Enhanced Enzyme Kinetic Stability by Increasing Rigidity within the Active Site*§

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Background: Improving the kinetic stability of enzymes is a key issue for protein engineers.

Results: Mutagenesis of residues with a high B factor located within 10 Å of the catalytic Ser105 residue enhances kinetic stability dramatically.

Conclusion: Increasing the rigidity of the flexible segment within the active site improves enzymatic kinetic stability.

Significance: Optimization of the active site may an alternative, efficient approach for enhancing protein stabilization.

Enzyme stability is an important issue for protein engineers. Understanding how rigidity in the active site affects protein kinetic stability will provide new insight into enzyme stabilization. In this study, we demonstrated enhanced kinetic stability of Candida antarctica lipase B (CalB) by mutating the structurally flexible residues within the active site. Six residues within 10 Å of the catalytic Ser105 residue with a high B factor were selected for iterative saturation mutagenesis. After screening 2200 colonies, we obtained the D223G/L278M mutant, which exhibited a 13-fold increase in half-life at 48 °C and a 12 °C higher Tm15. The temperature at which enzyme activity is reduced to 50% after a 15-min heat treatment. Further characterization showed that global unfolding resistance against both thermal and chemical denaturation also improved. Analysis of the crystal structures of wild-type CalB and the D223G/L278M mutant revealed that the latter formed an extra main chain hydrogen bond network with seven structurally coupled residues within the flexible α10 helix that are primarily involved in forming the active site. Further investigation of the relative B factor profile and molecular dynamics simulation confirmed that the enhanced rigidity decreased fluctuation of the active site residues at high temperature. These results indicate that enhancing the rigidity of the flexible segment within the active site may provide an efficient method for improving enzyme kinetic stability.

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† This article contains supplemental Table S1.

‡ These abbreviations used are: MD, molecular dynamics; RMSF, root mean square fluctuation; CalB, Candida antarctica lipase B; Tm15, the temperature at which enzyme activity is reduced to 50% after a 15-min heat treatment; C50, urea concentration at which an enzyme loses 50% activity upon incubation in urea for 24 h; pNP, p-nitrophenol; RMDSD, root mean square deviation; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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~490-fold higher than that of the wild-type protein. MD simulations have suggested that stepwise formation of an extensive hydrogen bond/salt bridge network of structurally coupled residues on the enzyme surface increases protein thermostability (19). Successful enhancement of \textit{B. subtilis} lipase thermostability using this approach led to widespread focus on this strategy (20–22). However, the analysis of enzymes larger and more complex than \textit{B. subtilis} lipase yielded inconclusive results. For example, when Kim \textit{et al.} (21) mutated the seven residues with the highest B factor on the surface of \textit{Candida antarctica} lipase B (CalB; composed of 317 residues that constitute a lid domain) the highest B factor on the surface of the complex than (20–22). However, the analysis of enzymes larger and more complex than \textit{B. subtilis} lipase yielded inconclusive results. For example, when Kim \textit{et al.} (21) mutated the seven residues with the highest B factor on the surface of \textit{Candida antarctica} lipase B (CalB; composed of 317 residues that constitute a lid domain) the highest B factor on the surface of this strategy (20–22). However, the analysis of enzymes larger and more complex than \textit{B. subtilis} lipase yielded inconclusive results. For example, when Kim \textit{et al.} (21) mutated the seven residues with the highest B factor on the surface of \textit{Candida antarctica} lipase B (CalB; composed of 317 residues that constitute a lid domain) the highest B factor on the surface of

Experimental Procedures

**Media and Reagents**—The CalB gene was synthesized at GenScript Crop (Nanjing, China). Restriction enzyme and T4 ligase were purchased from New England Biolabs (Ipswich, MA). PrimeSTAR polymerase was purchased from TaKaRa (Dalian, China). The QIAquick™ PCR purification kit was purchased from Qiagen (Hilden, Germany). \textit{Escherichia coli} Rosetta (DE3) competent cells and pET-22b were purchased from Novagen (Madison, WI). \textit{E. coli} was routinely cultured overnight at 37 °C in 2× YT broth containing Bacto tryptone (1.6%, w/v), Bacto yeast extract (1%, w/v), and sodium chloride (0.5%, w/v) or on 2× YT agar plates with (in both cases) 100 µg/ml ampicillin. All substrates were purchased from Sigma.

**In Silico Design Procedure**—We used the crystal structure of \textit{C. antarctica} lipase B (Protein Data Bank code 1TCA) to design thermostable CalB variants. For analyzing the flexibility of the protein, the B factor profile of Protein Data Bank code 1TCA was used. The amino acids located within 10 Å of the catalytic Ser105 residue, which showed a high B factor value, were chosen for saturation mutagenesis. Saturation mutagenesis libraries were created at amino acid positions Phe71, Asp223, Leu277, Leu278, Ala281, and Ile285.

In addition, regardless of the locations, the residues with the highest B factor values in CalB were also chosen as points for iterative saturation mutagenesis. Generally, the residues located on the protein surface have a higher B factor than residues in the protein core because few interactions are formed among surface residues. After deleting N-terminal and C-terminal amino acids, all sites were chosen on the surface of the protein (Arg249, Arg309, Arg242, Glu269, Leu219, Lys15, and Pro218).

**Recombinant Protein Expression and Purification**—CalB gene (wild type) was amplified using primers containing the restriction sites for Ncol and Xhol (supplemental Table S1). Polymerase chain reaction (PCR) amplification was carried out with PrimeSTAR polymerase and a temperature program consisting of 98 °C for 2 min; 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 1 min at 72 °C; and a final 10-min extension at 72 °C. The PCR product was digested with Ncol and Xhol and subsequently cloned into the vector pET-22b (pET22b-WT). pET22b-WT was transformed into the Rosetta (DE3) by electroporation. The expression and purification of recombinant protein followed the described method (33).

**Library Creation and Thermostability Screening**—The mutants were prepared by whole-plasmid PCR with the primer containing NNK (sense strand)/MNN (antisense strand) degeneracy at those target sites (supplemental Table S1). Here N represents any of the following A, T, G, or C; K represents G or T; M represents A or C. PCR was performed with PrimeSTAR polymerase and a temperature program consisting of 98 °C for 2 min; 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 7 min at 72 °C; and a final 10-min extension at 72 °C. The PCR products were digested with DpnI to remove the parent plasmid and purified with a PCR purification kit. The created libraries were electroporated into Rosetta (DE3) cells and selected for ampicillin.

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resistance. Cells were plated on 2× YT agar plates containing 100 μg/ml ampicillin and 0.5% (v/v) tributyrin emulsified in 0.4% (w/v) gum arabic followed by incubation at 37 °C for 8 h and then incubation at 15 °C for 24 h. The appearance of clear haloes around the colonies upon incubation indicates enzyme activity. All the clones that showed clear haloes were picked in 96-well plates containing 200 μl of 2× YT medium with 100 μg/ml ampicillin. After growth at 37 °C for 8 h, the cultures were used to inoculate fresh medium in an identical plate and incubated further for 3 h at 37 °C. After 1 mm isopropyl 1-thio-β-D-galactopyranoside was added, the cells were incubated for 24 h at 15 °C. Each saturation mutagenesis library contained 200 colonies.

Cells were harvested by spinning the plates at 4000 rpm for 30 min at 4 °C and lysed by a triple freeze-thaw from −80 °C, and then 200 μl of 50 mM sodium phosphate buffer (PBS, pH 7.5) was added in each well. The supernatant was divided into two identical 96-well PCR plates. One plate was incubated at high temperature for 15 min and cooled at 4 °C for 20 min followed by equilibration at room temperature for 15 min. The other plate was incubated under identical conditions except for incubation at high temperature. Screening of the thermostability of the mutants used pNP caprylate as the substrate, and hydrolysis was monitored at 405 nm for 2 min. The ratio of activity of each clone incubated at higher temperature versus control without incubation was taken as residual activity, which was used to identify the positive clones. A limited number of iterative saturation mutagenesis (18) experiments were performed in the next round by choosing the genes of some of the “best hits” as templates for randomizing at another amino acid position.

Enzyme Activity Assays—pNP caprylate was used to compare the activity of CalB variants. The ability of the enzyme to hydrolyze pNP caprylate was determined by measuring the absorbance of p-nitrophenol liberated using a UV-2550 spectrophotometer with a thermal control unit (Shimadzu, Kyoto, Japan). The reaction mixture consisted of 0.02 ml of 10 mM pNP caprylate as a substrate in acetonitrile and 0.97 ml of 50 mM PBS, pH 7.5 containing an appropriate amount (10 μl) of the enzyme solution. The enzyme reaction was performed for 1 min at 37 °C. The activities were determined photometrically at 37 °C, and the buffer was adjusted at 37 °C unless otherwise stated. One lipase unit in this assay is defined as the amount of enzyme that liberates 1 μmol of p-nitrophenol/min under these conditions.

The effect of temperature on the activity of lipase (1 μg/ml) was examined across the range 30–70 °C. The 50 mM PBS was adjusted to pH 7.5 at the respective temperature.

For the kinetic studies, the concentration of pNP caprylate increased from 50 to 1000 μM. The enzymatic activity of CalB variants was determined at different temperatures (293, 298, 303, 308, 313, 318, 323, and 328 K). Kinetic parameters $V_{\text{max}}$ and $K_m$ were acquired by fitting enzymatic activities as a function of substrate concentrations to the Michaelis-Menten equation using non-linear regression of the software Origin 8.0. The parameter $k_{\text{cat}}$ was obtained by using the following equation: $k_{\text{cat}} = V_{\text{max}}/E$ where $E$ is the molar concentration of the enzymes. Values of the free energy ($\Delta G^\circ$), enthalpy ($\Delta H^\circ$), and entropy ($\Delta S^\circ$) of activation at 308 K were calculated using the following equation: $\Delta G^\circ = RT\ln k_{\text{eq}}/h - \ln k_{\text{cat}}$, $\Delta H^\circ = E_a - RT$, and $\Delta S^\circ = \Delta G^\circ - \Delta H^\circ$ where $k_{\text{eq}}$ is the Boltzmann constant, $h$ is the Planck constant, and $R$ is the universal gas constant.

Thermal Inactivation and Unfolding—The CalB variants (0.1 mg/ml) were incubated at different temperatures for different time intervals from 0 to 150 min and then cooled on ice for 10 min. Their residual enzyme activities were assayed at 37 °C as described above. The data were fitted to first-order plots and analyzed with the first-order rate constants ($k_d$) determined by linear regression of $\ln$(residual activity) versus the incubation time ($t$). The time required for the residual activity to be reduced to half ($t_{\text{1/2}}$) of the CalB variants was calculated using the following equation: $t_{\text{1/2}} = ln 2/k_d$. The changes in transition state free energy ($\Delta G^\circ$) for inactivation between mutants and wild type were calculated using the following equation: $\Delta G^\circ = -RT \ln(k_{d\text{mutant}}/k_{d\text{wild type}})$ (34) where $T$ and $R$ are temperature and the gas constant (1.987 cal K$^{-1}$ mol$^{-1}$), respectively. $\Delta G^\circ$ of wild type was used as a reference value. $k_{d\text{wild type}}$ and $k_{d\text{mutant}}$ were inactivation rate constants of wild-type and mutant CalB, respectively.

Heat treatment of purified protein was carried out by incubating the protein in 0.2-ml PCR tubes in a programmable thermal cycler for precise temperature control. Proteins (25 μl of 0.1 mg/ml in 50 mM PBS, pH 7.5) were heated at different temperatures for 15 min and cooled at 4 °C for 20 min followed by equilibration at room temperature for 15 min. Samples were centrifuged to remove any aggregated protein before assaying for enzymatic activity. The activity at 37 °C was considered to be 100%; the residual activities were quantitatively measured after heating at different temperatures for 15 min. The $T_{50}^{15}$ value is the temperature at which enzyme activity is reduced to 50% after a 15-min heat treatment. The precise value was obtained by determination of the inflection point of a fit of the residual activities at certain temperatures to a sigmoidal plot (sigmoidal Boltzmann fit using Origin 8.0) (35).

Differential Scanning Calorimetry—All differential scanning calorimetry measurements were performed on a VP-Capillary differential scanning calorimeter (MicroCal, LLC) from GE Healthcare. Samples were degassed by stirring gently under vacuum prior to measurements. Protein samples were diluted to 2.5 mg/ml by 12.5 mM PBS, pH 7.5, and the corresponding buffer was used as a reference. Protein unfolding events were recorded between 25 and 85 °C with a scan rate of 2 K/min with an initial 21 min of equilibration at 25 °C. The scans were analyzed after subtraction of an instrument baseline recorded with buffer in both cells using the software package Origin provided by the manufacturer.

Urea-induced Unfolding—The enzyme inactivation was induced by incubating ~0.03 mg/ml proteins with a set of concentrations of urea for 24 h at room temperature. The residual activities of the enzymes were measured at 37 °C using pNP caprylate as the substrate in 50 mM PBS, pH 7.5 containing the corresponding concentration of urea. The $C_{50}^{15}$ value, indicating the concentration of urea in which 50% of enzymatic activity was retained, was calculated as $T_{50}^{15}$.
For fluorescence measurements, samples treated similarly were measured on an F-7000 fluorescence spectrophotometer (Hitachi, Japan) at 20 °C. Samples were excited at 282 nm to monitor the tryptophan emission with a slit width of 5 nm for both excitation and emission. Spectra were recorded from 300 to 400 nm at a scanning speed of 1200 nm/min. The scanning experiments were performed three times, and the processed data were given as the means of three measurements. The shift in wavelength maximum was used to monitor the conformational change at various denaturant concentrations. All the sample spectra were corrected for background fluorescence. The data for wild type and mutants were analyzed by a two-state model using Origin 8.0 according to the equation: 

\[ S_{\text{obs}} = (S_N + S_d e^{-(\Delta G - m d)/RT})/(1 + e^{-(\Delta G - m d)/RT}) \]

where \( S_{\text{obs}} \) is the observed spectroscopic signal, \( S_N \) and \( S_d \) are the signals for the native and unfolded species, respectively, \( \Delta G \) is the free energy of unfolding in the absence of denaturant, \( m \) is the partial derivative of \( \Delta G \) with respect to denaturant, and \( d \) is the concentration of denaturant. \( S_{\text{obs}} \) and \( d \) are the dependent and independent variables; \( S_N \), \( S_d \), \( \Delta G \), and \( m \) are treated as fitting parameters (36). The \( C_{\text{opt}} \), the urea concentration at the midpoint of urea-induced unfolding curve, was obtained using the following equation: 

\[ C_{\text{opt}} = \Delta G/m. \]

**Crystallization of CalB Wild Type and Mutants—** Crystals of CalB and its mutants were grown at 14 °C by the hanging drop, vapor diffusion method. All crystals were grown in 25% PEG 3350, 0.2 M sodium acetate, 0.1 M Bis-Tris, pH 6.5 and cryoprotected by the addition of 20% ethylene glycol (v/v) to the crystallization conditions. 1.5–1.6 Å-resolution data sets were collected by the addition of 20% ethylene glycol (v/v) to the cryoprotectant. The data for wild type and mutants were analyzed by a two-state model with Origin 8.0 according to the equation: 

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**RESULTS**

**Construction of Iterative Saturation Mutagenesis Libraries—**

CalB is a globular \( \alpha/\beta \)-hydrolase that is \( \sim 30 \times 40 \times 50 \) Å in size with a catalytic triad composed of Ser\(^{105}, \) Asp\(^{187}, \) and His\(^{224}. \) Its substrate binding pocket is an elliptical, steep funnel 9.5 × 4.5 Å in size. The catalytic Ser\(^{105} \) residue is located within the tight turn between \( \beta 4 \) and helix \( \alpha 4 \) at the bottom of the binding pocket. When bound, the substrate is oriented parallel to the long axis of the active site (39). To identify residues that play a role in enhancing the local stability of the CalB active site, we mutated amino acids based on two criteria: 1) location within a 10 Å radius around the Ser\(^{105} \) residue and 2) a relatively high B factor. As shown in Fig. 1, six residues with an average B factor over 10 were selected using B-FITTER (40) based on the published x-ray crystallography structure of CalB (Protein Data Bank code 1TCA). These residues were Leu\(^{278}, \) Ile\(^{285}, \) Leu\(^{277}, \) Ala\(^{281} \) (all located within the \( \alpha 10 \) helix), Phe\(^{23} \) (located in the loop connecting the \( \beta 3 \) strand and \( \alpha 3 \) helix), and Asp\(^{223} \) (located in the loop connecting the \( \alpha 8 \) and \( \alpha 9 \) helices) (Fig. 1). Saturation mutagenesis libraries of these residues were generated and screened for enhanced thermostability. To ensure reasonable coverage of the relevant protein sequence, 200 transformants were screened for each library (41). We found that amino acids 278, 281, and 285 were crucial for enzymatic activity because mutation led to a dramatic alteration in activity (data not shown). Heat treatment (55 °C for 15 min) identified several positive mutants in the Leu\(^{278} \) and Asp\(^{223} \) libraries. In particular, sequencing revealed that the most thermostable mutants were L278M and D223G.

To further improve enzyme stability, the gene encoding L278M was used as a template for iterative saturation mutagenesis at the other five sites (Ile\(^{285}, \) Leu\(^{277}, \) Ala\(^{281}, \) Phe\(^{21}, \) and Asp\(^{223} \)). Mutagenesis of Phe\(^{21} \) and Asp\(^{223} \) did not significantly impact enzymatic activity; however, mutagenesis of Leu\(^{277}, \) Ala\(^{281}, \) and Ile\(^{285} \) resulted in large fluctuations in enzyme activity. Heating these five libraries at 59 °C for 15 min revealed that the best variant was L278M/D223G from the L278M/D223X library. Additional iterative saturation mutagenesis of the remaining four residues using L278M/D223G as a template did not further improve thermostability. More importantly, the enzyme lost most of its activity following multiple mutations within the active site.
For comparison, residues located on the surface of CalB were also investigated using a similar method. Seven residues with high B factors were chosen for saturation mutagenesis, namely Arg<sup>249</sup>, Arg<sup>309</sup>, Arg<sup>242</sup>, Glu<sup>269</sup>, Leu<sup>219</sup>, Lys<sup>13</sup>, and Pro<sup>218</sup> (Fig. 1). The average B factors of these residues were much higher than those of residues within the active site. With the exception of those from the Pro<sup>218</sup> library, most of the mutants retained their catalytic activity (data not shown). No obvious thermostable variants were found in all the libraries examined under the same screening conditions.

**Kinetic, Dynamic, and Chemical Stability of the CalB Variants**—Irreversible thermal inactivation is an important parameter for describing the kinetic stability of enzymes. To assess this property, each variant was incubated at 48 °C and then cooled on ice for 10 min before the residual activity was measured at 37 °C with respect to the time of incubation. All traces of thermal inactivation were found to follow first-order kinetics. Calculation of the <em>t</em><sub>1/2</sub> and transition state free energy (<em>ΔG<em><sub>TS</sub></em></em>) of the CalB variants was based on the inactivation constant <em>k<sub>i</sub></em>. As shown in Table 1, the <em>t</em><sub>1/2</sub> of D223G/L278M at 48 °C was 49 min, which was about 13-fold higher than that of wild type. Thermodynamic analysis showed that the <em>ΔG<em><sub>TS</sub></em></em> of D223G/L278M, L278M, and D223G increased by 6.3, 4.6, and 3.1 kJ mol<sup>-1</sup>, respectively, compared with wild type. The higher <em>ΔG<em><sub>TS</sub></em></em> exhibited by D223G/L278M suggests that it possesses higher energy barriers against thermal inactivation, thus making it more stable than other single variants.

The temperature at which 50% of enzyme activity remains after incubation for 15 min (<em>T<sub>50</sub></em>) is another parameter used to evaluate kinetic stability. The activity of the CalB variants was assessed by incubating each enzyme from 37 to 65 °C. No significant differences in residual activity were observed with treatment below 40 °C (Fig. 2A). However, incubation at temperatures above 45 °C affected this activity. For example, at 50 °C, the residual activity of wild-type CalB was only 14% after treatment; however, D223G and L278M retained ~45% activ-

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**TABLE 1**

| CalB variants | <em>k</em><sub>i</sub> | <em>t</em><sub>1/2</sub> | <em>ΔG<em><sub>TS</sub></em></em> | <em>T<sub>50</sub></em> | <em>C<sub>50</sub></em> |
|---------------|----------------|----------------|----------------|--------------|----------------|
| Wild type     | 0.1816 ± 0.01 | 3.8 ± 0.2      | —              | 46.4 ± 0.3   | 2.07 ± 0.07    |
| D223G         | 0.052 ± 0.002 | 13.4 ± 0.6     | 3.1 ± 0.1      | 48.8 ± 0.3   | 2.75 ± 0.10    |
| L278M         | 0.0287 ± 0.0003 | 24.2 ± 0.3 | 4.6 ± 0.1      | 50.2 ± 0.1   | 2.87 ± 0.06    |
| D223G/L278M   | 0.0141 ± 0.0007 | 49.2 ± 2.5 | 6.3 ± 0.1      | 58.5 ± 0.3   | 2.90 ± 0.10    |

<sup>a</sup> <em>k</em><sub>i</sub> denotes the first-order rate constants of inactivation at 48 °C.

<sup>b</sup> <em>t</em><sub>1/2</sub> represents the half-life at 48 °C and is equal to <em>ln 2 / k</em><sub>i</sub>.

<sup>c</sup> <em>ΔG<em><sub>TS</sub></em></em> = —<em>RT</em> ln(<em>k</em><sub>i</sub><em><em>mutant</em></em> / <em>k</em><sub>i</sub><em><em>wild type</em></em>).

<sup>d</sup> Temperature at which enzymes lose 50% activity after incubation for 15 min.

<sup>e</sup> Urea concentration at which enzymes lose 50% activity after treatment for 24 h.

<sup>f</sup> This mutant was deactivated too rapidly to measure at 48 °C. The half-life was less than 5 min at only 45 °C.
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FIGURE 2. Thermally and urea-induced inactivation/unfolding profiles of wild-type and mutant CalB. A, thermal inactivation profiles of CalB mutants. Enzymes in 50 mM sodium phosphate buffer, pH 7.5 were incubated at various temperatures for 15 min and assayed for residual activity at 37 °C. The activity measured at 37 °C was considered to be 100%. Results from differential scanning calorimetric analysis of the CalB mutants. The scans were analyzed after subtraction of an instrument-derived baseline recorded with buffer in both cells as calculated using the Origin software package. C, the fluorescence spectra were measured at 37 °C. Activity measured in the absence of urea was considered to be 100%. ■, wild type; ●, D223G; ▲, L278M; ▼, D223G/L278M. The fitted curves for protein unfolding are shown as black lines. Error bars represent S.D.

TABLE 2
Thermodynamic properties of wild-type and mutant CalB

| Mutant          | $T_m$ (°C) | $\Delta H_u$ (kJ/mol) | $\Delta S_u$ (kJ/mol °K) | $C_m$ (M) |
|-----------------|------------|------------------------|--------------------------|----------|
| Wild type       | 56.0 ± 0.05| 114.5 ± 0.7            | 348.0 ± 2.3              | 2.9 ± 0.05|
| D223G           | 58.4 ± 0.1 | 104.4 ± 1.7            | 314.9 ± 5.2              | 3.1 ± 0.03|
| L278M           | 58.3 ± 0.2 | 87.7 ± 1.6             | 264.6 ± 4.7              | 3.3 ± 0.2 |
| D223G/L278M     | 59.6 ± 0.1 | 129.2 ± 1.6            | 388.4 ± 4.7              | 3.4 ± 0.1 |

$^a$ Midpoint of transition as determined by a red shift in fluorescence emission maxima.

ity, whereas D223G/L278M retained ~86% activity. The $T_m$ of D223G and L278M was higher by 2.4 and 3.8 °C, respectively, compared with wild type (Table 1). Unexpectedly, $T_m^{15}$ of the best variant, D223G/L278M, increased to 58.5 °C, which is 12 °C higher than that of wild type. This result demonstrates that a strong synergistic effect exists between D223G and L278M to enhance CalB kinetic stability, thereby leading D223G/L278M to greater kinetic stability than wild type.

To investigate the thermodynamic effect of the mutants, the melting temperature ($T_m$) of each CalB variant was measured by differential scanning calorimetry. Because a single peak appeared in the scans, we inferred that the wild type and three CalB variants underwent a single transition (Fig. 2B). As shown in Table 2, the $T_m$ values for D223G, L278M, and D223G/L278M were 2.4, 2.3, and 3.6 °C higher than that of wild type (56 °C), respectively. Unlike the change in kinetic stability ($T_m^{15}$), no synergistic effect between D223G and L278M was observed on the thermodynamic stability ($T_m$) of CalB.

To further examine changes in thermodynamic stability, the enthalpy ($\Delta H_u$) and entropy ($\Delta S_u$) of the unfolding process were calculated by non-linear fitting (Table 2). Although $\Delta H_u$ and $\Delta S_u$ of the single mutants decreased, both values increased in the double mutants. This result indicates that improving the stability of the double mutant was not due to a simple additive effect of the two single mutations.

In D223G/L278M, when the temperature was equal to $T_m$, $\Delta H_u$ and $T\Delta S_u$ canceled each other out. However, when the temperature was lower than $T_m$, $\Delta H_u$ became larger than $T\Delta S_u$, resulting in a more positive $\Delta G_m$ value that reflects enhanced stability. Therefore, mutants exhibiting a higher $\Delta H_u$ value may be more stable when $T < T_m$. This conclusion is consistent with the fact that a high $\Delta H_u$ value for D223G/L278M indicates that it has greater stability (e.g. longer $t_{1/2}$ and higher $T_m^{15}$) than other variants when $T < T_m$. Moreover, enhancement of $\Delta H_u$ contributed to increased thermodynamic stability of the double mutant.

To assess the chemical stability of the variants, urea-induced protein unfolding was performed. The maximum emission wavelength shift in intrinsic fluorescence was used as a measure of the global conformational change (Fig. 2C). The urea concentration at the unfolding curve midpoint ($C_m$) of wild-type CalB was 2.89 M. This value shifted 0.25, 0.43, and 0.55 M higher in concentration for D223G, L278M, and D223G/L278M.
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respectively (Table 2). The mutants also exhibited better chemical resistance than wild type in the urea-induced inactivation assay (Fig. 2D and Table 1). After treatment with 2.4 M urea for 24 h, wild-type CalB retained only 33% of its original activity, whereas all the mutants retained ~62% activity. The C50 value, which indicates the urea concentration that inhibits 50% of the enzymatic activity, increased 0.7, 0.8, and 0.8 M for D223G, L278M, and D223G/L278M, respectively. These results indicate that mutations within the active site could improve the thermal and chemical stability of CalB.

Catalytic Properties of CalB Variants—The specific activity of wild-type CalB and D223G, L278M, and D223G/L278M mutants was determined at temperatures ranging from 25 to 70 °C using pNP caprylate as the substrate. The optimal temperature for catalytic activity by the mutants increased to 57.5, 57.5, and 62.5 °C for D223G, L278M, and D223G/L278M, respectively, which is 2.5–7.5 °C higher than that of wild-type CalB (Fig. 3). Results from further analysis of the enzymatic activity of CalB variants at different temperatures are represented in the Arrhenius plot (ln \( k_{\text{cat}} \) versus 1/\( T \)) shown in Fig. 3 (inset). The activation energy (\( E_a \)) of the reactions was calculated from the slope of the Arrhenius plot (Table 3). D223G displayed an \( E_a \) similar to that of wild type, whereas the \( E_a \) value of L278M was greater. In contrast, the \( E_a \) of D223G/L278M (43.24 kJ mol\(^{-1}\)) was lower than that of wild type (45.23 kJ mol\(^{-1}\)), suggesting that the double mutation results in a weaker temperature dependence for enzymatic activity and a broader temperature range for catalytic activity (Fig. 3).

Using pNP caprylate as the substrate, we determined the kinetic parameters of wild-type CalB and its mutants. All mutants except D223G, which demonstrated a minor decrease in \( k_{\text{cat}} \), showed an increase in both \( k_{\text{cat}} \) and \( K_m \) compared with wild type (Table 3). L278M displayed the highest catalytic efficiency (\( k_{\text{cat}}/K_m \)) because its \( k_{\text{cat}} \) value was nearly 2.0-fold higher than that of wild type. The other two mutants, however, showed catalytic efficiency similar to that of wild type. Taken together, these results showed that the mutants possessed enhanced kinetic stability without sacrificing catalytic activity.

To provide additional insight into how the mutations affected CalB enzymatic activity, we calculated the free energy (\( \Delta G^\circ \)), enthalpy (\( \Delta H^\circ \)), and entropy (\( \Delta S^\circ \)) of activation (Table 3). \( \Delta G^\circ \) is the difference in free energy between the transition state intermediate and the reactant. The \( \Delta G^\circ \) value of each mutant was lower than that of wild type at 35 °C, suggesting that the rate of formation of mutant intermediates was faster than that of wild type.

Crystal Structure Analysis and MD Simulation—To gain insight into how the mutations affected CalB enzymatic activity, we performed x-ray crystallography on each mutant. The data for wild-type and mutant CalB (except D223G segment 141–146, which was missing) are summarized in Table 4. The resolution was 1.5, 1.6, 1.6, and 1.5 Å for wild-type CalB and D223G, L278M, and D223G/L278M mutants, respectively. Superimposing the mutant structure onto the wild-type structure revealed that the root mean square deviation (RMSD) of the Ca position of D223G, L278M, and D223G/L278M was similar (0.266, 0.063, and 0.121, respectively) (Fig. 4A). The D223G mutation was located in front of the catalytic His224 residue, whereas the L278M mutation resided in the middle of the long C-terminal a10 helix, which lines the channel leading into the active site (42). Compared with wild type, no obvious increase in interaction was observed in the mutant structures (Table 5). Even the most stable mutant, D223G/L278M, exhibited fewer hydrogen bonds and ionic interactions despite the additional aromatic-sulfur interaction. Therefore, our data indicate that enhanced stability in the mutants was not the result of a greater number of interactions. Instead, we found that the critical difference between the mutants and wild-type CalB involved the types of interactions introduced into the active site. The L278M mutation resulted in a slight shift of the main chain of surrounding residues that induced a new aromatic-sulfur interaction between Met778 and Trp104 while simultaneously eliminating the aromatic-sulfur interaction between Met298 and Tyr300 and introducing two new hydrogen bonds (Fig. 4, B and C, and Table 6). In D223G/L278M, the mutations caused tighter compaction of the a10 helix. Compared with wild-type CalB (Fig. 4B), the position of the main chain oxygen atom of Pro268, Lys271, Ala274, Ala275, and Ala279 moved ~0.2–0.4 Å, and the position of the main

![Temperature dependence of wild-type and mutant CalB](image)

**TABLE 3**

| Mutant         | \( K_m \) \( \mu M \) | \( k_{\text{cat}} \) min\(^{-1}\) | \( K_m \) \( \mu M \) | \( k_{\text{cat}}/K_m \) | \( E_a \) kJ mol\(^{-1}\) | \( \Delta G^\circ \) kJ mol\(^{-1}\) | \( \Delta H^\circ \) kJ mol\(^{-1}\) | \( \Delta S^\circ \) kJ mol\(^{-1}\) K\(^{-1}\) |
|----------------|-----------------------|--------------------------|------------------------|------------------------|--------------------------|------------------------------|------------------------------|-------------------------------|
| Wild type      | 9.5 ± 0.2             | 365 ± 10.5               | 38.4 ± 9               | 45.2 ± 0.3             | 70.9 ± 0.01              | 43.0 ± 0.3                   | -91.7 ± 0.09                 | -83.7 ± 0.09                  |
| D223G          | 10.7 ± 0.6            | 357 ± 39.5              | 33.4 ± 1.5             | 45.4 ± 0.02            | 68.6 ± 0.01              | 43.0 ± 0.02                  | -91.7 ± 0.09                 | -83.7 ± 0.09                  |
| L278M          | 11.1 ± 0.3            | 597 ± 85                | 54.0 ± 1.2             | 51.4 ± 0.2             | 67.4 ± 0.04              | 49.0 ± 0.2                  | -60.0 ± 0.90                 | -83.7 ± 0.09                  |
| D223G/L278M    | 14.6 ± 0.9            | 559 ± 7.2               | 38.4 ± 1.9             | 43.2 ± 0.2             | 67.5 ± 0.04              | 40.0 ± 0.2                  | -87.0 ± 0.80                 | -83.7 ± 0.09                  |

*The kinetic constants were determined at 35 °C using p-NP caprylate as the substrate.
chain nitrogen atom of Ala276, Leu277, and Ala279 shifted ~0.2–0.5 Å. Together with the change in the dihedral angle, these subtle structural adjustments induced a new hydrogen bond network involving six pairs of hydrogen bonds on segment 268–281 that strengthens its rigidity (Fig. 4C). The enhanced rigidity is reflected in the calculated RMSD values of the backbone atoms of the CalB variants (Table 4). The L278M mutant showed considerably greater structural rigidity than the WT and D223G/L278M enzymes. In wild-type CalB, fluctuation of this region was observed in L278M (data not shown). These data reflect a novel approach for enhancing the stability of CalB industrially. In this study, we conducted iterative saturation mutagenesis on the structurally flexible residues of the active site of CalB to demonstrate much greater fluctuation between 300 and 330 K than the mutants, which is consistent with the fact that the mutants possessed greater thermostability.

RMSF values often reflect the fluctuation of individual residues during the MD simulation process. As shown in Fig. 7, wild-type CalB possesses two major unstable regions, namely a segment of the α5 helix (142–148) and the initial portion of the α10 helix (267–286). The α5 helix resides on the lid domain, whose intrinsically high flexibility is beneficial for binding substrate and promoting catalysis. This helix exhibited high flexibility in wild-type CalB and the D223G/L278M mutant. Nevertheless, the behavior of the α10 helix was different between the two enzymes. In wild-type CalB, fluctuation of this region was higher at 330 K than at 300 K, suggesting that it is a highly flexible region during heat treatment. However, the D223G/L278M mutant showed considerably greater structural rigidity compared with wild type at both 300 and 330 K (Fig. 7), which is consistent with their relative B factor profiles (Fig. 5). These data reflect a novel approach for enhancing the stability of CalB that involves increasing the local rigidity of its active site.

**DISCUSSION**

Low stability often limits the usefulness of an enzyme industrially. In this study, we conducted iterative saturation mutagenesis on the structurally flexible residues of the active
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A

B

C

D

E

Wild type

D223G/L278M

Key
- Blue: Ligand bond
- Green: Non-ligand bond
- Red: Hydrogen bond and its length
- Orange: Corresponding atoms involved in hydrophobic contact(s)
- Pink: Non-ligand residues involved in hydrophobic contact(s)
site of *C. antarctica* lipase B to identify a very robust variant, D223G/L278M. Its kinetic stability was enhanced significantly as evidenced by a 13-fold increase in half-life at 48 °C and a 12 °C rise in $T_{50}^{15}$. Additionally, the dynamic and chemical stability of the mutant improved without sacrificing activity. To our knowledge, this is the first report demonstrating that increasing the rigidity of its active site can enhance the kinetic stability of an enzyme.

In our previous work, we found that local interaction at the N-terminal region or on the interdomain interface could significantly affect the stability of a hyperthermophilic acylpeptidase hydrolase from the archaeon *Aeropyrum pernix* K1 (4, 12). Koudelakova et al. (43) recently reported that the stability and resistance to organic cosolvent of haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064 could be adjusted by modifying the rigidity of the access tunnel. Because the kinetic stability of an enzyme is dependent on a subtle balance between the flexibility and rigidity of its active site, we reasoned that optimizing the rigidity of the active site may be an efficient method for enhancing kinetic stability of an enzyme. This modification may protect the enzyme against irreversible inactivation under harsh conditions. Moreover, it allows for

### TABLE 5

| Interactions potentially involved in the stability of wild-type and mutant CalB | Wild type | L278M | D223G/L278M |
|---|---|---|---|
| Hydrophobic interactions | 266 | 265 | 265 |
| Disulfide bonds | 375 | 371 | 371 |
| Main chain-main chain hydrogen bonds | 192 | 189 | 189 |
| Main side-chain side-chain hydrogen bonds | 121 | 120 | 112 |
| Imino interactions | 8 | 8 | 6 |
| Aromatic-aromatic interactions | 10 | 10 | 10 |
| Aromatic-sulfur interactions | 121 | 120 | 112 |
| Cation-π interactions | 4 | 3 | 3 |

### TABLE 6

| Extra main chain-main chain hydrogen bond interactions and shift of crucial atoms in CalB mutants compared with wild type | L278M | D223G/L278M |
|---|---|---|
| Extra MC-MC hydrogen bonds$^a$ | Distance change of correlated atoms$^b$ | Dihedrals angle change of correlated bonds$^c$ | 
| P268O$_{atom}$/271N$_{atom}$ (D-d = 3.44) | P268O$_{atom}$/271N$_{atom}$, 0.2 | P268O$_{atom}$/271N$_{atom}$, 0.2 |
| A276N$_{atom}$/A279N$_{atom}$ (D-d = 3.5) | A276N$_{atom}$/A279N$_{atom}$, 0.4 | A276N$_{atom}$/A279N$_{atom}$, 0.4 |
| K271N$_{atom}$/A274N$_{atom}$ (D-d = 3.3) | K271N$_{atom}$/A274N$_{atom}$, 0.1 | K271N$_{atom}$/A274N$_{atom}$, 0.1 |
| A279N$_{atom}$/A277O$_{atom}$ (D-d = 3.4) | A279N$_{atom}$/A277O$_{atom}$, 0.2 | A279N$_{atom}$/A277O$_{atom}$, 0.2 |
| A277O$_{atom}$/A279N$_{atom}$ (D-d = 3.4) | A277O$_{atom}$/A279N$_{atom}$, 0.5 | A277O$_{atom}$/A279N$_{atom}$, 0.5 |
| A275O$_{atom}$/L277N$_{atom}$ (D-d = 3.4) | A275O$_{atom}$/L277N$_{atom}$, 0.2 | A275O$_{atom}$/L277N$_{atom}$, 0.2 |
| A274O$_{atom}$/A276N$_{atom}$ (D-d = 3.3) | A274O$_{atom}$/A276N$_{atom}$, 0.2 | A274O$_{atom}$/A276N$_{atom}$, 0.2 |
| A276N$_{atom}$/A279N$_{atom}$ | A276N$_{atom}$/A279N$_{atom}$, 0.2 | A276N$_{atom}$/A279N$_{atom}$, 0.2 |
| K271N$_{atom}$/A274N$_{atom}$ | K271N$_{atom}$/A274N$_{atom}$, 0.3 | K271N$_{atom}$/A274N$_{atom}$, 0.3 |
| A279N$_{atom}$/A277O$_{atom}$ | A279N$_{atom}$/A277O$_{atom}$, 0.5 | A279N$_{atom}$/A277O$_{atom}$, 0.5 |
| L277O$_{atom}$/A279N$_{atom}$ | L277O$_{atom}$/A279N$_{atom}$, 0.1 | L277O$_{atom}$/A279N$_{atom}$, 0.1 |
| A275O$_{atom}$/L277N$_{atom}$ | A275O$_{atom}$/L277N$_{atom}$, 0.2 | A275O$_{atom}$/L277N$_{atom}$, 0.2 |

$^a$ Extra main chain-main chain hydrogen bond interactions induced by mutations. MC, main chain; N$_{atom}$, main chain nitrogen atom; O$_{atom}$, main chain oxygen atom; D-d, the distance between the proton donor and acceptor.

$^b$ Change in distance of the correlated atoms between wild-type and mutant CalB.

$^c$ Change in dihedral angle of correlated bonds between wild-type and mutant CalB.

### FIGURE 4

Three-dimensional structure superimposition and hydrogen bond analysis in the CalB mutants. A, structure alignment of wild-type (white) and mutant CalB. The D223G, L278M, and D223G/L278M mutants are depicted in red, green, and magenta, respectively. Crucial amino acids are shown as lines, whereas Ser$^{165}$ residue and the D223G and L278M mutants are shown in stick form. B, crucial amino acids in wild-type CalB. C, crucial amino acids in L278M. D, crucial amino acids in D223G/L278M. E, interactions between amino acid 278 and other residues in wild-type CalB and the D223G/L278M mutant. Crucial amino acids are shown in ball and line, whereas L278/M278 is shown in stick form. Yellow dashed lines indicate the extended network of hydrogen bonds.

### FIGURE 5

Relative B factor profiles for wild-type (black) and D223G/L278M (magenta) CalB. With the highest residue B factor set at 100%, the relative residue B factors for the entire protein were calculated. Structures of the 257–281 segment for wild-type CalB and D223G/L278M mutant are shown. The crucial amino acids are shown with lines, and the extra network of hydrogen bonds is shown as yellow dashed lines.
the generation of mutants that perform better at elevated temperatures.

Indeed, data from this study showed that CalB mutants with significantly enhanced stability could be created using this new strategy. The best mutant, D223G/L278M, showed a greater improvement in stability than most previously published work (21, 22). It should be noted that Zhang et al. (32) screened a random mutagenesis library to identify a CalB mutant (A281E) that exhibited as much as 20-fold greater thermostability at 70 °C. However, we did not find any stabilization effect at position Ala281 by site-directed mutagenesis or screening of the site saturation mutagenesis library (Table 1). Our results suggest that focusing the mutagenesis effort on the active site creates “smarter” libraries that are more likely to yield positive results. Moreover, this approach is more feasible than other methods that require an in-depth knowledge of the structural features of the enzyme.

Data from high resolution crystal structures of wild-type and mutant CalB also provided important insights into the source of stability in the mutants. No obvious changes in interaction were observed in the L278M and D223G single mutants. However, when both mutations were present, their synergistic actions introduced an extra hydrogen bond network on segment 269–281 that increased the rigidity of the segments (Fig. 4D and Table 6).

Long MD simulations of CalB in aqueous solvent confirmed high mobility in the regions lining the channel that lead into the active site, especially the α5 and α10 helices (42). We postulated that the segment between 268 and 287, which exhibited great flexibility, is susceptible to disruption at elevated denaturing conditions. This movement exposes the active site to the external environment, thereby inactivating the enzyme. Enhanced rigidity in this segment could help the enzyme maintain its correct conformation at high temperature, prevent exposure of the active site, and improve its kinetic stability.
This hypothesis was corroborated by relative B factor profiles and RMSF analysis (Figs. 5 and 7). The segment exhibited greater flexibility than other parts of the protein but displayed a notable decrease in flexibility (due to increased rigidity) in the presence of both mutations. This result demonstrates that even minimal structural modification can create sufficient conformational change to enhance thermostability. Our data also demonstrate the formation of an extra hydrogen bond network between atoms on the main chain of the protein rather than the side chains. For many years, enhanced enzymatic stability was achieved by introducing new side chain interactions because the main chain residues exhibited little change following mutagenesis (14, 19, 44). However, our results indicate that the accumulation of subtle hydrogen bond interactions between the main chain residues also contributes to enzyme stabilization. It should also be noted that the flexible secondary structure could be made even more rigid by further tightening the main chain interactions.

Enhancing stability while retaining enzymatic activity is a demanding task for protein engineering, particularly when improvements to stability are achieved through mutagenesis of residues within the active site. Because of high flexibility and the conservation of structure and sequences, any mutation within the active site can inactivate the enzyme. In this study, we successfully utilized a two-step screening method to increase the stability of CalB without sacrificing its activity. It is notable that the catalytic efficiency ($k_{cat}/K_m$) of all the mutants was not compromised in lieu of enhanced stability at all temperatures. Indeed, the relative B factor and RMSF profiles indicated that, with the exception of the flexible α10 helix (257–281), the backbone dynamic of the mutant did not change relative to the wild type (Figs. 5 and 7). This simultaneous improvement in stability and activity, which is reportedly uncommon, provides insight into how such mutations should be designed. While screening the iterative saturation mutagenesis libraries, we discovered that A281F and I285F exhibited even more specific activity than the wild type. The specific activity was 100.3 and 100 units/mg for A281F and I285F, respectively, compared with that of wild-type CalB, which was 20 units/mg. Both sites are located within the substrate-binding tunnel and are involved in enzyme-substrate binding. The introduction of more hydrophobic, larger side chains at these positions may facilitate substrate binding and/or catalysis, thereby leading to improved enzymatic activity. This result highlights the fact that mutagenesis of the structurally flexible residues within the active site can enhance kinetic stability and increase enzymatic activity.

CONCLUSIONS

In this study, we demonstrate the enhancement of C. antarctica lipase B kinetic stability by increasing the rigidity of its active site. Although some reports revealed that mutagenesis of high B factor sites/regions may enhance enzyme stability, our specific focus on the flexible residues within the active site allowed us to understand how local rigidity affects enzyme kinetic stability. The increased intermolecular interactions, particularly the main chain hydrogen bond network, contribute to higher kinetic stability and thus the maintenance of enzymatic activity at elevated temperatures. MD simulation in combination with analysis of the relative B factors suggests that a balance between flexibility (α5 helix) and rigidity (α10 helix) in the active site is essential for catalysis. Additional structural and biochemical assays will be performed on other enzymes to reveal the generality of this local stabilization strategy. Nevertheless, our results will help elucidate the properties that confer thermostability in enzymes and thus provide insight into how to design more efficient and thermostable biocatalysts.

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