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RESEARCH PAPER

# Structural Basis for the Substrate Specificity of 3-hydroxybutyrate Dehydrogenase

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**Abstract** The substrate specificity of 3-hydroxybutyrate dehydrogenase from *Alcaligenes faecalis* with a non-native substrate, levulinic acid, was studied by analysis of the enzyme-substrate molecular interactions. The relation between structural and kinetic parameters was investigated considering the catalytic mechanism of the enzyme. The effects of key positive mutations (H144L, H144L/W187F) on the catalytic activity of the enzyme were studied by employing a surface analysis of its interatomic contacts between the enzyme and substrate atoms. The results revealed that the alteration of hydrogen bond network and rearrangement of the hydrophobic interactions between the active site and substrate molecule are the key structural basis for the change of the substrate specificity of 3-hydroxybutyrate dehydrogenase toward levulinic acid. With this approach, the structural basis for the substrate specificity of the enzyme could be elucidated in a quantitative manner.

**Keywords:** 3-hydroxybutyrate dehydrogenase, interatomic contact surface, levulinic acid, molecular docking, substrate specificity

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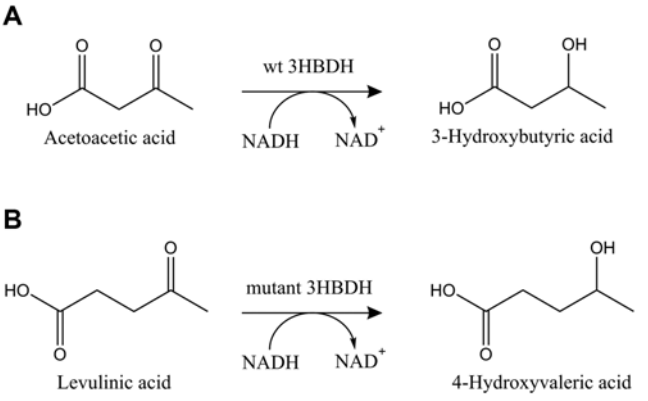
## 1. Introduction

Enzymes have been developed as environmentally friendly alternatives to traditional chemical catalysts [1]. However, the biotechnological application of the enzymes in their native form is limited by the finite repertoire of the enzymatic catalysis [2]. Engineering the substrate specificity of enzymes through protein engineering [3] is one of the promising approaches that could expand enzyme applications to the production of valuable chemicals, fuels, and pharmaceuticals [4-6].

The substrate specificity of 3-hydroxybutyrate dehydrogenase (3HBDH, EC 1.1.1.30) from *Alcaligenes faecalis* has been previously engineered to synthesize 4-hydroxyvaleric acid from levulinic acid (Scheme 1) [7]. 3HBDH is an $NAD^+$-dependent enzyme that catalyzes the reversible reaction between D-3-hydroxybutyrate and acetoacetate [8]. Levulinic acid is a promising chemical building block that can be obtained from cellulosic biomass [9], and 4-hydroxyvaleric acid can be used for value-added chemicals such as a monomer of bio-polyesters, a precursor of bio-fuels, industrial solvents and carbon-based chemicals [10,11]. In this regard, the enzymatic conversion of levulinic acid to 4-hydroxyvaleric acid is of special interest because the catalyzed reaction provides a useful basis for the bio-production of industrially valuable chemicals from cellulosic biomass.

In addition to the previously reported 3HBDH variants, additional mutants were generated in this study for a total of 16 variants to further investigate the structure-activity relation of the enzyme, and effects of the applied mutations on the enzyme's substrate specificity with the target substrate. Because the 3HBDH enzyme belongs to a family of short-chain dehydrogenase/reductases (SDRs), it follows the H-transfer mechanism for the catalysis [12,13]. The




**Scheme 1.** (A) The reduction of acetoacetate (production of 3-hydroxybutyric acid) by wild-type 3HBDH. (B) The reduction of levulinic acid (production of 4-hydroxyvaleric acid) by mutant 3HBDH.

donor-acceptor distance between the enzyme (or cofactor) and substrate is regarded as an important factor in the H-transfer of the reaction [14-16]. Therefore, donor-acceptor distance was selected as a key structural parameter to analyze the structure-activity relation of the system, and the structural basis of the enzyme-substrate specificity of 3HBDH for levulinic acid was analyzed by using this parameter.

The structural effects of the positive mutations were analyzed by interatomic contact analysis. The contact area of hydrogen bonds, hydrophobic interactions and hydrophobic-hydrophilic repulsions were incorporated to investigate the structural feature of the enzymatic system. The key enzyme-substrate interactions that facilitate the catalytic conversion of levulinic acid were identified, and the structural basis for the substrate specificity of 3HBDH was analyzed.

## 2. Materials and Methods

### 2.1. Model enzyme and reaction system

The enzymatic synthesis of 4-hydroxyvaleric acid from levulinic acid with 3HBDH has been attempted in our previous study [7]. Due to the low catalytic activity of the wild-type 3HBDH towards levulinic acid, the substrate specificity of 3HBDH was engineered and resulted in the H144L/W187F double mutant, which showed the highest catalytic activity towards levulinic acid [7]. To further investigate the structural effect of these mutations on the enzyme-substrate specificity, in this study, an additional 12 mutants (for a total of 16 variants) were constructed by focusing on the two substrate-binding residues, namely, H144 and W187. The relations between the kinetic and structural parameters of the mutants were investigated by using levulinic acid as a target substrate.

### 2.2. Materials

The codon-optimized synthetic 3HBDH gene was supplied by Bioneer (Daejeon, Korea). Restriction enzymes (NdeI and XhoI) and DNA ligase were purchased from Enzynomics (Daejeon, Korea). The primers were synthesized by Cosmogentech (Seoul, Korea). DNA purification and extraction kits and Ni-NTA agarose for His-tag protein purification were purchased from Qiagen (Valencia, CA, USA). Competent *E. coli* Top 10 and *E. coli* BL21 (DE3) cells were purchased from Invitrogen (Carlsbad, CA, USA) and from Novagen (Madison, WI, USA), respectively. Levulinic acid, lithium acetoacetate, NADH and ammonium formate were purchased from Sigma (St. Louis, MO, USA). A levulinic acid solution in salt form (as sodium levulinate) was prepared as a substrate for the enzyme assay by adding 20% NaOH to a 98+% levulinic acid solution (Sigma, USA) to reach a pH of 6.59. Because the pKa of levulinic acid is 4.59, the 2-pH unit difference caused by titrating with NaOH ensured that 99% of the levulinic acid was in the form of a sodium salt [17].

### 2.3. Enzyme mutation, expression and purification

The 3HBDH gene was PCR-amplified from the synthetic gene. The amplified fragments were digested by NdeI and XhoI and cloned into the multicloning site of the pET-22b(+) vector (Novagen, USA) to produce a recombinant plasmid that expressed the protein with an N-terminal 6-histidine tag. The mutants used in this study were constructed by using a modified QuikChange site-directed mutagenesis protocol [18]. All mutations were verified by DNA sequencing (Cosmogentech, Korea). For protein expression, the plasmids were chemically transformed into competent *E. coli* BL21 (DE3). The cells were induced by adding 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and cultured at 20°C for 24 h to express 3HBDH. The N-terminal His-tagged wild-type and mutant enzymes were purified as previously described [19]. The enzyme purity was estimated by 12% SDS-PAGE, and the enzyme concentration was determined by modified Bradford assay [20] according to the manufacturer's instructions (Bio-Rad, USA).

### 2.4. Kinetic analysis

To assay the enzyme activity, the acetoacetate and levulinic acid reduction was monitored by measuring the NADH oxidation rate with a UV-spectrophotometer (Varian, USA) at 340 nm. For the 3HBDH kinetic analysis, the $K_m$ and $k_{cat}$ values for each substrate were determined by using various concentrations of the substrates in 50 mM Bis-Tris-HCl buffer (pH 6.5) containing 0.25 mM NADH at 30°C. The kinetic parameters were determined from a Lineweaver-Burk

plot by using the initial rate data. All kinetic parameters presented in this study are mean values derived from triplicate measurements.

### 2.5. Molecular docking simulation

The molecular docking simulation was performed with a SYBYL-X package (Tripos, ver. 2.1). The crystal structure of 3HBDH from *Alcaligenes faecalis* (pdb code: 3VDR, [13]) was used to determine the best docking position of levulinic acid or acetoacetate at the binding pocket. All the structures of the substrates in this study were sketched by using the sketch module in the SYBYL-X package and minimized by using the Tripos force field [21] with the Gasteiger-Hückel charge [22] until the RMS gradient was < 0.05. The *in silico* mutation method was applied to generate mutant structures. The side chain conformation of the mutated residue was selected from the Lovell rotamer library [23] or the one that resulted in the fewest bumps with the rest of the molecule. The Surflex-Dock control files were defined by setting the *protomol*, a computational representation of the intended binding site to which putative ligands are aligned, generation options with a "Ligand" mode, a "Threshold" value of 0.5 and a "Bload" value of 3 Å. Surflex docking [24] of the substrate was performed using the Run-Multiple ligand option of Surflex-Dock, and the substrate bound forms of the enzyme structures were obtained.

### 2.6. Interatomic contact analysis

The structures of the enzyme-substrate complex generated from molecular docking simulations were used as input data of the web server of LPC/CSU (http://ligin.weizmann.ac.il/cgi-bin/lpccsu/LpcCsu.cgi) [25] for interatomic contact analysis. The surface areas of the interatomic contacts for hydrogen bonds, hydrophobic interactions and hydrophobic-hydrophilic repulsions between each heavy atom of the substrate with the active site atoms of the enzymes were obtained for structural investigation of the enzyme-substrate interactions.

## 3. Results and Discussion

### 3.1. Kinetic study of 3HBDH with levulinic acid

In our previous study, the H144L mutation generated the initial catalytic activity of 3HBDH towards levulinic acid, and a more increased activity was obtained through a double mutation of H144L/W187F [7,26]. In this regard, additional mutations and kinetic studies were conducted with a focus on the two residues, namely, H144 and W187. The H144 site was mutated with 5 hydrophobic residues (A, V, L, I, and F), and the H144L mutant exhibited the highest catalytic efficiency among them (Table 1). Starting with the H144L mutant, 5 double mutants were generated by applying the same mutations (A, V, L, I, and F) to the W187 residue. The 5 single hydrophobic single mutants for the W187 residue were also generated, and the kinetic parameters of all mutants were investigated. A total of 15 mutants (10 single and 5 double mutants) were obtained, and the kinetic parameters ($K_m$ and $k_{cat}$) were measured for each one, including the wild-type 3HBDH (Table 1). In this study, the data from 3HBDH variants with N-terminal His-tag were applied for the kinetic study due to the catalytically interfering effects of the C-terminal counterparts [26].

**Table 1.** The kinetic parameters of 3HBDHs with levulinic acid

| 3HBDH | $K_m$ (mM) | $k_{cat}$ (/min) | $k_{cat}/K_m$ (M/sec) |
|---|---|---|---|
| WT | 0.78 ± 0.08 | 0.36 ± 0.05 | 7.68 ± 1.12 |
| H144A | 9.87 ± 1.11 | 0.50 ± 0.07 | 0.84 ± 0.34 |
| H144F | 1.72 ± 0.24 | 0.95 ± 0.18 | 9.21 ± 2.24 |
| H144I | 2.81 ± 0.32 | 1.81 ± 0.30 | 10.69 ± 3.31 |
| H144L | 0.91 ± 0.09 | 3.61 ± 0.48 | 65.94 ± 8.42 |
| H144V | 3.84 ± 0.43 | 1.76 ± 0.24 | 7.66 ± 1.23 |
| W187A | 36.83 ± 4.54 | 0.35 ± 0.06 | 0.16 ± 0.02 |
| W187F | 0.63 ± 0.07 | 1.12 ± 0.18 | 29.69 ± 4.26 |
| W187I | 45.40 ± 4.71 | 0.83 ± 0.12 | 0.30 ± 0.05 |
| W187L | 13.49 ± 2.12 | 3.76 ± 0.48 | 4.65 ± 0.74 |
| W187V | 42.10 ± 3.83 | 0.76 ± 0.12 | 0.30 ± 0.05 |
| H144L/W187A | 13.03 ± 0.22 | 0.95 ± 0.18 | 1.22 ± 0.33 |
| H144L/W187F | 0.64 ± 0.07 | 9.83 ± 1.08 | 256.54 ± 30.34 |
| H144L/W187I | 25.76 ± 1.85 | 4.46 ± 0.54 | 2.89 ± 0.71 |
| H144L/W187L | 0.63 ± 0.09 | 2.67 ± 0.36 | 71.07 ± 14.25 |
| H144L/W187V | 25.57 ± 2.01 | 3.07 ± 0.42 | 2.00 ± 0.51 |

### 3.2. Relation between structural and kinetic parameters

The relation between $\Delta G_b$ and $K_m$ for the 16 3HBDHs with levulinic acid was investigated. The results revealed that the $\Delta G_b$ values are closely related to the $K_m$ values; the variants with negatively higher $\Delta G_b$ values have the smaller $K_m$ values (Fig. 1), which also indicates that the molecular docking simulation was conducted properly.

Considering the catalytic mechanism of 3HBDH, the donor-acceptor distances between the enzyme (or cofactor) and substrate are regarded as critical parameters that can be correlated with the catalytic activity of the enzyme [12,13]. In this regard, a new structural parameter ($D_{avg}$) was defined as follows.

$$D_{avg} = (d_1+d_2)/2 \quad \text{(Eqn. 1)}$$

where $d_1$ is the distance between the C4 atom of NADH and the C4 atom of levulinic acid, and $d_2$ is the distance between the oxygen atom of Tyr155 and the oxygen atom

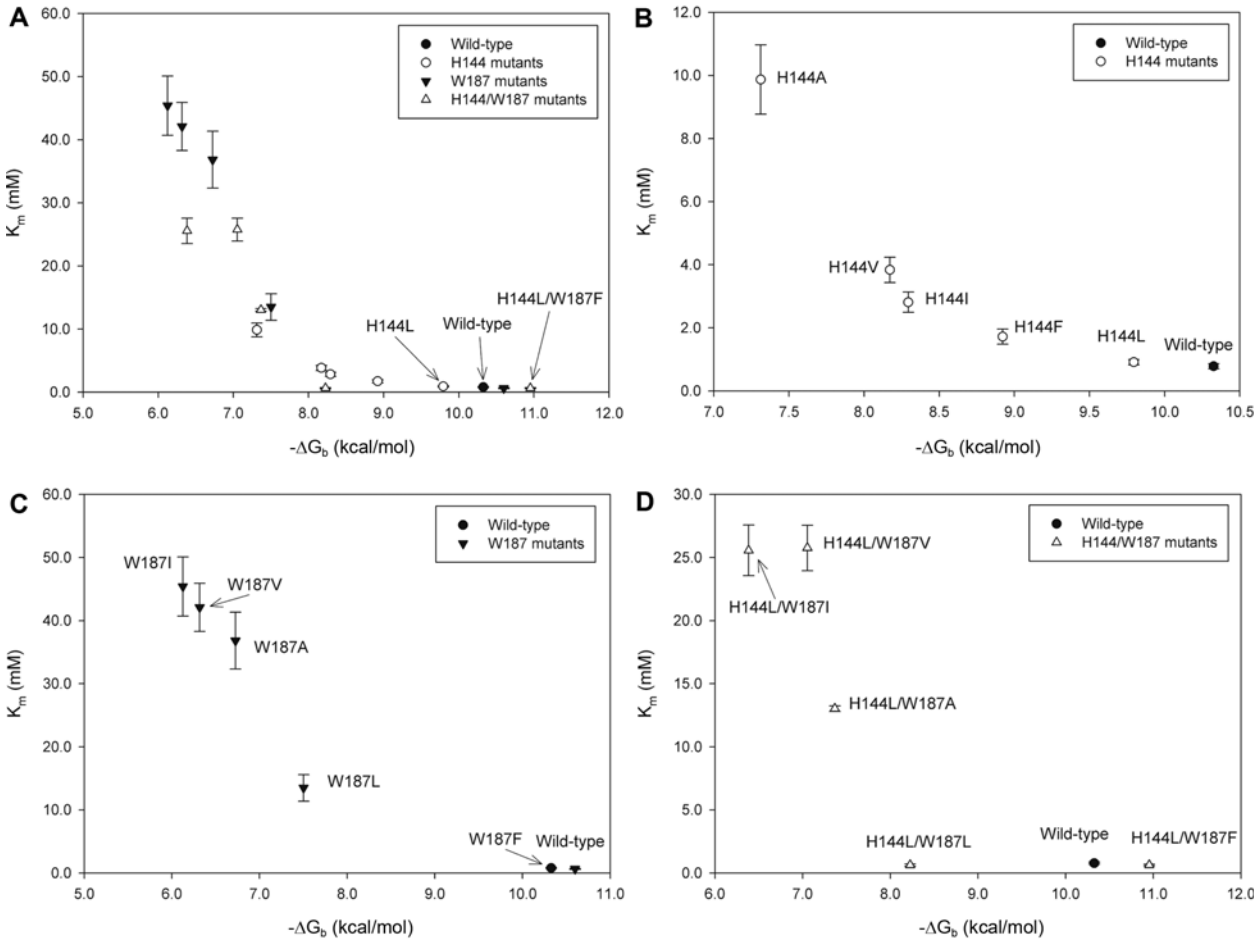
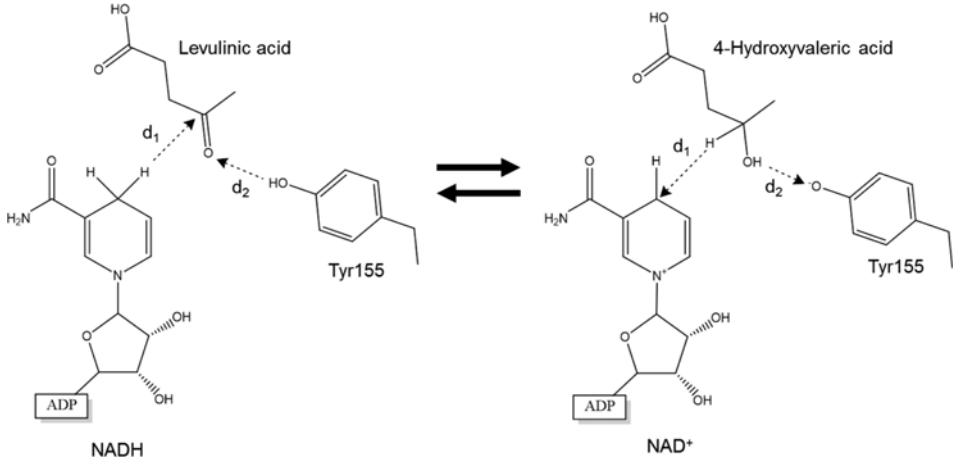



**Fig. 1.** Relation between $K_m$ and ($-\Delta G_b$) for the 16 3HBDH variants with levulinic acid (A) for all 16 3HBDHs, (B) for wild-type and H144 mutants, (C) for wild-type and W187 mutants, and (D) for wild-type and H144L/W187 mutants.




**Scheme 2.** Schematic diagram for the catalytic mechanism of 3HBDH with levulinic acid ($d_1$: The distance between the C4 atom of NADH and the C4 atom of levulinic acid, $d_2$: the distance between the oxygen atom of Tyr155 and the oxygen atom of the ketone group of levulinic acid).

of the ketone group of levulinic acid (Scheme 2).

In Eqn. 1, the average value ($D_{avg}$) of the two donor-acceptor distances ($d_1$ and $d_2$) was employed considering the H-transfer mechanism of the enzyme [12,13]. The relation between $D_{avg}$ and $k_{cat}$ in the 16 3HBDHs was investigated to study the structure-activity association of

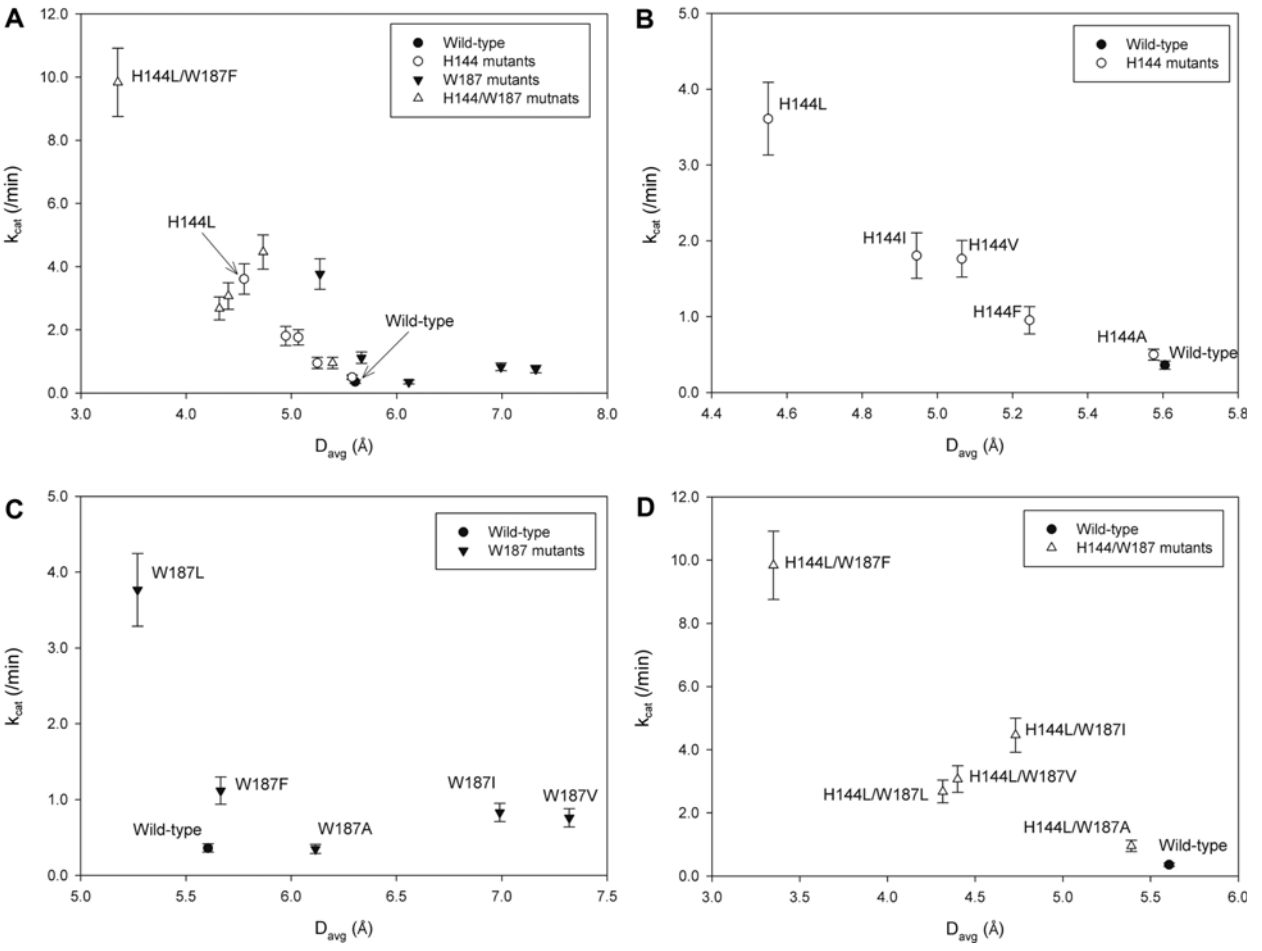



**Fig. 2.** The relation between $k_{cat}$ and $D_{avg}$ for the 16 3HBDH variants with levulinic acid (A) for all 16 3HBDHs, (B) for wild-type and H144 mutants, (C) for wild-type and W187 mutants, and (D) for wild-type and H144L/W187 mutants.

the enzyme with levulinic acid. The results revealed that the $D_{avg}$ values are closely related to the $k_{cat}$ values; the shorter the donor-acceptor distances between the enzyme (or cofactor) and substrate, the higher the catalytic turnover rate of the 3HBDH enzyme (Fig. 2). In addition, because the $\Delta G_b$ and $D_{avg}$ are related to $K_m$ and $k_{cat}$, respectively, the correlation between the $(-\Delta G_b/D_{avg})$ and $k_{cat}/K_m$ was also investigated. The results indicated that the $(-\Delta G_b/D_{avg})$ value is positively related to the catalytic efficiency of the system (Fig. 3). These findings indicate that $\Delta G_b$, $D_{avg}$ and $(-\Delta G_b/D_{avg})$ could be used as potential parameters for structure-activity relation of the enzyme. However, there must be additional factors other than donor-acceptor distance and binding affinity to elucidate the whole principle of enzymatic catalysis. More fundamental factors, therefore, such as electrostatic preorganization [27] and conformational dynamic effects [28], should be considered by employing additional approach, such as quantum mechanics/molecular mechanics (QM/MM) and/or molecular dynamics (MD) *etc*. On the other hand, quantitative structure-activity relationship (QSAR) approach could be another option for the study of structure-activity relation of enzymes [29].

### 3.3. Validation of the approaches with acetoacetate

Structural analyses with acetoacetate, the native substrate of 3HBDH, were performed to validate the approaches used in this study. The molecular docking for acetoacetate was carried out in the same way as for the levulinic acid case. The ($K_m \sim -\Delta G_b$), ($k_{cat} \sim D_{avg}$) and ($k_{cat}/K_m \sim (-\Delta G_b/D_{avg})$) correlations were investigated for the 8 representative 3HBDHs with acetoacetate, and similar results were obtained as in the case of levulinic acid (Figs. 4A, 4B, and 4C). These results indicate that the correlations are not limited to the specific levulinic acid example.

### 3.4. Interatomic contact analysis of 3HBDH with levulinic acid

An enzyme-substrate interatomic contact analysis was performed to analyze the applied mutation effects on the substrate specificity of the enzyme for levulinic acid. The

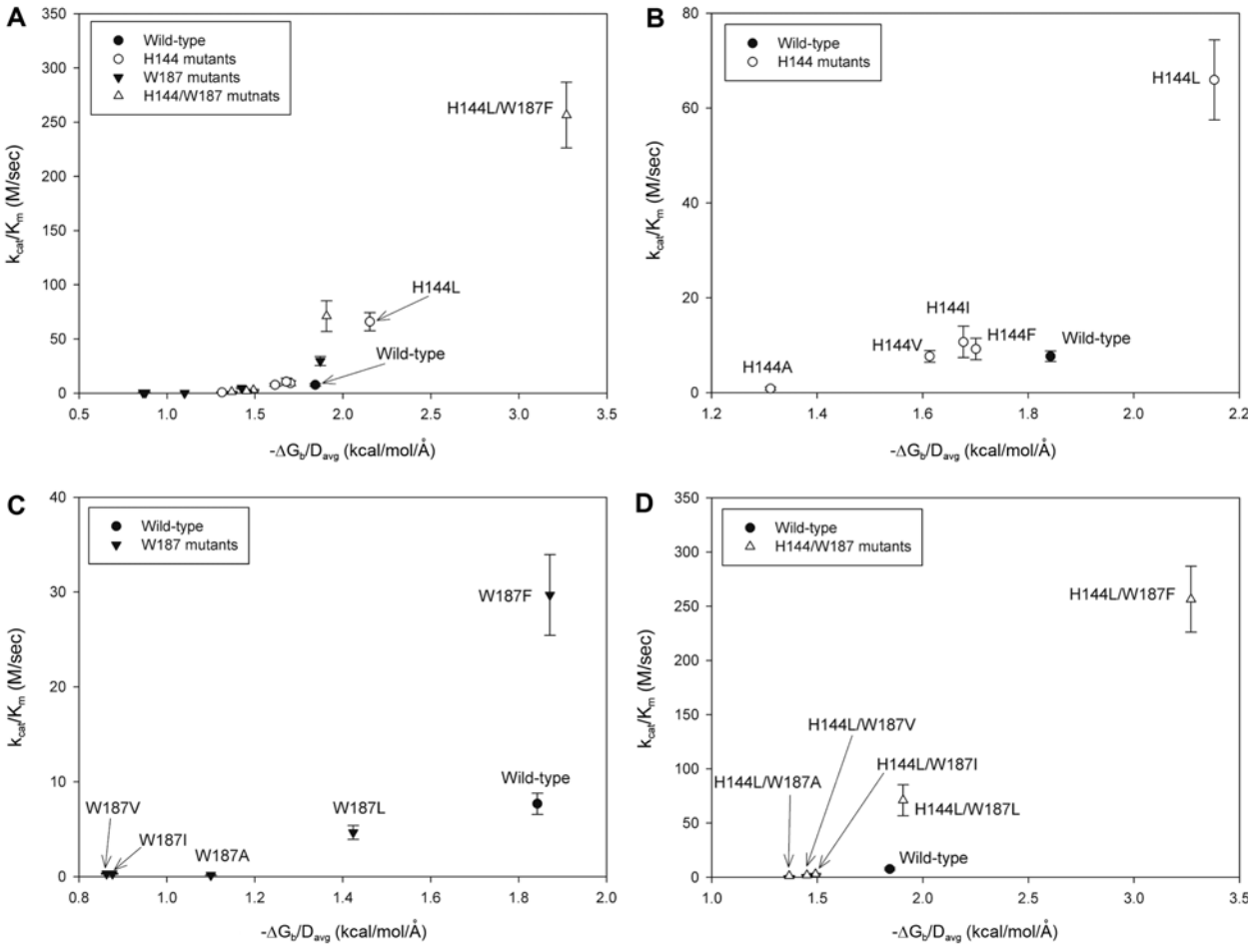



**Fig. 3.** The relation between $k_{cat}/K_m$ and $(-\Delta G_b/D_{avg})$ for the 16 3HBDH variants with levulinic acid (A) for all 16 3HBDHs, (B) for wild-type and H144 mutants, (C) for wild-type and W187 mutants, and (D) for wild-type and H144L/W187 mutants.

three key variants of 3HBDH, namely, the wild-type, H144L and H144L/W187F, were selected to study the structural characteristics of the enzyme-substrate interactions. The interatomic contact area for hydrogen bonds, hydrophobic interactions and hydrophobic-hydrophilic repulsions were investigated to identify the key enzyme-substrate interactions that facilitate the catalytic conversion of levulinic acid.

When the wild-type 3HBDH was changed into the H144L mutant, the hydrogen bond between substrate atom 6 and H144 disappeared (the atom numbers of levulinic acid are noted in Figs. 5D, 6A, and 6B). In addition, the substituted L144 residue generated a hydrophobic-hydrophilic repulsion interaction between the L144 residue and atom number 8 of the substrate (Fig. 5C). This repulsion pushed atom 8 towards S142, and it generated a hydrogen bond between atom 8 and the residue (Fig. 6B). Throughout this procedure, the C4 atom (atom number 2) of levulinic acid moved towards the C4 atom of NADH, and the distance between atom 8 and the oxygen atom of Y155 became shorter. Therefore, the donor-acceptor distances of the catalysis were decreased, and the catalytic activity increased. However, the H144L mutation changed the hydrogen bonding pattern of the substrate carboxyl group with the substrate binding residues of the enzyme (Fig. 6B); the hydrogen bond contact area of atom 6 was increased (from 22.9 to 41.7 Å$^2$), and that of atom 7 was decreased (from 31.7 to 9.2 Å$^2$) (Fig. 5A). This structural change rearranged the binding mode of levulinic acid in a manner that was favorable to the corresponding catalysis by decreasing the donor-acceptor distances (Fig. 7A).

When the H144L mutant was changed to the H144L/W187F mutant, the space in the active site was increased because of the smaller size of the F rather than that of W (Figs. 6B, 6C, and 7B). This change reduced the hydrophobic contact area of substrate atom numbers 1, 3, 4 and 5 (Fig. 5B), and it adjusted the binding mode of the substrate to become more favorable to catalysis (Fig. 7B). The donor-acceptor distances became shorter (Figs. 2 and 7B) through the disappearance of the hydrophobic-hydrophilic

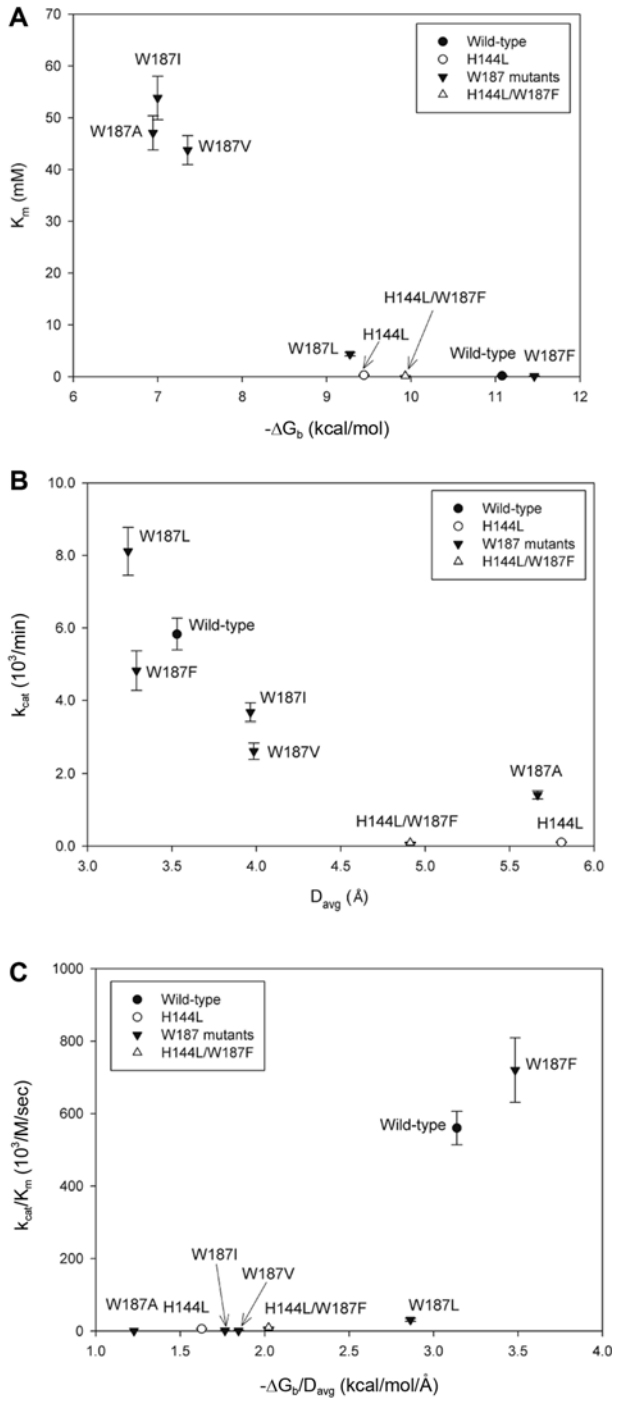



**Fig. 4.** The relation between kinetic parameters and structural parameters for the 8 3HBDHs with acetoacetate, (A) Relation between $K_m$ and ($-\Delta G_b$), (B) Relation between $k_{cat}$ and $D_{avg}$, (C) Relation between $k_{cat}/K_m$ and ($-\Delta G_b/D_{avg}$).

repulsion of atom 8 (Fig. 5C) while retaining the hydrogen bond contact area of atom 8 (Fig. 5A). The mutation also changed the hydrogen bonding pattern of the carboxyl group of the substrate with the substrate binding residues of the enzyme (Fig. 5A), but the spatial coordinate of the

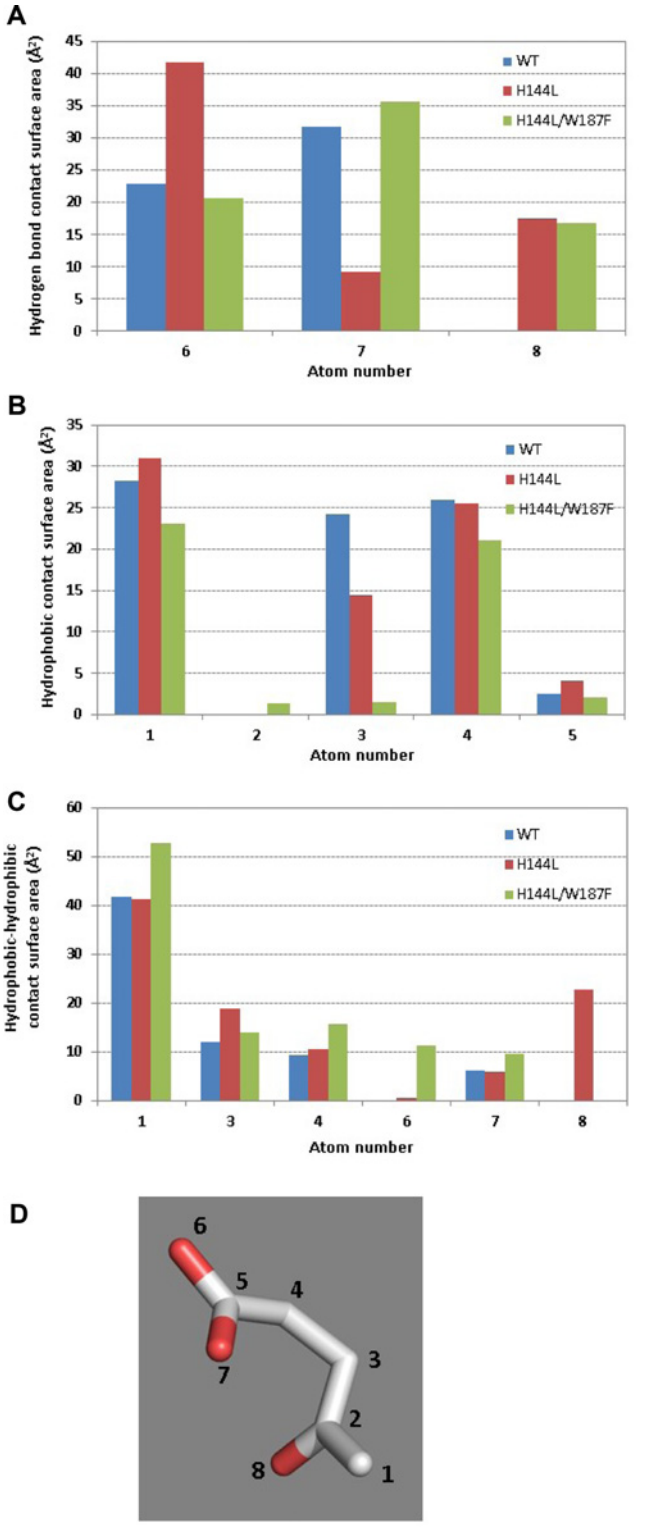



**Fig. 5.** The interatomic contact surface area for levulinic acid with (A) hydrogen bond contact surface area between the substrate and enzyme (no values with atom numbers 1, 2, 3, 4, and 5), (B) hydrophobic contact surface area between the substrate and enzyme (no values with atom numbers 6, 7, and 8), (C) hydrophobic-hydrophilic contact surface area between the substrate and enzyme (no values with atom number 5), and (D) atom numbers assigned to the levulinic acid.

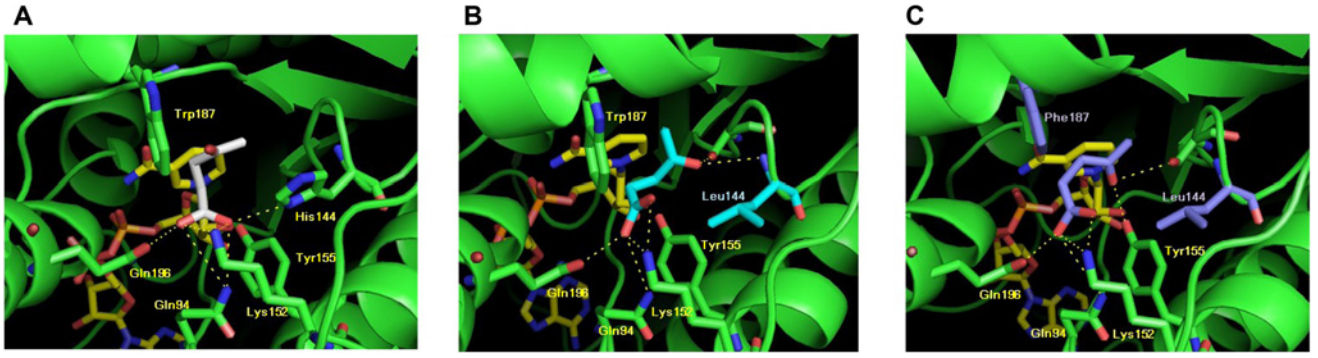



**Fig. 6.** The hydrogen bonding network of levulinic acid in the active site of 3HBDHs obtained from docking simulations (A) Wild-type (white stick: levulinic acid) (B) H144L (cyan stick: levulinic acid and mutated residue), and (C) H144L/W187F (purple stick: levulinic acid and mutated residues), common descriptions; yellow dotted line: hydrogen bond, yellow stick: NADH, red atom: oxygen, blue atom: nitrogen.

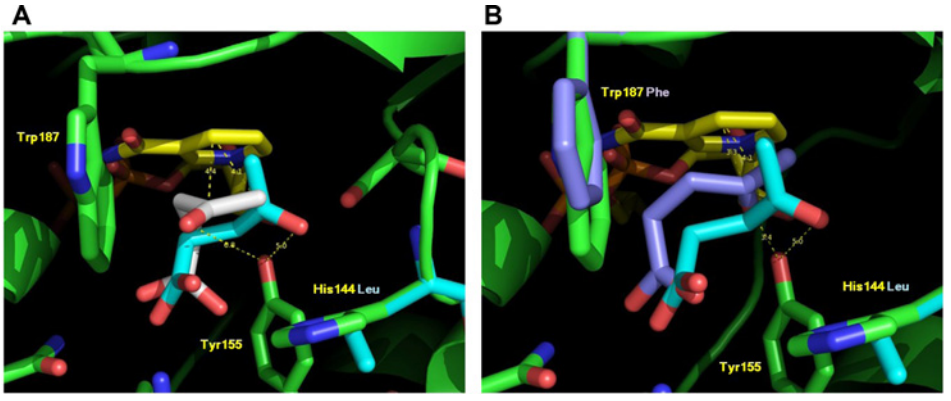



**Fig. 7.** The binding poses of levulinic acid in the 3HBDHs active site according to the mutations was as follows: (A) The mutation of the wild-type to H144L (white stick: levulinic acid, cyan stick: levulinic acid and mutated residue), (B) The mutation of H144L to H144L/W187F (cyan stick: levulinic acid and mutated residue, purple stick: levulinic acid and mutated residues); common descriptions; yellow dotted line: donor-acceptor distances in the catalytic mechanism, yellow stick: NADH, red atom: oxygen, blue atom: nitrogen.

carboxyl group was almost unchanged (Figs. 6B and 6C).

However, the binding affinity ($K_m$ and $\Delta G_b$) of the 3 enzyme variants (Fig. 1 and Table 1) showed no significant differences. The $K_m$ values for the wild-type, H144L and H144L/W187F were 0.78, 0.91 and 0.64 mM, and the $\Delta G_b$ for the variants are -10.3, -9.8, and -11.0 kcal/mol, respectively. These values indicate that the major mutational effect that improved the catalytic efficiency of the enzyme is not the increased binding affinity, but the increased catalytic turnover rate ($k_{cat}$). In other words, the decreasing donor-acceptor distances are the major mutational effects that improved the catalytic efficiency of the enzyme towards levulinic acid.

In summary, the H144L mutation increased the catalytic activity by changing the hydrogen bond network between the substrate and the active site, and the additional W187F mutation further enhanced the activity by providing extra space to adjust the binding mode of the substrate with rearranged hydrophobic interactions. In both cases, the mutations enhanced the enzyme-substrate binding affinity and the catalytic activity by decreasing the donor-acceptor distances, which is the key structural basis of the improved catalytic efficiency of the enzyme with levulinic acid.

## 4. Conclusion

The substrate specificity of 3HBDH variants with levulinic acid was studied by employing molecular docking simulation and interatomic contact analysis. The relation between structural and kinetic parameters was investigated in a quantitative manner. The results revealed that the alteration of the hydrogen bond network and rearrangement of the hydrophobic interactions between the active site and substrate molecule are the key structural basis for the change of the substrate specificity of 3HBDH toward levulinic acid.

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