Interdomain Hydrophobic Interactions Modulate the Thermostability of Microbial Esterases from the Hormone-Sensitive Lipase Family*

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Microbial hormone-sensitive lipases (HSLs) contain a CAP domain and a catalytic domain. However, it remains unclear how the CAP domain interacts with the catalytic domain to maintain the stability of microbial HSLs. Here, we isolated an HSL esterase, E40, from a marine sedimental metagenomic library. E40 exhibited the maximal activity at 45 °C and was quite thermostable, with a half-life of only 2 min at 40 °C, which may be an adaptation of E40 to the permanently cold sediment environment. The structure of E40 was solved to study its thermostability. Structural analysis showed that E40 lacks the interdomain hydrophobic interactions between loop 1 of the CAP domain and α7 of the catalytic domain compared with its thermostable homologs. Mutational analysis showed that the introduction of hydrophobic residues Trp202 and Phe203 in α7 significantly improved E40 stability and that a further introduction of hydrophobic residues in loop 1 made E40 more thermostable because of the formation of interdomain hydrophobic interactions. Altogether, the results indicate that the absence of interdomain hydrophobic interactions between loop 1 and α7 leads to the thermostability of E40. In addition, a comparative analysis of the structures of E40 and other thermostable and thermostable HSLs suggests that the interdomain hydrophobic interactions between loop 1 and α7 are a key element for the thermostability of microbial HSLs. Therefore, this study not only illustrates the structural element leading to the thermostability of E40 but also reveals a structural determinant for HSL thermostability.

Hormone-sensitive lipases (HSLs)² exist widely in microorganisms, plants, and animals. Microbial HSLs are classified into two subfamilies: the GTSAG motif subfamily and the GDSAG motif subfamily (1, 2). The first crystal structure of microbial HSLs, brefeldin A esterase from Bacillus subtilis, was solved in 1999 (3). Since then, the structures of 26 microbial HSLs have been solved (4). Although only one structure from the GTSAG motif subfamily has been reported, most microbial HSL structures are from the GDSAG motif subfamily. Microbial HSLs are composed of two distinct domains: the N-terminal CAP domain and the C-terminal catalytic domain (5, 6). The CAP domain mainly comprises of two α-helices and a loop between the two α-helices. The catalytic domain shows the canonical α/β hydrolase fold (7). HSLs utilize a catalytic mechanism known as the catalytic triad, a hydrogen bond network of Ser-His-Asp/Glu (8, 9). In the hydrolysis of esters, the catalytic Ser residue acts as a nucleophile to attack the carbonyl carbon of the susceptible ester, whereas the Asp/Glu residue interacts with the His residue to facilitate the proton transfer from the catalytic Ser to His, thereby enhancing the nucleophilicity of Ser (2, 10, 11). The conserved HGG motif of microbial HSLs, which is involved in the formation of the oxyanion hole, is in close proximity to the catalytic triad. The oxyanion hole participates directly in the catalytic process by stabilizing the tetrahedral intermediate of the reaction (12). Both the catalytic triad and the oxyanion hole are located in the catalytic domain.

Most HSL esterases tend to form oligomers in solution. Structural analyses of the HSL oligomers from the GDSAG motif subfamily reveal that multiple hydrogen bonds and hydrophobic interactions involving the β8 of the catalytic domain contribute to their oligomerization and that oligomerization is a key element for the thermostability of E40.

Background: The effect of interdomain interactions on the thermostability of microbial hormone-sensitive lipases (HSLs) remains unclear.

Results: The absence of interdomain hydrophobic interactions between loop 1 and α7 leads to the thermostability of E40, a thermolabile HSL esterase.

Conclusion: Interdomain hydrophobic interactions are a key element for the thermostability of microbial HSLs.

Significance: Our study is helpful for protein engineering of thermolabile HSLs.

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The atomic coordinates and structure factors (code 4XVC) have been deposited in the Protein Data Bank (http://wwpdb.org).

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2 The abbreviations used are as follows: HSL, hormone-sensitive lipase; pNP, p-nitrophenyl; PDB, Protein Data Bank.
Structural Insight into Microbial HSL Thermostability

EXPERIMENTAL PROCEDURES

Screening and Sequence Analysis of Lipolytic Enzymes from a Metagenomic Library—Seven fosmid clones showing lipolytic activity have been identified from a fosmid library constructed from South China Sea surface sediment (2, 22). The gene sequences encoding lipolytic enzymes were determined through the construction of subcloning libraries and subsequent sequencing as previously described (2). One of the identified genes encoding a lipolytic enzyme was named E40. Phylogenetic analysis of E40 and its homologous sequences was performed using the MEGA 6.0 (23). Multiple sequence alignment was carried out using MUSCLE (24). SignalP 4.0 (25) was used to identify the potential signal peptide sequence.

Gene Cloning and Mutagenesis—The E40 gene was amplified via PCR using the E40-containing fosmid DNA as a template. The amplified fragment was ligated with the vector pET28a to construct the recombinant plasmid pET28a-E40. Mutants S202W, I203F, S202W/I203F, and M3 were created by a modified QuickChange™ site-directed mutagenesis method (26) using plasmid pET28-E40 as the template. Mutant M3+S202W/I203F with multiple mutations in several regions was created by the same method with the plasmid of mutant M3 as the template. All the recombinant plasmids were verified by sequencing.

Protein Expression and Purification—The WT E40 protein and all mutants were expressed in Escherichia coli BL21 (DE3) cells and induced by the addition of 1 mm isopropyl-β-D-thiogalactopyranoside at 20 °C for 20 h. Cells were collected and disrupted by sonication in 50 mm Tris-HCl buffer (pH 8.0). The resulting extract was first purified by nickel-nitrilotriacetic acid resin (Qiagen) and then by ion exchange chromatography on a Source 15Q column (GE Healthcare) with a linear gradient of 0–0.5 M NaCl. The eluted enzyme fractions were further purified by gel filtration chromatography on a Superdex 200 column (GE Healthcare) at a flow rate of 0.5 ml/min using 10 mm Tris-HCl buffer (pH 8.0) containing 100 mm NaCl as the running buffer. The column was calibrated in the same buffer with the following protein size standards from GE Healthcare: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa). The void volume of Superdex 200 column was determined with blue dextran 2000 (2,000 kDa) and thyroglobulin (669 kDa).

Biochemical Characterization—p-nitrophenyl butyrate (pNPC4) was used as the substrate for enzymatic activity assays. E40 activity was assayed by the method previously described for esterase E25 (2), except that the reaction temperature for E40 was 45 °C. Substrate specificity assays were performed with several pNP derivatives different in acyl chain length (C2–C16) (Sigma). The optimum temperature (T_{opt}) was determined in a range of 0 to 70 °C at pH 8.0. For the thermal stability assay, the enzyme was preincubated at temperatures ranging from 0 to 50 °C for 1 h, and the residual activity was measured at 45 °C. The half-life (t_{1/2}) of thermal inactivation of WT E40 and its mutants was determined by plotting ln(A_{405}) versus incubation time and deduced by linear regression. The optimum pH of E40 was determined at 45 °C in the Britton-Robin-
Enzyme kinetics assays were carried out in 50 mM Tris-HCl buffer (pH 7.5) using \[ p \text{NPC4} \] at concentrations from 0.05 to 3.0 mM. Kinetic parameters were calculated by nonlinear regression fit directly to the Michaelis-Menten equation using the Origin8 software. The overall secondary structures of WT E40 and its mutants were investigated at 25 °C using a J-810 CD spectropolarimeter (JASCO). CD spectra were collected from 200 to 250 nm at a scanning rate of 200 nm/min with a path length of 0.1 cm. Proteins for CD spectroscopy assays were at a concentration of 0.3 mg/ml in 50 mM Tris-HCl buffer (pH 8.0).

Crystallization, Data Collection, and Structure Determination—WT E40 for crystallization was diluted to 2.0 mg/ml in 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. Crystals suitable for x-ray diffraction were obtained using the hanging drop, vapor diffusion method. Before crystallization, E40 was incubated with 0.5 mM PMSF for 1 h at 4 °C to yield the complex E40-benzylsulfonyl (E40-PMS). E40-PMS crystals grew at 4 °C in the buffer containing 0.15M (NH4)2SO4, 0.1M MES (pH 5.8), and 12% (w/v) PEG 4000. All the x-ray diffraction data were collected on the BL17U1 Beamline at Shanghai Synchrotron Radiation Facility using Area Detector Systems Corporation Quantum 315r. The initial diffraction data sets were processed by the HKL2000 program (27). The crystal structure of E40-PMS complex was solved by molecular replacement using the EstE5-PMS structure (PDB code 3H18) as the starting model. Subsequent refinement was performed using Coot (28) and Phenix (29). All structure figures were generated using PyMOL software. The PISA (protein interactions, surfaces, and assemblies) server (30) was used to deduce the dimerization interface of E40. The protein interactions calculator (31) was used to analyze intraprotein interactions of E40 and other HSL esterases.

Accession Code—The nucleotide sequence encoding E40 has been deposited in the GenBank database with the accession number KP696774. The structure of E40-PMS has been deposited in the Protein Data Bank (PDB) with the accession number 4XVC.

FIGURE 1. Phylogenetic tree of representative lipolytic sequences from the microbial HSL family. The tree was built by the neighbor joining method with a Jones–Taylor–Thornton matrix-based model using 217 amino acid positions. Bootstrap analysis of 500 replicates was conducted, and values above 50% are shown. Homologs from family VII were used as outgroups. Sequences having crystal structures are indicated by solid circles.
RESULTS

E40 Is a Thermolabile Esterase Belonging to the Bacterial HSL Family—Five genes encoding lipolytic enzymes were previously identified from the metagenomic library constructed from a sediment sample from the South China Sea (2, 22). In this study, one of these genes, E40, was studied. E40 is 894 bp in length and encodes an HSL enzyme (E40) with 297 amino acid residues. E40 lacks an N-terminal signal peptide sequence according to the SignalP 4.0 prediction. Phylogenetic analysis suggested that E40 belongs to the GDSAG motif subfamily of the HSL family (Fig. 1). Sequence analysis showed that E40 has a catalytic triad formed by Ser145, Glu239, and His269 (Fig. 2). The catalytic Ser145 is located in the conserved GDSAG motif.

The recombinant E40 expressed in E. coli BL21 (DE3) showed an efficient hydrolysis toward short chain pNP esters (C4–C10) with the highest activity toward pNPC4 but had limited activity toward long chain pNP esters (C12-C16) (Fig. 3A), indicating that E40 is an esterase. The optimal temperature for E40 activity was 45 °C. E40 was quite thermolabile. It retained only 17% activity after 1 h of incubation at 30 °C and lost all activity after 6 min of incubation at 40 °C (Fig. 3B). The maximal activity of E40 toward pNPC4 was observed at pH 8.0, and 90% of activity was retained over a wide pH range of 5.0–9.0 (Fig. 3C). E40 activity could be stimulated by 0.5 M NaCl by 1.7-fold (Fig. 3D), consistent with its marine origin. E40 was severely inhibited by PMSF, indicating that E40 is a serine hydrolase, similar to other HSLs. Cu2+, Fe2+, and Zn2+ had strong inhibitory effect on E40 activity (Table 1).

Overall Structure of Esterase E40—To study the thermolabile mechanism of E40, the crystal structure of E40-PMS complex was solved at 2.0 Å resolution by molecular replacement using the EstE5-PMS structure (52% sequence identity with E40, PDB code 3H18) as the starting model. Some data collection and statistics are summarized in Table 2. E40 crystals belong to the P1 space group, with eight molecules per asymmetric unit. Eight molecules are loosely packed in the E40 crystals. However, gel
filtration analysis showed that E40 formed tetramers in solution (Fig. 4A). PISA analysis (30) suggested that the oligomerization interface of E40 cannot result in the formation of stable oligomers, which suggests that tetramers of E40 should be loosely aggregated in solution. The overall structure of E40 monomer is similar to those of resolved HSL esterases, showing a root mean square deviation of 0.83 Å (for 248 Cα/H9251 positions) to the closest structure 3H18. Like other HSLs, E40 monomer contains a CAP domain (Met1–Ile45) and a catalytic domain (Gln46–Gly297) (Fig. 4B). Residues Gly76 and Gly77 within the conserved HGG motif comprise the oxyanion hole that is involved in substrate binding for HSL esterases. The catalytic triad composed of residues Ser145, Glu239, and His269 is below the oxyanion hole. In the structure of E40, the Oγ of the nucleophilic Ser145 is covalently bound to the sulfate moiety of the PMS inhibitor.

**Comparative Structural Analysis of Esterase E40 with Other HSL Esterases**—Many thermostable HSLs can form stable oligomers both in crystal structure and in solution, and it has been demonstrated that oligomerization contributes to the thermal stability of these HSLs (9, 13, 16). In contrast, although E40 was tetramers in solution, the oligomeric E40 was quite thermostable (Fig. 3B), indicating that the formation of tetramers has little contribution to stabilizing the protein structure of E40, most likely due to the loose aggregation of the E40 tetramers. To reveal the structural basis for E40 thermolability at the monomer level, E40 structure was compared with those of two
monomeric thermostable HSL esterases: Est2 and AFEst (Fig. 5 and Table 3). Compared with Est2 (32, 33) and AFEst (19), the distance between the loop between α1-α2 (hereafter called loop 1) in the CAP domain and the catalytic domain of E40 is much larger than that in Est2/AFEst (Fig. 5), implying that there may be less interactions between the CAP domain and the catalytic domain in E40 than in Est2/AFEst. By protein interactions calculator analysis (31), we compared the interactions between the CAP domain and the catalytic domain in E40 and other thermolabile and thermostable esterases, including thermolabile mesophilic EstFa_R (21), thermostable mesophilic rPPE (34, 35), and thermophilic Est2 (33), AFEst (19), and PestE (9), all of which have the CAP and catalytic domains similar to E40 in length (Tables 3 and 4). Among these HSLs, mesophilic enzymes have less interdomain interactions than their thermophilic homologs. As shown in Table 4, the interdomain interactions of these enzymes are dominated by hydrophobic interactions, and mesophilic HSL enzymes, including E40, have less interdomain hydrophobic interactions than their thermophilic homologs, suggesting that the number of interdomain hydrophobic interactions seems to be related to the thermal stability of microbial HSLs.

To further reveal the role of interdomain hydrophobic interactions in HSL enzyme stability, a comparative analysis of the interdomain hydrophobic interactions in HSLs was carried out. The CAP domain of HSLs mainly contains loop 1, loop 2, and loop 1. Both thermolabile and thermostable HSLs have interdomain hydrophobic interactions between the α1 and α2 of the CAP domain and the catalytic domain (Table 4). However, the interdomain hydrophobic interactions between loop 1 of the CAP domain and the catalytic domain are quite different in thermolabile and thermostable HSLs (Fig. 6 and Table 4). For thermostable enzymes, including mesophilic rPPE and thermophilic Est2, PestE, and AFEst, loop 1 of the CAP domain forms interdomain hydrophobic interactions with α7 of the catalytic domain. Loop 1 of thermostable HSLs is rich in hydrophobic residues, and one or two of these hydrophobic residues, which are variable hydrophobic residues with large side chains, are involved in the interdomain interactions with α7. Residues in α7 involved in these interactions are two adjacent hydrophobic residues: Trp and Phe, both of which are aromatic bulky residues and conserved in thermostable HSLs (Figs. 2 and 6). More-
over, these two hydrophobic residues in α7 also form interdomain hydrophobic interactions with the α2 of the CAP domain. In contrast, thermolabile enzymes, including E40 and EstFa_R, lack interactions between loop 1 and α7. In α7 of EstFa_R, the corresponding residue Phe is retained at position 213, whereas Leu32 and Leu36 in the α2 of the CAP domain. The selected HSL esterases all have CAP and catalytic domains similar to E40 in length. For EstFa_R, residues 1–51 are the CAP domain, and residues 52–313 are the catalytic domain; for AFEst, residues 1–54 are the CAP domain, and residues 55–311 are the catalytic domain; and for PestE, residues 1–49 are the CAP domain, and residues 50–313 are the catalytic domain.

### TABLE 3
Comparison of biochemical characteristics between E40 and other HSL esterases

| Enzyme      | Source                  | pH optimum  | T<sub>opt</sub> | t<sub>1/2</sub> (40 °C) | Substrate optimum | Km<sup>sub</sup> | k<sub>cat</sub> | k<sub>cat</sub>/Km<sup>sub</sup> | Oligomer in solution | Sequence identities to E40 | PDB code | References |
|-------------|-------------------------|-------------|-----------------|--------------------------|-------------------|-----------------|---------------|-----------------------------|--------------------------|------------------------|----------|------------|
| E40         | Metagenomic             | 6.0         | 45 °C           | 2 min                    | Substrate optimum | 0.24            | 27            | 1.0 x 10<sup>5</sup>            | Tetramer                  | 100%       | 4XVC     | This paper |
| EstE1       | Metagenomic             | 7.0         | 95 °C           | 30 min                    | Substrate optimum | 0.7            | 1.6 x 10<sup>5</sup>            | 2.3 x 10<sup>5</sup>            | Dimer                    | 35%        | 2C7B     |            |
| PestE       | Pyrobaculum calidifonti | 7.0         | 90 °C           | 56 min                    | Substrate optimum | 0.044          | 2.6 x 10<sup>5</sup>            | 6.0 x 10<sup>4</sup>            | Dimer                    | 34%        | 3ZWQ     | Ref. 9    |
| Est2        | Alcaligenes faecalis    | 7.1         | 70 °C           | 30 min                    | Substrate optimum | 0.011          | 6.0 x 10<sup>5</sup>            | 3.0 x 10<sup>5</sup>            | Monomer                  | 31%        | 1EQV     | Refs. 32   |
| rPPE        | Pseudomonas putida      | 5.5–6.5     | 50 °C           | 51 min                    | Substrate optimum | 1.41           | a             | a                          | Monomer                  | 30%        | 4OB8     | Refs. 34, 35 |
| AFEst       | Archaeoglobus fulgidus  | 6.5–7.5     | 80 °C           | 26 min                    | Substrate optimum | 0.011          | 9.1 x 10<sup>5</sup>            | 3.0 x 10<sup>5</sup>            | Monomer                  | 29%        | 1JJ      | Refs. 19, 36 |
| Sto-Est     | Sulfolobus tokodaii     | 7.5–8.0     | 75 °C           | 40 min                    | Substrate optimum | 0.53           | 240            | 4.0 x 10<sup>5</sup>            | Dimer                    | 28%        | 3AIK     | Refs. 15, 37 |
| EstFa_R     | Ferrophilum acidophilum | 5.0         | 50 °C           | <1 min                    | Substrate optimum | 0.32           | 175            | a                          | Monomer                  | 27%        | 3WJ2     | Ref. 21   |

### TABLE 4
Comparison of the interactions between the CAP and catalytic domains of E40 and other HSL esterases

All interactions were calculated based on protein interactions calculator analysis. For comparison, the loop connecting the CAP domain and the catalytic domain is also assigned to the CAP domain. The selected HSL esterases all have CAP and catalytic domains similar to E40 in length. For EstFa_R, residues 1–51 are the CAP domain, and residues 52–313 are the catalytic domain; for Est2, residues 1–49 are the CAP domain, and residues 47–310 are the catalytic domain; and for PestE, residues 1–49 are the CAP domain, and residues 50–313 are the catalytic domain.

### Interdomain interactions

|                      | E40 | EstFa_R | rPPE | E40 | EstFa_R | rPPE | E40 | EstFa_R | rPPE | E40 | EstFa_R | rPPE |
|----------------------|-----|---------|------|-----|---------|------|-----|---------|------|-----|---------|------|
| Total hydrophobic interactions within 5 Å | 26  | 22  | 25  | 35  | 39  | 37  | 26  | 22  | 25  | 35  | 39  | 37  |
| Hydrophobic interactions between α1 and the catalytic domain | 14  | 8  | 8  | 11  | 15  | 13  | 14  | 8  | 8  | 11  | 15  | 13  |
| Hydrophobic interactions between loop 1 and the catalytic domain | 3  | 7  | 9  | 7  | 12  | 14  | 3  | 7  | 9  | 7  | 12  | 14  |
| Main chain-main chain hydrogen bonds | 1  | 3  | 1  | 3  | 2  | 1  | 1  | 3  | 1  | 3  | 2  | 1  |
| Main chain-side chain hydrogen bonds | 3  | 12  | 11  | 9  | 14  | 11  | 3  | 12  | 11  | 9  | 14  | 11  |
| Side chain-side chain hydrogen bonds | 7  | 7  | 5  | 10  | 8  | 9  | 7  | 7  | 5  | 10  | 8  | 9  |
| Ionic interactions within 6 Å | 5  | 5  | 2  | 4  | 3  | 2  | 5  | 5  | 2  | 4  | 3  | 2  |
| Aromatic-aromatic interactions within 4.5–7 Å | 1  | 1  | 1  | 4  | 1  | 1  | 1  | 1  | 1  | 4  | 1  | 1  |
| Cation-pi interactions within 6 Å | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |

*a* No interaction detected.

**Improving E40 Stability by Introducing Hydrophobic Residues in Loop 1 and α7**—To support our hypothesis that the low thermal stability of E40 might be attributed to the lack of interdomain hydrophobic interactions between loop 1 and α7, several mutants with introduced hydrophobic residues in loop 1 and/or α7 were constructed to introduce interdomain hydrophobic interactions between loop 1 and α7 in E40 (Table 5). In the thermophilic Est2, there are hydrophobic interactions between residues Tyr<sup>22</sup> and Leu<sup>26</sup> in loop 1 and residues Trp<sup>213</sup> and Phe<sup>214</sup> in α7 (Fig. 6). Based on comparative analysis of the structures and sequences of E40 and Est2 (Figs. 2 and 6), five mutants of E40 were constructed, which were classified into three types: (a) mutant M3 with mutation in loop 1 (residues Arg<sup>22</sup>–Thr<sup>24</sup> of E40 replaced by Tyr<sup>22</sup>–Leu<sup>26</sup> of Est2 to introduce hydrophobic residues Tyr and Leu in loop 1); (b) mutants with mutation in α7 to replace Ser<sup>202</sup> with Trp and/or Ile<sup>203</sup> with Phe, including S202W, I203F, and S202W/I203F; and (c) mutant M3 + S202W/I203F with mutations in both loop 1 and α7 (Table 5). Both S202W/I203F and M3 + S202W/I203F formed tetramers in solution (Fig. 4A), just as with WT E40, which suggests that these mutations had no impact on E40 oligomerization.

The thermostability of the mutants was compared with that of WT E40 by measuring the half-life (<i>t</i>_<i>1/2</i>) at 40 °C and/or 50 °C (Table 6). WT E40 had a <i>t</i>_<i>1/2</i> of ~2 min at 40 °C. Mutant M3 with two introduced hydrophobic residues (Tyr<sup>22</sup> and Leu<sup>26</sup>) in loop 1 had a similar <i>t</i>_<i>1/2</i> to WT E40, and a lower <i>T</i>_<i>opt</i> (35 °C) than WT E40 (45 °C), suggesting that an introduction of hydrophobic residues Tyr<sup>22</sup> and Leu<sup>26</sup> in loop 1 may result in the low thermostability of E40.
residues only in loop 1 has little improving effect on E40 thermostability.

In contrast, several mutations with introduced hydrophobic residues in α7, including S202W, I203F, and S202W/I203F, caused significant increases in $t_{1/2}$ (15-1000 min). Moreover, except that S202W mutation had no impact on $T_{\text{opt}}$, the other two mutations in α7 all led to an increase of $T_{\text{opt}}$ to 60 °C. I203F ($t_{1/2}$ of 241 min at 40 °C) was more stable than S202W ($t_{1/2}$ of 15 min at 40 °C), suggesting that Phe$^{203}$ is more important than Trp$^{202}$ in stabilizing the protein structure. In the structure of E40, Ile$^{203}$ protrudes into the substrate-binding pocket and has no direct interaction with the CAP domain (Fig. 7A). However, there are hydrophobic interactions between Ile$^{203}$, Val$^{200}$, and Ile$^{81}$ within the catalytic domain, and Ile$^{81}$ has hydrophobic interactions with Tyr$^{32}$ in the α2 from the CAP domain. Therefore, Ile$^{203}$ interacts indirectly with Tyr$^{32}$ through residues Val$^{200}$ and Ile$^{81}$. Compared with Ile, Phe has a larger side chain. When Ile$^{203}$ is replaced by Phe, the large side chain of Phe$^{203}$ shortens its distances to surrounding hydrophobic residues, which can strengthen the hydrophobic interactions of Phe$^{203}$.
with Val^{80} and Ile^{81} and may lead to the formation of interdomain hydrophobic interactions between Phe^{203} and Tyr^{12}. The strengthened intraprotein hydrophobic interactions in the I203F mutant would make its protein structure more rigid than WT E40. Different from Ile^{203}, the side chain of Ser^{202} in the structure of E40 protrudes out into the solution (Fig. 7A). When the hydrophilic Ser^{202} is replaced by the hydrophobic Trp, the side chain of Trp^{202} might also protrude out into the solution in a similar orientation to Ser^{202}, thereby producing limited interactions with surrounding residues. Thus, Trp^{202} is less important than Phe^{203} for the stability of E40. Double mutant S202W/I203F was much more thermostable than the single-point mutants S202W and I203F (Table 6), indicating that the hydrophobic interactions formed between Trp^{202} and Phe^{203} in mutant S202W/I203F can strengthen the hydrophobic interactions of Phe^{203} with the residues from the CAP and the catalytic domains, which we take as evidence that mutant S202W/I203F has a more rigid structure than S202W or I203F. These mutational results indicated that Trp^{202} and Phe^{203} in α7 are key residues for the thermostability of these E40 mutants.

By further introducing hydrophobic residues Tyr^{22} and Leu^{25} in loop 1 of mutant S202W/I203F, mutant M3 + S202W/I203F was created. M3 + S202W/I203F was more thermostable than S202W/I203F (Table 6), suggesting that M3 + S202W/I203F may form hydrophobic interactions between loop 1 and α7 because of the introduction of the hydrophobic residues Tyr^{22} and Leu^{25} and therefore has a more rigid structure than S202W/I203F.

Analysis of the Effect of the Mutations on E40 Catalytic Activity—In addition to the impact on protein thermostability, the introduced hydrophobic residues also influence the catalytic activity of E40 (Table 6). Mutation in loop 1 (M3) caused reductions in both $k_{cat}$ and substrate affinity and almost abolished the enzyme activity. CD spectroscopy analysis showed that the secondary structures of mutant M3 exhibited obvious deviation from that of WT E40 (Fig. 7B), suggesting that the introduction of hydrophobic residues in loop 1 resulted in structural changes in E40 and thus led to enzyme inactivation. Single-point mutations S202W and I203F in α7 affected the $k_{cat}$, but not the $K_m$. S202W reduced the activity by a half, whereas I203F increased the activity by 3.0-fold. Double mutant S202W/I203F showed increases in both $k_{cat}$ and substrate affinity, with activities increased by 2.6-fold. In the structure of E40, Ile^{203} forms hydrophobic interactions with residues Val^{80} and Ile^{81} (Fig. 7A). Because Val^{80} and Ile^{81} are in the same loop with residues Gly^{76} and Gly^{77} that comprise the oxyanion hole, different hydrophobic interactions exerted by Ile^{203} or Phe^{203} on this loop in mutants S202W, I203F, and S202W/I203F may result in different changes in both the $k_{cat}$ and the $K_m$. Mutant M3 + S202W/I203F with multiple mutations in both loop 1 and α7 had similar $k_{cat}$ and $K_m$ to mutant S202W/I203F but was more stable, suggesting that the interactions formed between loop 1 and α7 affect protein stability, but not enzyme activity. In contrast to mutant M3, the secondary structures of mutant M3 + S202W/I203F exhibited little deviation from that of WT E40 (Fig. 7B), indicating that the hydrophobic interactions formed between the hydrophobic residues introduced in loop 1 and α7 may stabilize the enzyme structure.

In summary, our structural, mutational, and biochemical analyses indicated that the absence of hydrophobic interactions between loop 1 of the CAP domain and α7 of the catalytic domain is a main element for the thermostability of E40. The introduction of hydrophobic residues in α7 significantly increased E40 stability by strengthening both intradomain and interdomain hydrophobic interactions, which also affected the catalytic efficiency and the substrate affinity possibly by interacting with the hydrophobic residues near the oxyanion hole. After the introduction of hydrophobic residues in α7, a further introduction of hydrophobic residues in loop 1 of the CAP domain could stabilize E40 further by the formation of extra interdomain hydrophobic interactions.

DISCUSSION

The High Thermolability of Esterase E40 Is an Adaptation of the Enzyme to the Permanently Cold Environment—The gene encoding the HSL esterase E40 was isolated from a surface sediment sample from the South China Sea at a water depth of 154 m, and the temperature of the sample was reported to be 17.3 °C (22). Therefore, wild E40 should be in a permanently cold marine environment. Our results showed that E40 activity could be stimulated by 0.5 M NaCl by 1.7-fold (Fig. 3D). E40 had a maximal activity at 45 °C and was quite unsta-
able at temperatures over 30 °C but stable at 20 °C or below (Fig. 3B). At 20 °C, E40 still retained 40% of the maximal activity (Fig. 3B). These results reflect the adaptation of the thermolabile E40 to the marine permanently cold environment it inhabits.

Interdomain Hydrophobic Interactions Are a Key Element for the Thermostability of Microbial HSLs at the Protein Monomer Level—It has been reported that oligomerization and ion pair networks and hydrophobic interactions on/within the catalytic domain contribute to the stability of thermostable HSLs (9, 13, 17, 20). However, thermostable HSLs have been seldom studied. In this study, we gained insight into the structural basis for the thermostability of E40, which belongs to the GDSAG motif subfamily of the HSL family. The results indicated that the absence of the interdomain hydrophobic interactions between α7 of the catalytic domain and loop 1 of the CAP domain is a main element leading to the thermostability of E40 (Fig. 6 and Table 6). Mesophilic EstFa_ R is also a thermolabile HSL esterase of the GDSAG motif subfamily (21), which also lacks hydrophobic interactions between α7 and loop 1 (Fig. 6). In contrast, all thermostable HSLs of the GDSAG motif subfamily, including mesophilic rPPE, and thermophilic Est2, PestE, AFEst, and EstE1, have interdomain hydrophobic interactions between α7 and loop 1 (Fig. 6). Moreover, structural analysis of the thermostable mesophilic esterase E25, the only structure of the GTSAG motif subfamily of the HSL family, revealed that hydrophobic interactions are also present between α7-like region (α10) from the catalytic domain and the loop between α2 and α3 from the CAP domain (2). Therefore, interdomain hydrophobic interactions between α7 or the α7-like region of the catalytic domain and the loop of the CAP domain seem to be a thermostability determinant for microbial HSLs at the protein monomer level. Thus, our study reveals a key structural element for the thermostability of microbial HSLs. This finding is helpful for the protein engineering targeting the thermostability improvement of thermostable HSLs with industrial potential.

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