at present we still do not have sufficient
information to design the structure-based engineering of PhaB for the activity enhancement.

Therefore, at the initial step in the present study, we attempted to enhance the enzymatic activity of PhaB using a non-structural strategy of a random mutagenesis of PhaB and high-throughput screening of the activity-enhanced PhaB mutants. This method is applicable without structural information, and is a useful means of identifying regions which exert an effect on the enzymatic activity. In the next step, the wild-type (WT) PhaB and certain highly active mutants were subjected to crystal structure analysis. The structural features of the beneficial sites and comparative analysis of the structure of the WT and activity-enhanced mutants were expected to provide insight into the regions in PhaB contributing to the reaction rate as well as the reaction mechanism of this enzyme.

A technical barrier impeding this goal had been the design of a high-throughput screening method for selecting highly active PhaB mutants. Our group has performed extensive *in vitro* evolution of PhaC for the purposes of increasing its activity (11-12) and altering its substrate specificity (13-14). The *in vivo* screening method used in these studies is based on polymer accumulation in recombinant *Escherichia coli* expressing the randomly mutated *phaC* gene together with the *phaAB* genes, and grown on Nile red-containing agar plates (15-17). The presence of Nile red-stained hydrophobic polymer granules in the cells allowed visualization of the polymer content in terms of the relative fluorescent intensity of the colonies. Namely, the colonies with a brighter fluorescence were suggested to be highly active mutants of the targeted enzyme. However, this method was not exclusively applicable to the engineering of PhaB, because the activity of the WT
PhaB achieved approximately 60 wt% polymer accumulation in *E. coli* (15, 17), which resulted in the saturated fluorescence and prevented the selection of mutants with a higher activity. Thus, the optimizing condition of the plate assay should allow for easy identification of the positive candidates from the other mutants and even the WT enzyme.

To meet this challenge, we replaced PhaA with AcAc-CoA synthase (AACS), which was recently isolated from terpenoid-producing *Streptomyces* sp. strain CL190 (18). AACS acted as a less efficient AcAc-CoA supplying enzyme than PhaA, and recombinant *E. coli* harboring the AACS gene, together with the WT *phaB* and *phaC* genes, accumulated a small amount of P(3HB) (19). The low basal polymer accumulation level, i.e. the weak fluorescence in the colonies expressing the WT *phaB* gene, would be preferable for isolating colonies emitting a brighter fluorescence. In fact, this approach successfully retrieved highly active mutants of PhaB. The kinetics and crystal structure of the obtained mutants are discussed.

**MATERIALS AND METHODS**

**Plasmid constructions and screening of phaB gene.** The *phaB* gene of *R. eutropha* was amplified under error-prone conditions (20) using pGEM"CAB as a template and a pair of primers, 5'-TTCCGGGGCTCGAGCGGTG-3’ and 5'-CTCAAGCTAGCATGCAACG-3’. The restriction sites used for the construction are underlined. The amplified fragment was digested with *XhoI*/EcoT22I and inserted into *XhoI*/EcoT22I sites of pGEM"CAACSB, which harbors AcAc-CoA synthase gene (AACS) together with the *phaB* and *phaC* genes (19) to replace the *phaB* gene with its mutants. The
plasmids were introduced into *E. coli* JM109 and cells were grown on LB agar plates containing glucose and Nile red (20). Colonies emitting enhanced fluorescence on a transilluminator were chosen as potential mutants of high activity.

To express the selected *phaB* mutant genes in *C. glutamicum*, the plasmids pPSCAB(Q47L) and pPSCAB(T173S) were constructed as follows. The *AACS* gene in the selected pGEM"CAACSB was replaced with the *phaA* gene from *R. eutropha* using the *PstI*/XhoI sites. The resultant plasmids pGEM"phaCAB(Q47L) and pGEM"phaCAB(T173S) were digested with *Csp45I/BamHI*, and a 4.3-kb fragment was inserted into the *BstEII/BamHI* site of pPSPTG1 harboring the *cspB* promoter (21) to yield pPSCAB(Q47L) and pPSCAB(T173S), respectively. pPSCAB (21) bearing the WT *phaB* gene was used as a control.

For enzyme purification, the selected *phaB* gene mutants as well as the wild-type gene were amplified using a pair of primers; 5'-GTGGGATCCACTCAGCGCATTGCG-3' and 5'-GCCAAGCTTTCAGCCCATATGCAG-3'. The restriction sites used for the construction are underlined. The amplified fragments were digested with *BamHI/HindIII* and inserted into pQE30 (Qiagen, USA) to construct the gene encoding the N-terminal His-tag fusion of PhaB (pQEphaB).

**Polymer productions and analysis.** For polymer production in *E. coli*, the engineered cells harboring pGEM"CAACSB were grown on LB medium containing 2% glucose and 100 µg/L ampicillin at 37°C for 48 h. Cells were lyophilized and the P(3HB) content was determined by HPLC, as described previously (22).

Transformation of *C. glutamicum* ATCC 13803 was performed by electroporation, as
described previously (21). The recombinant cells harboring pPSCAB (WT, Q47L and T173S) were grown on CM2G medium at 30°C for 12 h, then transferred into MMTG medium (23) containing 6% glucose and further cultivated at 30°C for 72 h. P(3HB) content was determined by HPLC, as described (22).

**PhaB activity in crude extract.** The PhaB activity in the crude extract of *C. glutamicum* harboring pPSCABs was measured as well. Cells grown on MMTG medium under the same condition for P(3HB) production were harvested at 24 h. The both cells were suspended in 125 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. The soluble fraction was used for enzymatic assay. The reaction mixture contained 125 mM Tris-HCl buffer (pH 8.0), 5 mM AcAc-CoA (SIGMA), 20 mM NADPH (Oriental Yeast Co., LTD, Japan) and 3.125% (vol./vol.) crude extract. The decrease in absorbance at 340 nm was monitored at 30°C to measure the consumption rate of NADPH. The activity was normalized by the protein concentration determined using Bradford assay (Biorad).

**Purification of PhaB and kinetic analysis.** *E. coli* BL21(DE3) harboring pQEphaB (the WT and mutants) and pREP4 bearing lacI repressor gene (Qiagen, USA) was grown on LB medium containing 100 µg/L ampicillin and 25 µg/L kanamycin at 25°C for 2.5 h. Then a final 1.0 mM IPTG was added and cells were further cultivated for 10 h. Purification of the N-terminal His-tag fusion of PhaB was performed using His-Bind Resin (Novagen), as described previously (24). The eluted solution was replaced with 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl using a PD10 column (GE Healthcare) and stored at -80°C until analysis. The assay was carried out at 30°C using 125 mM Tris-HCl (pH 8.0) containing 130 - 180 ng enzyme, and varied concentrations of AcAc-CoA (4.2 to
Engineering of NADPH-dependent acetoacetyl-CoA reductase

12.5 µM) and NADPH (25 to 125 µM).

**Crystallization and structural analysis.** The His-tag fusion of PhaB was further purified on a HiLoad 26/60 Superdex 200-pg column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl (pH 8.0) and 200 mM sodium chloride. The crystals of PhaB were obtained from a buffer containing 0.1 M MES (pH 7.1), 1.6 M ammonium sulfate and 10% 1,4-dioxane. Crystals of PhaB complexed with NADP⁺ and AcAc-CoA were obtained from the same buffer containing 0.9 mM NADP⁺ and 0.9 mM AcAc-CoA. Crystals of the Q47L mutant and T137S mutant were grown from a buffer composed of 0.6 – 1.2 M sodium / potassium tartrate, 0.16 – 0.20 M lithium sulfate and 0.1 M CHES (pH 8.9 – 9.9). X-ray diffraction experiments were performed at SPring-8 (Harima, Japan) and Photon Factory (Tsukuba, Japan) under the proposal number of 2010B1460 and 2011G012, respectively. The X-ray diffraction data set was collected under cryogenic conditions (100 K). Crystals were soaked in a mother liquor containing 20% glycerol and flash-cooled in a stream of liquid nitrogen. The diffracted data were indexed, integrated and scaled using the HKL2000 program package (25) or the XDS (26). The statistical data are shown in Table S1.

The structure of PhaB was determined by the molecular replacement method by means of the MOLREP program (27) using the structure of FabG from *E. coli* (PDB ID 1I01) as the search probe. To monitor the refinement, a random 5% subset was set aside for calculation of the R-free factor. After several cycles of manual model fitting and building with Coot (28) and refinement with REFMAC5 (29), individual atomic coordinate refinement and individual ADP refinement were performed using phenix.refine (30). The
atomic coordinates of WT PhaB, mutant and ternary complex have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3VZP, 3VZR, and 3VZS). The refinement statistical data are summarized in Table S1.

**Particle size analysis.** The PhaB particle size in solution was analyzed with dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern). PhaB at 5 mg/ml was filtered and then analyzed.

**RESULTS**

**Selection of beneficial PhaB mutants from the mutant library.** Recombinant *E. coli* harboring the mutagenized *phaB* gene, together with the *AACS* and *phaC* genes, were grown on Nile red-containing plates for the screening of beneficial PhaB mutants. Out of the approximately twenty thousand mutant clones, we isolated two colonies emitting a brighter fluorescence. The selected mutants produced 7.0 ± 0.3 and 6.8 ± 0.2 wt% P(3HB), respectively, while the WT produced 6.0 ± 0.3 wt% under the same conditions. These results suggest that the selected mutants were highly active. The gene sequence of the mutants revealed that the PhaB mutants bore Gln47Leu (Q47L) and Thr173Ser (T173S) substitutions, respectively.

**Enhanced P(3HB) production using highly active PhaB mutants in engineered *C. glutamicum*.** The effect of the selected PhaB mutants on *in vivo* P(3HB) production was evaluated using *C. glutamicum*, which is a GRAS (Generally Recognized As Safe) platform for microbial polyester production (21, 31), because P(3HB) accumulation in recombinant *E. coli* expressing the wild-type *phaCAB* genes was close to saturation (15,
Indeed, the recombinant *C. glutamicum* harboring the two mutants exhibited increased \( \text{P(3HB)} \) accumulation (Fig. 1A) and the PhaB activities were correlated with the polymer content (Fig. 1B). This result indicated that the selected PhaB mutants were highly active and exerted a beneficial effect on \( \text{P(3HB)} \) production in the *C. glutamicum* strain.

**The PhaB mutants exhibited an enhanced turnover rate.** The kinetics of the selected mutants was analyzed using the N-terminal His-tag fusion forms of PhaB and its mutants expressed in *E. coli*. The homogeneously purified PhaB and its mutants using one step Ni-column affinity chromatography were directly used in the analysis. The activity of the recombinant PhaB and its mutants was measured using the varied concentrations of the two substrates, AcAc-CoA and NADPH. The kinetic parameters of PhaBs were determined (Table 1) based on Lineweaver–Burk plots (Fig. S1). The Q47L and T173S mutants had 2.4 and 3.5-fold higher \( k_{\text{cat}} \) values than the WT enzyme, respectively. The increase in \( k_{\text{cat}} \) was associated with increases in the \( K_{M(\text{NADPH})} \) and \( K_{M(\text{AcAcCoA})} \) values. The \( k_{\text{cat}}/K_M \) values of the Q47L mutant were elevated for \( k_{\text{cat}}/K_{M(\text{NADPH})} \), while \( k_{\text{cat}}/K_{M(\text{AcAcCoA})} \) was lower than that of WT PhaB. In contrast, the \( k_{\text{cat}}/K_{M(\text{AcAcCoA})} \) value of T173S was higher than the WT, although \( k_{\text{cat}}/K_{M(\text{NADPH})} \) was decreased by this mutation. These results suggest that the effects of the two mutations on the interaction between the enzyme and the two kinds of substrates were different. Namely, the Q47L mutation may improve the recognition of NADPH rather than AcAc-CoA, while T173S may contribute to the AcAc-CoA recognition. In addition, kinetic analysis provided insight into the reaction mechanism. The lines produced from the Lineweaver–Burk plot intersected (Fig. S1), suggesting that PhaB had a ternary-complex rather than a ping–pong mechanism.
Crystal structure analysis of PhaB and certain highly active mutants. In order to obtain a better understanding of the beneficial mutation effects, the PhaB crystal structure was determined at a resolution of 1.8 Å by a molecular replacement method using the structure of 3-ketoacyl-ACP reductase (FabG) as a search probe. Four molecules of PhaB were contained in an asymmetric tetramer with the point group being 222, which was observed also for FabG (Fig. 2A and B). The tetramer of PhaB was superimposed onto the tetrameric FabG, with an RMSD of 1.85 Å for the 865 Cα atoms (Fig. S1). DLS analysis revealed a radius of approximately 43.6 Å for PhaB in solution, which is in good agreement with the tetrameric structure in the crystal. These observations indicated that PhaB exists as a tetramer in solution, as is the case for FabG.

The crystal structures of the Q47L and T173S mutants were determined at a resolution of 2.0 and 2.9 Å, respectively. A single asymmetric unit contained two molecules, and these formed a crystallographically evident tetramer. The tetrameric structures of these mutants were superimposable onto that of the WT PhaB (0.62 Å for the 974 Ca atoms of Q47L and 0.71 Å for the 975 Ca atoms of T173S), although the α7–8 regions and their continuous loops exhibited conformational changes (Fig. 2C). The temperature factor of the α7–8 regions of the WT structure were significantly high compared with other regions (Fig. 2D), suggesting that this region has an intrinsically flexible characteristic. DLS analysis revealed a radius of 44.8Å and 44.5 Å for the Q47L and T173S mutants, respectively, clearly indicating that each of these mutants formed a tetramer in solution.

Taken together, it can be concluded that these mutants maintained a tetramer structure that...
was identical to the WT enzyme, and thus there is a reason other than drastic structural change for the increase in the enzymatic activity due to these mutations.

PhaB formed a ternary complex with AcAc-CoA and NADPH. In order to obtain an understanding of the contribution of the mutations to the enzymatic activity, the crystal structure of PhaB in complex with AcAc-CoA and NADP$^+$ was determined. In the structure obtained, an obvious electron density pattern corresponding to these substrates was observed in a large cavity found in all four molecules in a single asymmetric unit, and both NADP$^+$ and AcAc-CoA were present (Fig. 3A and B). This structure strongly supports the finding that the reaction mechanism is effected via a ternary complex as proposed by the kinetic analysis.

The NADP$^+$ molecule was bound non-covalently in the cavity encircled by loops between $\beta 1 - \alpha 1$, $\beta 2 - \alpha 2$, $\beta 3 - \alpha 3$, $\beta 4 - \alpha 4$, $\beta 5 - \alpha 5$ and $\beta 6 - \alpha 7$. The corresponding region of FabG was also used for the NADP(H)-binding pocket (32). NADP$^+$ was directly recognized by Arg40, Gly60-Asn61, Gly90-Thr92 and Pro183-Val191 (Fig. 3C). It should be noted that Pro183-Val191 corresponds to $\alpha 8$ and its continuous loop, which exhibits flexibility in the apo form (Fig. 2C and D).

An AcAc-CoA molecule was found adjacent to the NADP$^+$ binding site. The nicotinamide ring of NADP$^+$ was in contact with the AcAc moiety, which explains the catalytic reduction of AcAc-CoA. AcAc-CoA is recognized by Ser140, Thr92, Asp94, Gln147-Tyr153, Gly184, Tyr185 and Arg195. Gly184, Tyr185 and Arg195 are located in a flexible portion of $\alpha 7 - 8$ (Fig. 2C, D, Fig. 3C, and D), suggesting that the flexibility of this region may have a significant role in both AcAc-CoA and NADP(H) recognition.
Discussion

The kinetic analysis indicated that the T173S mutant exhibited a higher $k_{cat}/K_{M(\text{AcAcCoA})}$ than the WT PhaB, whereas $k_{cat}/K_{M(\text{NADPH})}$ was lower than the WT. This result shows that the reaction with AcAc-CoA was accelerated by the T173S mutation. This effect was interpreted based on the crystal structure. The residue at the beneficial site 173 of the adjacent subunit (subunit A) was shown to be located close to the adenylyl moiety of AcAc-CoA, although direct interaction was not observed (Fig. 3D). Moreover, T173 of another adjacent subunit (subunit B) interacted with the Pro207 residue located at the root of the $\alpha7 – 8$ using the $\gamma2$ atom, which is absent in the T173S mutant. Although the interaction between Ser173 and Pro207 was maintained in the structure of T173S, the N atom of Ser173, instead of $\gamma2$, interacted with Pro207. These facts suggest that a change in the interaction with Pro207, due to a substitution at position 173, may alter the flexibility of the $\alpha7 – 8$ region in the adjacent protomer, and influence the interaction toward AcAc-CoA as a consequence.

On the other hand, kinetic analysis of the Q47L mutant showed that the mutation had an enhanced $k_{cat}/K_{M(\text{NADPH})}$ value compared with WT PhaB, while $k_{cat}/K_{M(\text{AcAcCoA})}$ was comparatively lower. This suggests that Q47L influenced the interaction of PhaB with NADPH. This change in kinetics was also evident by the 3-dimensional structure. Based on the crystal structure, Q47 was found to be located in the $\alpha2$ helix. The loop between $\beta2$ and $\alpha2$ contributed to NADP$^+$ recognition through Arg40 (Fig. 3C). Furthermore, the temperature factor in this region, as well as $\alpha7 – \alpha8$, was higher than in the others (Fig.
These results suggest that the substitution for Q47 may alter the flexibility of this region so as to be preferable for the interaction with NADP(H). Taking these results together, it was concluded that the selected mutations influenced the recognition of NADPH and/or AcAc-CoA indirectly via alterations in the mobility of α2 and α7–8. The crystal structure data did account for the beneficial effects of the mutations. However, it is worth noting that the mutations were unpredictable from the protein structure, which may indicate the need for a combined strategy of structural and non-structural based enzyme engineering.

The $K_{M(AcAcCoA)}$ and $K_{M(NADPH)}$ values of the native PhaB isolated from R. eutropha were previously reported to be 5 and 19 μM, respectively (33). $K_{M(AcAcCoA)}$ was consistent with the result of the present experiment, while the $K_{M(NADPH)}$ value of native PhaB was lower than the recombinant protein. This disagreement in the kinetic parameters could be caused by the difference in the host strain used for PhaB expression and also the presence of His-tag at the N-terminal of the protein. Moreover, it is reported that R. eutropha possesses multiple PhaB isologs (34). Therefore, the native PhaB isolated from R. eutropha might be composed of multiple proteins having very similar physical properties, which could be the cause of the differences in the kinetic parameters.

The potential variety in the mutated residue was estimated by the database search (http://blast.ncbi.nlm.nih.gov/), which retrieved PhaB homologs assigned as AcAc-CoA reductase (Fig. 4). Fig. 4 indicates alignment of PhaB homologs, which were chosen based on the variety of residues 47 and 173, namely many similar sequences are omitted. According to the alignment of these homologs, residue 173 was found to be located in a
highly-conserved region, which suggests that the region around the 173 position plays an
important role in PhaB activity. In contrast, the \( \alpha_2 \) region that included residue 47 was not
highly conserved. The position was occupied by a limited variety of amino acid residues,
such as Gln, Glu or Asp, but not Leu. In fact, \( \alpha_2 \) was not directly in contact with the
substrate and thus was presumably tolerant to further modification.

**Conclusions**

In this study, two novel activity-enhanced mutants of PhaB exhibited enhanced
\( k_{\text{cat}}/K_{M(\text{NADPH})} \) and \( k_{\text{cat}}/K_{M(\text{AcAcCoA})} \) values, respectively. The design of the plate assay-based
high-throughput screening, namely the effect of AACS on suppressed P(3HB) production,
was a key to obtaining the beneficial mutants. These mutants should be applicable to
obtaining higher P(3HB) production in various platforms, including microbes and plants, if
the monomer-supplying step is rate-limiting, as observed here for recombinant *C. glutamicum*. In addition, the crystal structure analysis of PhaB revealed that the flexible
regions in PhaB contributed to the activity. The structural information reasonably explained
the effect of the mutations on the enzymatic activity, and provides a clue to design the
structure-based engineering of this enzyme, as well as future mutation/selection or other
strategies along similar lines to the one employed in this study.

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REFERENCES


Engineering of NADPH-dependent acetoacetyl-CoA reductase


rearrangements establish a catalytically competent active site and a proton relay conduit in FabG. Structure 12:417-428.


**FIGURE LEGENDS**

**FIG. 1.** P(3HB) production in recombinant C. glutamicum harboring the engineered phaB genes. (A) Time course of P(3HB) accumulation. Circle: wild-type PhaB, triangle: Q47L, square: T173S. (B) Correlation between the enzymatic activity and P(3HB) content in recombinant C. glutamicum. Black bar: enzymatic activity (left axis). Gray bar: P(3HB) content (right axis). The PhaB activity was measured at 24 h. All data is the average of at least three trials.

**FIG. 2.** Crystal structure of the PhaB and mutants. (A) Ribbon diagram of the PhaB monomer. The ribbon model is colored according to the sequence, from blue at the N-terminus to red at the C-terminus. (B) Tetrameric structure of PhaB. The model is colored according to the subunit. Q47 (purple) and T137 (cyan) are also shown as spherical models. (C) Structure superimposition among wild-type (red), Q47L (blue), and T137S (orange). For clarity, only a monomer of the superimposed tetramer is shown. (D) Temperature factor of wild type PhaB. The tube model is colored according to the
temperature factor, from blue at 20 to red at 50. The width of the tube also corresponds to temperature factor. In short, red thick region implies high flexibility and blue thin region low. Q47 is also shown as green sticks. The flexible $\alpha_7 - 8$ and $\alpha_2$ are indicated.

**FIG. 3.** Crystal structure of PhaB – AcAc-CoA – NADP$^+$ ternary complex. (A) The Fo-Fc map (contoured at 1.5s) of AcAc-CoA and NADP$^+$. AcAc-CoA (yellow) and NADP$^+$ (green) are also shown as sticks. (B) Ribbon diagram of the tetramer in complex with AcAc-CoA and NADP$^+$. The bound AcAc-CoA (yellow) and NADP$^+$ (green) are shown as spherical models. (C) Close-up view of the substrate-binding site. The bound substrates and their recognition residues are shown in stick models. The flexible $\alpha_7 - 8$ region and $\beta_2 - \alpha_2$ are colored purple and brown, respectively. (D) T173 in the adjacent subunits located around the AcAc-CoA binding site. T173 residues in the adjacent subunits are shown as blue spheres. P207 interacting with T173 is shown as a purple sphere. The ribbon diagrams are colored according to the subunits, although the flexible $\alpha_7 - 8$ regions and their continuing loops are colored purple.

**FIG. 4.** Partial alignment of PhaB from *R. eutropha* and its homologous enzymes. Asterisk indicates the beneficial sites. Secondary structure was shown based on the crystal structure of PhaB from *R. eutropha*. The residue 47 locates in the non-conserved $\alpha$-helix, while residue 173 locates in the random coil. The position 47 is often occupied with hydrophilic residues. The position 173 is mostly occupied with Thr, while some PhaB homologs possess Ser residue at this position.
Figure 1
Fig. 2
Fig. 3
Figure 4.
Figure S1. Lineweaver Burk plots for the wild-type and mutated acetoacetyl-CoA reductase (PhaB). NADPH concentrations are 25 (diamond), 31 (square), 42 (triangle), 62.5 (cross) and 125 µM (asterisk), respectively.
### Table S1. X-ray data collection and refinement statistics

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<td>C2</td>
<td>C2221</td>
<td>C222</td>
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<tr>
<td>Unit-cell parameters (Å, °)</td>
<td>a = 67.7, b = 123.7, c = 260.8</td>
<td>a = 93.6, b = 88.6, c = 70.7, β = 104.24</td>
<td>a = 70.7, b = 185.9, c = 75.5</td>
<td>a = 67.4, b = 123.4, c = 260.2</td>
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<tr>
<td>Number of molecule in an asymmetric unit</td>
<td>4</td>
<td>2</td>
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<td>4</td>
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<tr>
<td>No. of observed reflections</td>
<td>721056</td>
<td>159130</td>
<td>67176</td>
<td>330241</td>
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<tr>
<td>No. of unique reflections</td>
<td>100490</td>
<td>37627</td>
<td>11342</td>
<td>58459</td>
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<tr>
<td>Completeness † (%)</td>
<td>97.7 (89.2)</td>
<td>99.4 (97.8)</td>
<td>99.8 (99.3)</td>
<td>96.9 (98.7)</td>
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</tr>
<tr>
<td>&lt;(I&gt;/&lt;σ(I))&gt; † (%)</td>
<td>17.85 (3.86)</td>
<td>7.07 (3.07)</td>
<td>7.57 (2.15)</td>
<td>25.14 (7.86)</td>
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</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th>R-factor / R-free (%)</th>
<th>19.2 / 23.8</th>
<th>23.5 / 26.7</th>
<th>18.9 / 24.0</th>
<th>16.2 / 20.7</th>
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<tbody>
<tr>
<td>No. of atoms</td>
<td>8195</td>
<td>4075</td>
<td>3668</td>
<td>8469</td>
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<td>ligands</td>
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<td>0</td>
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<td>water</td>
<td>692</td>
<td>407</td>
<td>0</td>
<td>627</td>
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<tr>
<td>R.m.s.d bond lengths (Å)</td>
<td>0.007</td>
<td>0.002</td>
<td>0.005</td>
<td>0.03</td>
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<tr>
<td>R.m.s.d bond angles (°)</td>
<td>0.98</td>
<td>0.61</td>
<td>0.84</td>
<td>0.77</td>
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<tr>
<td>Average B-factor</td>
<td>21.3</td>
<td>14.0</td>
<td>24.3</td>
<td>19.6</td>
<td></td>
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</tbody>
</table>

| Ramachandran plot † (%) | favored | 98 | 98 | 97 | 98 |
| outiers      | 0       | 0 | 0 | 0 | 0 |

*Values in parentheses are for outermost shell.
†Ramachandran statistics were calculated with MolProbity.