Engineering of the Conformational Dynamics of an Enzyme for Relieving the Product Inhibition

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Supporting Information

ABSTRACT: Enzymes with a product inhibition are generally considered a limiting step of the metabolic pathway in producing valuable compounds. Most attempts to relieve the product inhibition have relied on mutations at the product-binding site of enzymes. However, such an approach has resulted in a severe decrease in the catalytic activity, mainly owing to the shared binding site of product with substrate. Herein, we present the modulation of the conformational dynamics of chorismate-pyruvate lyase (CPL) for relieving the product inhibition without a decrease in the catalytic efficiency. CPL is a key enzyme in the biosynthesis of diverse aromatics but incurs a severe product inhibition owing to a strong product binding. On the basis of a structural analysis and molecular dynamics simulations, two key residues were identified for increasing the conformational dynamics of the flaps and thereby facilitating the product release. The designed mutants exhibited almost an 8-fold reduction in product inhibition and a 3-fold higher catalytic rate in comparison to the wild type. We demonstrate that mutation at two key residues leads to a significant increase in the conformational dynamics of the flaps, enhancing the product release through an opening of the flaps and thereby relieving the product inhibition.

KEYWORDS: conformational dynamics, chorismate-pyruvate lyase, product release, product inhibition, molecular dynamics simulation

INTRODUCTION

The product inhibition of enzymes in a cellular metabolic pathway is one of the key mechanisms in controlling the biosynthesis of metabolites.1 From a practical standpoint, however, enzymes with a product inhibition are considered a limiting step of the metabolic pathway in producing valuable products. Many attempts to relieve the product inhibition of enzymes have relied on mutations at the product-binding site of enzymes.2−5 In most cases, however, such mutations have resulted in a severe decrease in the catalytic activity,2−5 and this seems to be due to the shared binding site of product with substrate. Mutations at the product-binding site are likely to cause deleterious effects on the binding of the substrate, making it difficult to avoid a trade-off between the catalytic efficiency and product tolerance.6,7

Conformational dynamics of the protein plays a key role in enzyme catalysis, influencing the essential steps during enzyme catalysis, including substrate recognition and binding, allosteric regulation, the formation of an enzyme−substrate complex, and product release.8−12 Among the individual steps, substrate binding and product dissociation are considered to commonly determine the overall catalytic rate of an enzyme. Recent studies have shown the connection between the conformational dynamics of proteins and a dissociation of the ligand and product.13−17 In particular, the kinetic analysis of the conformational dynamics of maltose binding protein (MBP) through single-molecule fluorescence resonance energy transfer (smFRET) measurements showed that the ligand dissociation is determined by the intrinsic opening rate of the protein, while the ligand-binding interface remains intact.14−15 Despite many advances in understanding the fundamental aspect of the protein dynamics,16−20 a practical implementation of such knowledge to the design of enzymes remains a challenge.

Chorismate-pyruvate lyase (CPL; EC 4.1.3.40) catalyzes the cleavage of a C−O bond in chorismate, yielding 4-hydroxy-benzoate (4HB) and pyruvate (Scheme 1), as the first-step enzyme in a ubiquinone biosynthetic pathway.21 CPL is a key enzyme in the biosynthesis of diverse aromatic compounds, including aromatic amino acids, hydroquinone, protocatechuate, vanillin, and vitamins.22−25 However, CPL undergoes a severe competitive inhibition by 4HB owing to a strong binding of 4HB to the enzyme,26−28 limiting the biosynthetic pathways for producing aromatic compounds. Herein, we present the engineering of the conformational dynamics of CPL to increase
the product release for relieving the product inhibition, without a
decrease in the catalytic efficiency. On the basis of a structural
analysis and molecular dynamics simulations, we identi
cified two key residues on the flaps overlaying the product-binding site for
enhancing the conformational dynamics of the flaps and thereby
the product release, without a decrease in the catalytic efficiency.
We constructed the mutants and investigated their kinetic
properties as well as the mutational effects on the product
binding and conformational dynamics of the flaps.

RESULTS AND DISCUSSION
Selection of Mutation Sites on the Basis of a Structural
Analysis. The structural analysis of CPL showed that 4HB is
bound internally to the enzyme through five hydrogen bonds and
hydrophobic contacts and is covered by two overlaying
flaps (Figure 1A), resulting in a severe product inhibition by 4HB in a
competitive manner.26−29 It was reported that CPL has a product
inhibition constant ($K_p$) of 2.1 μM, which is about 13 times lower
than the Michaelis constant for substrate, $K_m$.25,26 We
first attempted to weaken the interactions between the product and
enzyme through a site-directed mutagenesis at the product-
binding site to increase the product release and consequently relieve the product inhibition. However, we observed that such
mutations resulted in a serious loss in catalytic activity, and this
seems to be due to detrimental effects on the structural integrity
around the substrate/product binding site. It is likely that the
residues at the product-binding site also play a role in the binding of
substrate, considering the competitive product inhibition
(Table S1 in the Supporting Information).27,28 With an
inspiration from our previous study on the effect of conformational
dynamics of a protein on binding and dissociation of a
ligand,13,14 we intended to modulate the conformational dynamics around the flap regions to increase the intrinsic
opening rate of CPL and thereby facilitate the product release
without perturbing the binding of substrate. To this end, we first
selected the mutation sites around the flaps on the basis of the
following considerations: (1) to minimize the distortion in the
secondary structures of the flaps, the candidate residues were
placed within the loop regions of the flaps and (2) to avoid a
dramatic change in the main chain dynamics, proline and glycine
were excluded. On the basis of these considerations, we selected
four residues (Leu31, Glu32, Asp33, and Ser34) on flap 1 and
five residues (Lys109, Leu110, Lys112, Thr113, and Leu115) on flap
2 (Figure 1B). The selected residues are located on either the
loop region of flap 1, spanning from 30 to 34, or that of flap 2,
spanning from 108 to 115. In particular, Leu31 appears to form a
hydrophobic patch together with the residues (W28, M35, R38,
and F39) on flap 1 (Figure S1A in the Supporting Information).
Asp33 and Ser34 make a hydrogen bond with Arg117 and
Leu115 of flap 2, respectively (Figure S1B). Glu32, Lys109,
Leu110, Lys112, and Thr113 apparently have no interactions
with the other residues. Although Leu115 is thought to make

Figure 1. Structural analysis of product-bound chorismate-pyruvate lyase (CPL) and the selection of candidate residues. (A) Structure of CPL in
complex with the product, 4HB. Flaps 1 and 2 are colored in red and blue, respectively. The inset shows the interactions between 4HB and the enzyme.
Five hydrogen bonds and their interacting residue are shown by the blue dashed lines and labeled. The product-binding site is presented in gray. (B)
Selected residues for modulating the dynamics of the flaps. The Cα positions of each residue on the structure are presented through the spheres and
labeled.
hydrophobic contact with the core β-sheet, it was selected owing to its direct hydrophobic contact with 4HB, by which flap2 could be held (Figure S1C).

**Identification of Key Residues on the Basis of MD Simulations.** To identify the key residues that appear to increase the conformational dynamics around the flap regions, we conducted MD simulations for the wild type and alanine substitutions of each candidate on the basis of the product-bound structure (PDB: 1G1B) and assessed the changes in the dynamics by the root-mean-square fluctuation (RMSF) of the α-carbons (Figure S2 in the Supporting Information). As a result, a distinctive change in RMSF around flap 1 was observed from two substitutions of L31 and D33 in comparison to the wild type and other alanine mutants (Figure S2). Thus, both L31 and D33 were considered key residues that can induce the conformational dynamics around the flaps by mutations. To determine the best substitutions for L31 and D33, we further conducted MD simulations for 20 substituted amino acids at positions 31 and 33 and found that alanine mutations at both sites resulted in the most significant change in RMSF (Figure S3 in the Supporting Information). From the differences in RMSF between the wild type and mutants L31A and D33A (ΔRMSF, Figure 2A), the largest change in the mobility of the main chains near 1 Å (ΔRMSF) was clearly observed over the residues from D33 to Q41 of flap 1 for both mutants, whereas the change in flap 2 was negligible. The region spanning from D33 to Q41 corresponds to the loop and second helix of flap 1, which have a high proximity to 4HB (Figure 2B,C). Thus, both mutants can be expected to weaken the product binding to the enzyme through the increased dynamics of flap 1. On the basis of these analyses, we finally selected L31A and D33A as the best mutants for enhancing the product release by increasing the conformational dynamics of flap 1. The E32A mutant, which shows negligible changes in dynamics, was additionally selected because it can provide insight into whether the mutational effects of L31A and D33A come from changes in the dynamics (Figure S2) or from the shortened side chains.

**Construction and Kinetic Analysis of the Variants.** To directly assess the mutational effect on the product inhibition, we constructed L31A, E32A, D33A, and L31A/D33A variants through a site-directed mutagenesis and determined the $K_p$ of 4HB for wild-type and mutant enzymes (Table 1). All mutants were expressed with a solubility and stability comparable with the wild type. The L31A and D33A mutants were shown to have significantly increased $K_p$ values of 27.2 and 29.4 μM, respectively, which correspond to almost an 8-fold enhancement in comparison to the wild-type enzyme (3.6 μM). The increased inhibition constant indicates a reduction in the product inhibition of the mutants. E32A showed a $K_p$ value similar to that of the wild type, implying that a reduction in the product inhibition of L31A and D33A mutants mainly stems from the change in the conformational dynamics of flap 1 rather than from the shortened side chains. The double mutant (L31A/D33A) also exhibited a $K_p$ value similar to that of the wild type, indicating a negligible synergistic effect by both mutations. Overall, it is plausible that an increase in the dynamics of flap 1 resulting from the L31A and D33A mutations led to a significant reduction in product inhibition.

In order to analyze the mutational effect on the binding equilibrium between product and enzyme, we determined the dissociation constant ($K_d$) of 4HB for both wild-type and mutant enzymes by isothermal titration calorimetry (ITC) (Figure S4 in

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**Figure 2.** Change in the dynamics in the flap regions of the enzyme through MD simulations. (A) Differences in RMSF between the wild type and mutants (L31A and D33A). The RMSF values of both mutants were compared with that of the wild type over all of the residues. Flaps 1 and 2 are presented through the red and blue boxes, respectively. Superposed snapshots of the MD simulations of (B) L31A and (C) D33A mutants. Flaps 1 and 2 are presented through the dashed red and blue lines, respectively. The product, 4HB, is shown in cyan. The changes in RMSF of each α-carbon are shown using a different color (note the color scale).
The 3-fold increase in subsequent increase in the concentration of the free enzyme. It seems to result from the enhanced product release and binding site, resulting in a decrease in the substrate binding the basis of these, the mutations seem to also a to be shared with the substrate in CPL, and the mutations were competitive product inhibition, the product binding site is likely toffi

calculated according to the equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_m (1 + [P]/K_p) + [S]} \]

where \( V_0 \) is the initial reaction rate, \( V_{\text{max}} \) the maximum reaction rate, \( K_m \) the Michaelis constant, [S] substrate concentration, [P] product concentration, and \( K_p \) the product inhibition constant.

To investigate the effect of mutations on the enzyme kinetics, we determined the kinetic parameters of the mutants using a coupled assay protocol\(^{25} \) (Table 2). The turnover numbers (\( k_{\text{cat}} \)) of both the L31A and D33A mutants increased by about 4-fold in comparison to those of both the wild type and E32A, which seems to result from the enhanced product release and subsequent increase in the concentration of the free enzyme. The 3-fold increase in \( K_m \) of the L31A and D33A mutants indicates a decrease in the substrate binding affinity in comparison to both the wild type and E32A. Considering the competitive product inhibition, the product binding site is likely to be shared with the substrate in CPL, and the mutations were shown to increase the dissociation constant of the product. On the basis of these, the mutations seem to also affect the substrate binding site, resulting in a decrease in the substrate binding affinity. However, it is noteworthy that the catalytic efficiencies (\( k_{\text{cat}}/K_m \)) of the L31A and D33A mutants remained comparable to that of the wild type, which was achieved through compensation by the increased \( k_{\text{cat}} \). The double mutant (L31A/D33A) exhibited a significant decrease in catalytic efficiency in comparison to the wild type and E32A, probably owing to a low structural stability. The melting temperature of this double mutant was estimated to be 41.8 °C, which is lower than that of the wild type (Table S3 in the Supporting Information), supporting our hypothesis.

**Conformational Dynamics of the Flaps in the Variants.** To obtain some insight into how a single mutation significantly relieves the product inhibition, we analyzed in more detail the structure of the product-binding site resulting from the MD simulations for the L31A and D33A mutants (Figure 3). The product-binding site of the wild type was shown to remain almost closed during the initial state of the MD simulation due to the overlying flaps (Figure 3A). Both the L31A and D33A mutants were also found to have a conformation similar to that of the wild type during the initial state of the MD simulations (transparent cartoons and sticks in pink, Figure 3B,C). However, as the conformational dynamics of the flap 1 increase in the course of the MD simulations, the product-binding sites of both mutants are shown to be opened up (transparent surfaces in gray, Figure 3B,C). This change seems to result from the weakened structural constraint of the flap 1 by the mutations. On the basis of the structural analysis, L31 forms a hydrophobic patch together with the residues (W28, M35, R38, and F39) on flap 1, and D33 makes a hydrogen bond with the main-chain amide of R117 on flap 2 (Figure 3A). The mutation of L31 and D33 to alanine would thus weaken the structural constraint of flap 1, leading to an increase in the conformational dynamics of the flap 1 and consequently the opening of the product-binding site. We also investigated the mutational effects on the hydrogen bond distance between amino acid residues and 4HB and product-binding energy for the L31A and D33A mutants. Interestingly, the distance of the hydrogen bond between the main-chain amide of M35 and the hydroxyl group of 4HB was shown to significantly increase in both mutants in comparison to that in wild type (Figure 3D, upper) and in other 4HB-binding residues (Figure S5 in the Supporting Information), exhibiting a close correlation with the change in the product-binding energy (Figure 3D, lower). This result could be explained by the mutational effects on the conformational dynamics of flap 1 and consequently the product-binding energy. An increase in the conformational dynamics of flap 1 through the mutations leads to the change in the distance between the main-chain amide of M35 and the hydroxyl group of 4HB from 3.5 Å to 6.7 Å, resulting in the hydrogen bond breaking and accordingly a decrease in the product-binding energy (Figure 3E). The hydroxyl group of 4HB has been known to make a significant contribution to a strong binding of 4HB to the enzyme,\(^{25} \) and the hydrogen-bond breaking seems to cause a decrease in the product-binding energy by about 50 kJ/mol on the basis of the MD simulation results (Figure 3D, lower). This value is comparable to the energy level that would be necessary to release 4HB from the enzyme through the flap opening (10 kcal/mol = 41.8 kJ/mol).\(^{29} \) It is therefore likely that the L31A and D33A mutations increased the conformational dynamics of flap 1, thereby enhancing the product release through an opening of the product-binding site and reduced product-binding energy, eventually relieving the product inhibition.

To confirm the above explanations, we constructed other mutants, L31V and D33S, which were expected to restore the effects of their corresponding alanine mutation by generating a hydrophobic effect and a hydrogen bond to a certain extent.

### Table 1. Product Inhibition Constants of the Wild Type and Its Variants

<table>
<thead>
<tr>
<th>mutant</th>
<th>( K_m (\mu M)^a )</th>
<th>fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>3.6 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>L31A</td>
<td>27.2 ± 0.8</td>
<td>7.6</td>
</tr>
<tr>
<td>L31V</td>
<td>4.5 ± 0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>D33A</td>
<td>29.4 ± 2.1</td>
<td>8.2</td>
</tr>
<tr>
<td>E32A</td>
<td>4.6 ± 1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>L31A/D33A</td>
<td>4.1 ± 0.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Values represent the mean and standard deviations during the duplicate experiments. \( K_m \) values of the wild type and its variants were calculated according to the equation

\[ V_0 = \frac{V_{\text{max}} [S]}{K_m (1 + [P]/K_p) + [S]} \]

### Table 2. Kinetic Parameters of the Wild Type and Its Variants

<table>
<thead>
<tr>
<th>mutant</th>
<th>( k_{\text{cat}} ) (s(^{-1}))(^a)</th>
<th>( K_m (\mu M)^a )</th>
<th>( k_{\text{cat}}/K_m ) (M(^{-1}) s(^{-1}))(^b)</th>
<th>fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.7 ± 0.02</td>
<td>31.4 ± 2.8</td>
<td>2.0 × 10(^6)</td>
<td>1.0</td>
</tr>
<tr>
<td>L31A</td>
<td>2.3 ± 0.01</td>
<td>73.3 ± 4.7</td>
<td>3.1 × 10(^6)</td>
<td>1.5</td>
</tr>
<tr>
<td>L31V</td>
<td>0.7 ± 0.1</td>
<td>33.6 ± 4.4</td>
<td>2.2 × 10(^4)</td>
<td>1.1</td>
</tr>
<tr>
<td>D33A</td>
<td>2.3 ± 0.1</td>
<td>83.7 ± 7.3</td>
<td>2.0 × 10(^4)</td>
<td>1.0</td>
</tr>
<tr>
<td>D33S</td>
<td>ND(^b)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L31A/D33A</td>
<td>1.1 ± 0.2</td>
<td>163.3 ± 18.6</td>
<td>7.0 × 10(^7)</td>
<td>0.3</td>
</tr>
<tr>
<td>M35G</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E32A</td>
<td>0.7 ± 0.1</td>
<td>35.1 ± 5.4</td>
<td>1.9 × 10(^4)</td>
<td>1.0</td>
</tr>
</tbody>
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\(^a\)Kinetic values represent the mean and standard deviations in the duplicate experiments. \(^b\)ND: not determined because of an insoluble expression of the enzyme.

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respectively. Interestingly, all of the kinetic constants and $K_p$ values of L31V were comparable to those of both the wild type and E32A (Tables 1 and 2). This result supports our hypothesis that an L31A mutation perturbs the hydrophobic patch on flap 1, increasing the dynamics of the flaps, and consequently facilitates the product release and decreases the product inhibition. We attempted to compare the D33A mutant with D33S but failed owing to the insoluble expression of D33S. However, a comparison with E32A (Tables 1 and 2) implies that a decrease in the product inhibition of the D33A mutant can be mainly attributed to the increased conformational dynamics of the flaps through a perturbation of the hydrogen bond, rather than the shortened side chain. To analyze the role of M35 in the product binding, an M35G mutant was also constructed. Both the M35G and D33S mutants were expressed in an insoluble form, however, which may be due to a destruction of the hydrophobic interface (M35G) and an unexpected structural change (D33S).

Figure 3. Modulation in conformational dynamics around the product-binding site through mutations. (A) Product-binding site of the wild type during the initial state of the MD simulation. The amino acid residues involved in the product binding and the interaction with L31 and D33 are shown. The product-binding site is presented with a transparent surface in gray. Hydrophobic contacts of L31 are shown by a transparent surface in violet. Conformational dynamics in the product-binding sites of (B) L31A and (C) D33A at their maximum open states through MD simulations. The mutation sites and the interacting residues are presented. The positions of each flap and the mutation sites in the initial states of the MD simulations are presented with transparent cartoons and sticks. (D) Changes in the distance of the hydrogen bond between M35 and 4HB (upper) and the product-binding energy (lower) in the wild type and its variants during the MD simulations. (E) Detailed changes in the distance of the hydrogen bond between the hydroxyl group of 4HB and the main-chain amide of M35 of the D33A mutant. The distance of the hydrogen bond during the initial state (blue dashed line) and the maximum open state (red dashed line) are presented along with the lengths.

Figure 4. Production of 4HB from chorismate by wild type and its mutants: (A) production profile; (B) yield of 4HB from chorismate by wild type and its variants. The reaction was carried out by incubating wild type or its mutants (0.5 μg/mL) in 50 mM Tris-HCl buffer (pH 7.5) containing 150 μM chorismic acid with 1 mM DTT for 30 min at room temperature. The reaction was stopped by adding 80 μL of 1 N HCl, and the concentrations of 4HB and chorismic acid were determined by HPLC. Details are provided in the experimental procedures of the Supporting Information.
Production Rate and Yield of the Mutants. According to the rate equation presented in Table 1, an increase in the $K_p$ value results in an enhancement in the reaction rate. To examine the effect of the reduced product inhibition on the reaction rate, we determined the production rate and yield of 4HB from chorismate by both the wild type and the mutants (Figure 4A). The D33A and L31A mutants showed significantly increased production rates of 4HB in comparison to the wild type, while the production rate by the double mutant (L31A/D33A) was shown to be lower than the wild type. For quantitative comparison of the production rates, we determined the yields of 4HB for both the wild type and the mutants (Figure 4B). The comparison of the production rates, we determined the yields shown to be lower than the wild type. For quantitative comparison of the production rates, we determined the yields of 4HB for both the wild type and the mutants (Figure 4B). The L31A and D33A mutants exhibited maximum yields of 81% and 68% (mol/mol), respectively, which correspond to about 2.6- and 3-fold higher yields than the wild type. These results indicate that a relieved product inhibition by the mutations significantly enhanced the catalytic activity of the enzyme.

Taken together, we demonstrated that our strategy leads to a significant reduction in the product inhibition through the enhanced conformational dynamics of the enzyme without a decrease in the catalytic efficiency, consequently leading to a 3-fold increase in the overall reaction rate. Despite many advances in understanding the fundamental aspects of the protein dynamics, a practical implementation of such knowledge to the design of enzymes remains a challenge. Our results will provide some insights into applying the conformational dynamics of a protein to the strategy of enzyme design.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.catal.6b02793.

Experimental procedures including details of MD simulations, structural analysis of the product binding site of CPL, RMSFs of the candidates, distance changes of hydrogen bonds with 4HB during the MD simulations, multiple sequence alignments of the CPLs, kinetic parameters of the other variants, and melting temperatures of the single and double mutants of key residues (PDF)

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Notes

The authors declare no competing financial interest.

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