Conversion of a \textit{Rhizopus chinensis} lipase into an esterase by lid swapping

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Abstract In an effort to explore the feasibility of converting a lipase into an esterase by modifying the lid region, we designed and characterized two novel \textit{Rhizopus chinensis} lipase variants by lid swapping. The substrate specificity of an \textit{R. chinensis} lipase was successfully modified toward water-soluble substrates, that is, turned into an esterase, by replacing the hydrophobic lid with a hydrophilic lid from ferulic acid esterase from \textit{Aspergillus niger}. Meanwhile, as a comparison, the lid of \textit{R. chinensis} lipase was replaced by a hydrophilic lid from \textit{Rhizomucor miehei} lipase, which did not alter its substrate specificity but led to a 5.4-fold higher catalytic efficiency ($k_{\text{cat}}/K_{m}$) toward \textit{p}-nitrophenyl laurate. Based on the analysis of structure-function relationships, it suggests that the amphipathic nature of the lid is very important for the substrate specificity. This study provides new insight into the structural basis of lipase specificities and a way to tune the substrate preference of lipases.—Yu, X-W., S-S. Zhu, R. Xiao, and Y. Xu. \textbf{Conversion of a \textit{Rhizopus chinensis} lipase into an esterase by lid swapping.} \textit{J. Lipid Res.} 2014. 55: 1044–1051.

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Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are two most attractive hydrolyses used in various industries, such as food manufacturing, new biological materials, biosensors, and biomedicine (1–3). Lipases are capable of hydrolyzing the ester bonds of water-insoluble substrates at the interface between substrate and water (4). Esterases hydrolyze solutions of water-soluble short acyl chain esters. All lipases as well as the majority of the esterases share the $\alpha/\beta$ hydrolase fold (5). Although lipases and esterases show close relations in structure, these enzymes exhibit different chain-length specificity, which is the only valid criterion for differentiation of these two closely related enzymes. Other criteria, such as interfacial activation, the existence of a lid, and hydrophobicity of the substrate binding region, were also used previously with several exceptions; although these features are not distinctive of lipases, they certainly play a crucial role in determining substrate specificity (6, 7).

Through comparison of structural features of sequence-related esterases and lipases, lipases display a statistically significant enhanced occurrence of hydrophobic amino acid residues in the vicinity of their active site (8, 9). Moreover, most lipases contain an $\alpha$-helical lid covering the catalytic center, while most esterase enzymes have either no lid or a small lid (10). At the lipid-water interface, the lipase’s active site is exposed by the movement of the helical lid, and lid opening appears to further strengthen the hydrophobic pattern (8). The interfacial activation of \textit{Burkholderia cepacia} lipase studied by molecular dynamic simulations revealed that the lid switches from an open to a closed conformation in aqueous media, while the reverse motion occurs in an organic environment (11). Several studies showed that the alternation of the chain-length specificity of lipases toward short-chain fatty acids can be achieved by rational design of the substrate binding site by introducing polar residues (12, 13). Lid swapping is another way to study the lid function in the research of \textit{Candida antarctica} lipase B, \textit{Candida rugosa} isolipases, lipoprotein lipase, and endothelial lipase (6, 7, 14). However, there are no examples of converting a lipase into an esterase by modifying the lid region. Herein, we describe the conversion of a lipase into an esterase by lid swapping, choosing a thermostable lipase from \textit{Rhizopus chinensis} CCTCC M201021 (S4-3) as the test enzyme.

\textit{Rhizopus} lipases have been widely used in food industries (15, 16). For example, in the dairy industry, lipase-mediated

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Abbreviations: AnFaeA, ferulic acid esterase from \textit{Aspergillus niger}; \textit{fNPL}, \textit{p}-nitrophenyl laurate; RML, \textit{Rhizomucor miehei} lipase; ROL, \textit{Rhizopus oryzae} lipase; S4-3, a thermostable lipase from \textit{Rhizopus chinensis} CCTCC M201021; S4-3M, an S4-3 lipase variant generated from the lid of S4-3 swapped with that from RML; S4-3N, an S4-3 lipase variant generated from the lid of S4-3 swapped with that from AnFaeA.

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hydrolysis of milk fat and other endogenous esters is believed to be the key reaction to produce special products with characteristic flavor profiles, which is based on the different substrate specificities of lipases (17–19). Rhizomucor miehei lipase (RML) exhibits the substrate selectivity toward ρ-nitrophenyl decanoate (C10) and myristate (C14) (20), and Rhizopus oryzae lipase (ROL) shows the highest specificity toward tricaprylin (C8) and triolein (C18) (13). The thermostable variant S4-3 (21) from R. chinensis CCTCC M201021 (22) shows the chain-length specificity toward tricaprylin (C8) and triolein (C12). However, the enzyme specificity with regard to the chain length in the substrate is not necessarily optimal for the manufacture of a particular product. Therefore, the molecular manipulation of lipases to alter chain-length specificity is obviously of industrial and theoretical interest.

In this research, in an effort to explore the feasibility of converting a lipase into an esterase by modifying the lid region, we designed and characterized two novel R. chinensis lipase variants by lid swapping. The R. chinensis lipase S4-3 was successfully converted into an esterase by replacing the hydrophobic lid with a hydrophilic lid from ferulic acid esterase from Aspergillus niger (AnFaeA). Meanwhile, as a comparison, the lid of S4-3 replaced by a hydrophobic lid from RML was also studied.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions

Escherichia coli DH5α was used as the cloning host. Pichia pastoris GS115 (His’, Novagen) was used as the heterologous expression host. Plasmid pPIC9K (Novagen) was used as gene expression vector. The recombinant plasmid pPIC9K-proRCLS4+3 contained the gene coding the thermostable lipase S4-3 (21).

The recombinant P. pastoris was enrichment-cultivated in buffered glycerol-complex medium [10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/l yeast nitrogen base (YNB), 4 × 10−3 g/l biotin, and 10 g/l glycerol] and induction-cultivated in buffered methanol-complex medium [10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/l YNB, 4 × 10−3 g/l biotin, and 5 g/l methanol]. E. coli was cultivated in Luria-Bertani medium [10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl]. P. pastoris GS115 was grown and maintained in yeast extract peptone dextrose medium (YPD) [10 g/l yeast extract, 20 g/l peptone, and 20 g/l dextrose]. The minimal dextrose medium plate (20 g/l dextrose, 100 mM potassium phosphate (pH 6.0), 13.4 g/l YNB, and 4 × 10−3 g/l biotin) was used for selection of His’ transformants. Transformation of P. pastoris GS115 was transformed with 1 µg of SaI-linearized expression vector by electroporation. The His’ transformants were obtained on minimal dextrose medium plate. The resistant genetic transformants were screened on YPD-G418 plate. The insertion and methanol metabolism were checked by PCR. The PCR amplifications were carried out according to Invitrogen’s recommendations with genomic DNA and primers complementary to the 5’ and 3’ regions of the AOX1 gene.

Expression and purification of lipases

P. pastoris His’ transformants were cultured in 25 ml of buffered glycerol-complex medium shaken at 30°C and 250 rpm in 250 ml glass flasks. When cultures reached an optical density at 600 nm (OD600) of ~6.0, the cells were centrifuged and resuspended in 100 ml buffered methanol-complex medium to an OD600 of 1.0, shaken at 28°C and 250 rpm in 500 ml glass flasks for 120 h. The cultures were supplemented with 5 g/l methanol every 12 h to induce the expression of lipase.

After induction, the lipases from the culture supernatant were harvested by centrifugation at 8,000 g at 4°C for 20 min and then concentrated and interchanged with 10 mM phosphate buffer (pH 8.0) by ultrafiltration through a 10 kDa membrane (Millipore). Then, the concentrated solutions were loaded onto a HiTrap SP FF column (Pharmacia, 5 × 5 ml) equilibrated with 20 mM acetic acid-sodium acetate buffer (pH 5.0), and eluted with 0–0.5 M NaCl in the same buffer. Fractions containing lipase activities were pooled, concentrated, and chromatographed on a HiTrap Phenyl HP (Pharmacia, 5 × 5 ml) equilibrated in 50 mM sodium phosphate buffer (pH 7.5) containing 1.6 M ammonium sulfate. Lipases were then eluted in an ammonium sulfate concentration gradient ranging from 1.6 to 0 M in 50 mM sodium phosphate buffer (pH 7.5). Protein fractions with lipase activities were collected and dialyzed against phosphate buffer (pH 8.0). The purified enzymes were stored at 4°C. Protein fractions collected from the previous steps were analyzed by SDS-PAGE. Protein concentrations were determined using the Bradford assay. Bovine serum albumin was used as a standard.

Influence of pH and temperature on activity and stability

The enzymatic activities for hydrolyses of ρ-nitrophenyl esters were measured according to Kordel et al. (24). Optimal pH was determined by examining the activity of the enzyme using ρ-nitrophenyl palmitate (pNPP) as substrate at 40°C in the following buffers: 50 mM citrate (pH 5.0–5.5), 50 mM phosphate (pH 6.0–8.0), 50 mM Tris–HCl (pH 8.5–9.0), and 50 mM carbonate (pH 9.5–10.0). The pH stability was determined by incubating 0.2 U lipase solution in buffers (50 mM, pH 5.0–10.0) for 1 h at 25°C and analyzing the residual activity. Optimal temperature was determined by measuring the enzyme activity using pNPP as substrate.
for S4-3N using various concentrations of \( \beta \)-nitrophenyl laurate (\( \beta \)NPL), \( \beta \)-nitrophenyl acetate, or \( \beta \)-nitrophenyl propionate as substrate according to the method described by Burdette et al. (26).

### 3D structure simulation

A 3D model of \( R. \ chinensis \) lipase in a closed conformation was built by SWISS-MODEL protein automated modeling program (27) on the basis of the crystal structure of ILGY (crystal structure of lipase II from \( R. \ niveus \) solved with a resolution of 2.20 Å) (28). The structure of \( R. \ chinensis \) lipase in an open form was modeled by SWISS-MODEL based on the 3D crystallographic structure of the inhibited \( R. \ miehei \) lipase (PDB id: 4TGL) (29). The structures were energy minimized by molecular dynamics for 10 ps at 298 K.

### RESULTS

#### Sequence alignment and lid swapping

Homology modeling of the \( R. \ chinensis \) lipase S4-3 shows an identity of 81.1% compared with the crystal structure of ILGY. The secondary structure of \( R. \ chinensis \) lipase consists of nine \( \alpha \)-helices and eight \( \beta \)-strands, and the catalytic triad of Ser145-His257-Asp204 is shielded by a mobile lid in the “closed” form. The lid in \( R. \ chinensis \) lipase S4-3 consists of 6 amino acids (underlined, \( \text{TNSFRSAIT} \)) plus 3–5 amino acids as two hinges around the lid. The superimposed \( \alpha \)-\( \beta \)-structures of S4-3, RML (PDB id: 3TGL), and AnFaeA (PDB id: 1USW) (Fig. 1) show the close structural relations between these three structures, except the big difference in the lid region between AnFaeA and S4-3/RML. The lids of S4-3 and RML in their \( \alpha \)-\( \beta \)-structures are in a closed form, which shields the active site and the substrate binding region. However, the lid of AnFaeA is kept in an open form, and AnFaeA also does not demonstrate interfacial activation (30).

#### Lipase substrate specificity

Chain-length specificity of the purified enzyme was analyzed by using various fatty acid esters of triglycerides as substrates. The enzymatic activities for hydrolyses of triglycerides were determined by the spectrophotometric assay at 430 nm using the formation of copper soaps for the detection of free fatty acids (25). One unit (1 U) of lipase activity was defined as the amount of enzyme needed to liberate 1 \( \mu \)mol of free fatty acids per minute under defined reaction conditions.

#### Interfacial kinetics

The values of interfacial kinetic parameters \( k_{\text{a}} \) and \( k_{\text{cat}} \) were determined in a heterogeneous medium reacted under the condition of pH 8.0 and 40°C for S4-3 and S4-3M and of pH 8.0 and 45°C in RML.
the catalytic properties of the lipase. The lid of S4-3 was swapped with that from AnFaeA or RML to generate the variant S4-3N or S4-3M, respectively.

Enzymatic properties of S4-3, S4-3M, and S4-3N

S4-3, S4-3M, and S4-3N were constructed and extracellularly expressed under the control of the methanol-inducible alcohol oxides promoter in P. pastoris GS115. The parent S4-3 and the variants S4-3M and S4-3N were purified to electrophoretically homogeneous states at the final step as shown in Fig. 3. Purified lipases were used for determination of their properties (Fig. 4). The activities and stabilities of S4-3, S4-3M, and S4-3N were measured over a pH range of 5.0–10.0 at 40°C. They all showed the optimum activities at pH 8.0 (Fig. 4A). However, S4-3N was more active below pH 6.0 compared with S4-3 and S4-3M, and the activity of S4-3N was >85% in the range of pH 7.5–9.0 while for S4-3 only in the range of pH 8.0–8.5, and a sharp decrease below or above pH 8 was observed for S4-3M. S4-3M and S4-3N were stable over a broader pH range, which preserved 80% residual activity at the pH range of 6.5–8.5, whereas S4-3 only maintained 80% residual activity at pH 7.5–8.5 (Fig. 4B). The effects of temperature on lipase activities were examined at temperatures between 20°C and 70°C (Fig. 4C). S4-3N was most active at 45°C, which was 5°C higher than the optimum temperatures of S4-3M and S4-3. In addition, S4-3N maintained higher activity at temperatures above 45°C compared with S4-3M and S4-3. S4-3M and S4-3N were both less thermostable than S4-3 (Fig. 4D). After incubation at 40°C for 1 h, S4-3 retained ~100% residue activity, but the residual activities of S4-3M and S4-3N were only 45% and 38%, respectively.

Substrate specificity

The lid in lipase plays an important role in modulating not only stability and activity but also substrate specificity. A comparative study of fatty acid chain-length specificity was carried out by measuring the initial rate of hydrolysis of various fatty acid esters of triglycerides by S4-3, S4-3M, and S4-3N. As shown in Table 1, specific activities were obtained for the purified lipases toward triacetin, tributyrin, tricaprin, tricaprylin, trilaurin, tripalmitin, and tristearin (C2–C18). The relative activities (activity toward trilaurin equals 100%) are shown in Fig. 5. For the lid swapped with the lid from AnFaeA, the specific activity of S4-3N toward the substrates with fatty acid chain longer than C6 reduced significantly by 6- to 410-fold. On the contrary, the specific activity of S4-3N toward short-chain substrates increased.

Fig. 3. SDS-PAGE of S4-3N (A), S4-3M (B), and S4-3 (C) in each purification procedure. M, protein marker; 1, supernatant; 2, ultrafiltration; 3, HiTrap SP FF; 4, HiTrap Phenyl HP.

Fig. 4. The pH profile (A), pH stability (B), temperature profile (C), and thermal stability (D) of S4-3, S4-3M, and S4-3N.

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by 7.2-fold (C3) and 38.0-fold (C2), respectively. Accordingly, S4-3N displayed an altered substrate preference toward short-chain fatty acid esters, which exhibited a 4,440-fold higher relative activity toward the triacetin (C2)/trilaurin (C12) activity ratio compared with the parent. For the variant S4-3M, we found that replacement of the lid with that from RML caused a 1.5- to 3.3-fold increase in the specific activity toward those substrates (C2, C6, C8, C12, and C16) and a 40% reduction toward tristearin (C18) compared with the corresponding activity of the parent. Comparing the relative activity toward various substrates tested (Fig. 5), S4-3M and S4-3 both exhibited higher substrate specificities toward tricaprylin (C8) and trilaurin (C12), while S4-3N mostly preferred short-chain substrates (C2–C6).

**Interfacial kinetic parameters**

Interfacial kinetic parameters $K_m^*$ and $k_{cat}$ were used to determine the intrinsic substrate fatty acyl specificity of lipase catalysis. By measuring the interfacial kinetic parameters toward short- and long-chain fatty acid esters, we attempted to obtain more insight into the impact by lid swapping. As shown in Table 2, when the long-chain fatty acid ester $p$NPL (C12) was used as the substrate, the $K_m^*$ value of S4-3M increased subtly, while the $K_m^*$ value of S4-3N was 4.8 times higher than that of the parent, which indicated that the affinity between S4-3N and the substrate $p$NPL decreased significantly. The $k_{cat}^*$ value of S4-3M increased by 6.2 times, and the value for S4-3N decreased by 6.0 times compared with that of the parent, which suggested that the introduced new lid interfered with the interaction between the catalytic active site and the long-chain fatty acid ester. On the other hand, in agreement with the shift of the substrate specificity of S4-3N toward short-chain fatty acid esters [$p$-nitrophenyl acetate (C2) and $p$-nitrophenyl propionate (C3)], the $k_{cat}^*/K_m^*$ value of S4-3N was about 3 times higher than those of the parent and S4-3M, and according to the low $K_m^*$ value of S4-3N, the affinity between S4-3N and short-chain fatty acid esters was much stronger than those of the parent and S4-3M. The $k_{cat}^*/K_m^*$ value of S4-3N toward $p$-nitrophenyl acetate (C2) or $p$-nitrophenyl propionate (C3) was one magnitude higher than that toward $p$NPL (C12).

**DISCUSSION**

Most lipases, including S4-3, have a lid domain that covers the catalytic triad, and the movement of an $\alpha$-helical lid at the lipid-water interface created a large hydrophobic patch around the catalytic triad, resulting in activation of lipase. In this paper, the hydrophobic lid of S4-3 was replaced with that of homologs to explore the feasibility of converting a lipase into an esterase and to evaluate the role of the lid in the modulation of catalytic properties, including pH and temperature properties, kinetics, and substrate specificity. In order to minimize structure perturbation, we chose two closely structure-related enzymes, AnFaeA and RML, with a lid in the same size for lid swapping. AnFaeA is an esterase with a hydrophilic lid kept in an open form, which hydrolyzes the ester bonds between hydroxycinnamic acids and polysaccharide or lignin. From a different genus, RML is the closest related lipase with a hydrophobic lid, which showed similar substrate selectivity toward $p$-nitrophenyl decanoate (C10) and myristate (C14) compared with S4-3 (20), but the reported catalytic efficiency of RML was much higher than that of lipases from *Rhizopus* sp. (31). Insertion of the lid from AnFaeA or RML into the S4-3 backbone did not change much of the optimum pH, the pH stability, and the optimum temperature. However, the lid swapping did negatively affect the thermostability of the variants. The results suggested that the lid swapping destabilized the structure of the variants; however, the subtle change of the structures could not be judged from the far-UV circular dichroism spectra of the enzymes (data not shown). A site-directed mutagenesis study also demonstrated that mutations in the lid region destabilized the protein (32). The most apparent changes by lid swapping were that the replacement of the S4-3 lid with that of AnFaeA shifted the specificity toward short-chain substrates (C2–C6) compared with that of the parent (C12), while the replacement of the S4-3 lid with that of RML enhanced the catalytic efficiency toward long-chain fatty acid substrate.

**Table 1.** Lipase activity of S4-3, S4-3M, and S4-3N against the triglyceride substrates

| Triacylglycerols   | S4-3 (U/mg) | S4-3M (U/mg) | S4-3N (U/mg) |
|--------------------|-------------|--------------|--------------|
| Triacetin (C2)     | 14.2 ± 0.5  | 47.4 ± 5.6   | 532.9 ± 35.9 |
| Tributyrin (C3)    | 66.3 ± 1.2  | 54.4 ± 3.7   | 476.2 ± 32.8 |
| Tricaprin (C6)     | 2,550.6 ± 58.2 | 7,911.6 ± 230.9 | 422.9 ± 45.3 |
| Tricaprylin (C8)   | 8,394.6 ± 250.0 | 14,196.1 ± 259.8 | 258.4 ± 24.8 |
| Trilaurin (C12)    | 9,504.2 ± 280.1 | 14,374.4 ± 330.1 | 120.1 ± 15.7 |
| Tripalmitin (C16)  | 5,858.9 ± 78.2 | 8.2 ± 2.1   |
| Tristearin (C18)   | 678.5 ± 34.5 | 5.9 ± 1.3   |

The reaction conditions were pH 8.0 and 40°C for S4-3 and S4-3M, and pH 8.0 and 45°C for S4-3N, respectively.
ping, the mutation Phe95Tyr in S4-3N introduced a polar residue in the substrate binding groove, which probably partially contributed to the high C2/C12 activity ratio and the high catalytic efficiency toward short-chain fatty acid esters. In addition, the substitution of positively charged Arg87 with noncharged Thr disrupted the original salt bridge between Arg87 and Asp61, which might destabilize the conformation of the variant.

The lid swapped with the hydrophilic lid from RML did not change the substrate specificity of the parent S4-3. As shown in Fig. 6, the exposed open lid showed the same hydrophobic pattern, which is crucial for the interaction with the water-insoluble substrates. However, the lid swapping resulted in the enhancement of the catalytic efficiency toward \( p \)-nitrophenyl caprylate \((p\text{-NPL}(C12))\), which was another interesting issue of the impact of the lid. The \( k_{\text{cat}}^* \) for S4-3M toward \( p \)-nitrophenyl esters increased considerably by 6.2 times with a small increase of \( K_m^* \), accounting for an increase in \( k_{\text{cat}}^*/K_m^* \). It was very interesting because the \( k_{\text{cat}}^* \) value of the chimera S4-3M with a lid from RML was comparable with that of RML toward \( p \)-nitrophenyl caprylate \((p\text{-NPL})\). The different amino acid composition of the RML lid might be one of the contributors to the high catalytic efficiency. As shown in Fig. 6, the ligand residues Thr83, Ala89, Asp92, Met93, and Phe95 in the lid-open form of S4-3 were predicted to reside in the substrate-binding groove. The corresponding residues in RML were Ser82, Trp88, Asp91, Leu92, and Phe94, among which Asp91 and Phe94 were conserved.

After replacement with the lid from RML, one important difference in the substrate-binding region of S4-3M was the substitution of Thr83 (S4-3) to Ser82 (RML). This residue was proposed to act in the formation of the oxyanion hole, which stabilized a negatively charged oxygen in the tetrahedral intermediates of the covalent bound substrate (36, 37). From molecular dynamics, it was evident

### Table 2. Interfacial kinetic parameters of S4-3, S4-3M, and S4-3N against \( p \)-nitrophenyl esters

| Enzymes   | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_m \) (\( \mu \text{M} \)) | \( k_{\text{cat}}^*/K_m^* \) (s\(^{-1}\)) | \( k_{\text{cat}}^* \) (M\(^{-1}\) s\(^{-1}\)) | \( K_m^* \) (\( \mu \text{M} \)) | \( k_{\text{cat}}^*/K_m^* \) (M\(^{-1}\) s\(^{-1}\)) | \( k_{\text{cat}}^* \) (M\(^{-1}\) s\(^{-1}\)) | \( K_m^* \) (\( \mu \text{M} \)) | \( k_{\text{cat}}^*/K_m^* \) (M\(^{-1}\) s\(^{-1}\)) |
|-----------|----------------------------------|-------------------------------|------------------------------------------|-------------------------------|------------------------|------------------------------------------|-------------------------------|------------------------|------------------------------------------|
| S4-3      | 27.2 ± 2.8                       | 398 ± 9                       | 6.8 ± 10\(^i\)                           | 5.9 ± 0.9                     | 1,620 ± 12            | 3.6 ± 10\(^i\)                           | 7.8 ± 0.8                     | 1,550 ± 15            | 5.0 ± 10\(^i\)                           |
| S4-3M     | 168.4 ± 2.2                      | 455 ± 12                      | 3.7 ± 10\(^i\)                           | 6.2 ± 0.7                     | 1,580 ± 15            | 3.9 ± 10\(^i\)                           | 6.9 ± 0.6                     | 1,650 ± 18            | 4.2 ± 10\(^i\)                           |
| S4-3N     | 4.5 ± 0.7                        | 1,903 ± 15                    | 2.4 ± 10\(^i\)                           | 21.3 ± 0.6                    | 320 ± 10              | 6.7 ± 10\(^i\)                           | 23.1 ± 0.7                    | 350 ± 12              | 6.6 ± 10\(^i\)                           |

The reaction conditions were pH 8.0 and 40°C for S4-3 and S4-3M, and pH 8.0 and 45°C for S4-3N, respectively.

The available X-ray structures of free ROL, RML coupled with substrate-like inhibitor, and AnFaeA give a hint of the molecular basis of substrate-enzyme interactions (28, 29, 33). AnFaeA is an esterase that has high substrate specificity toward hydrophilic feruloyl esters (34). Moreover, the lid in AnFaeA is always held in an open form without showing interfacial activity (34). The overall structure between S4-3 and AnFaeA is very similar with a backbone RMSD of 2.9 Å, while the deviation of the lid is about 7.5 Å. As shown in Fig. 2, the lid of AnFaeA was more hydrophilic with 85.7% polar residues compared with 57.1% polar residues in the lid of S4-3. Figure 6 shows that upon activation in S4-3N, 80% polar residues of the open lid were exposed on the protein surface, which constituted one side of the substrate binding region, while in the parent S4-3 only 40% polar residues was observed. The variant S4-3N swapped with the AnFaeA lid exhibited substrate specificity toward short-chain substrates (C2-C3) (Table 2). It suggested that the variant S4-3N with a hydrophilic lid favored binding of relatively hydrophilic substrates such as \( p \)-nitrophenyl acetate and \( p \)-nitrophenyl propionate. Besides the significant increase in the hydrophilicity of the lid, replacement of key residues appears worthy of discussion. Residues Thr83, Arg87, Asp92, and Phe95 in the lid of S4-3 were important residues involved in the catalytic process. Thr83 and Asp92 were conserved in S4-3 and AnFaeA, while Arg87 and Phe95 in S4-3 corresponded to Thr and Tyr in AnFaeA, respectively. According to the literature, in ROL upon activation Arg87 approached Asp61 to form a salt bridge that stabilized the open conformation (35), and Phe95 located at the end of the hydrophobic substrate-binding groove is important for the substrate specificity of ROL (13). After lid swapping, the mutation Phe95Tyr in S4-3N introduced a polar residue in the substrate binding groove, which probably partially contributed to the high C2/C12 activity ratio and the high catalytic efficiency toward short-chain fatty acid esters. In addition, the substitution of positively charged Arg87 with noncharged Thr disrupted the original salt bridge between Arg87 and Asp61, which might destabilize the conformation of the variant.

The lid swapped with the hydrophilic lid from RML did not change the substrate specificity of the parent S4-3. As shown in Fig. 6, the exposed open lid showed the same hydrophobic pattern, which is crucial for the interaction with the water-insoluble substrates. However, the lid swapping resulted in the enhancement of the catalytic efficiency toward \( p \)-nitrophenyl (C12), which was another interesting issue of the impact of the lid. The \( k_{\text{cat}}^* \) for S4-3M toward \( p \)-nitrophenyl increased considerably by 6.2 times with a small increase of \( K_m^* \), accounting for an increase in \( k_{\text{cat}}^*/K_m^* \). It was very interesting because the \( k_{\text{cat}}^* \) value of the chimera S4-3M with a lid from RML was comparable with that of RML toward \( p \)-nitrophenyl caprylate (31). The different amino acid composition of the RML lid might be one of the contributors to the high catalytic efficiency. As shown in Fig. 6, the ligand residues Thr83, Ala89, Asp92, Met93, and Phe95 in the lid-open form of S4-3 were predicted to reside in the substrate-binding groove. The corresponding residues in RML were Ser82, Trp88, Asp91, Leu92, and Phe94, among which Asp91 and Phe94 were conserved. After replacement with the lid from RML, one important difference in the substrate-binding region of S4-3M was the substitution from Thr83 (S4-3) to Ser82 (RML). This residue was proposed to act in the formation of the oxyanion hole, which stabilized a negatively charged oxygen in the tetrahedral intermediates of the covalent bound substrate (36, 37). From molecular dynamics, it was evident

**Fig. 6.** Proposed substrate binding regions of S4-3, S4-3M, and S4-3N in an open form. The substrate binding region of S4-3 consists of the amino acids Thr83, Ala89, Asp92, Met93, Phe95, Val112, Li146, Phe178, Ile205, Thr206, Val209, Phe210, Phe211, Phe212, and Ile245, which were colored in green, purple, and yellow. The nonpolar residues in the lid regions were colored in purple, and the polar residues in the lid regions were colored in yellow. The marked number of the residues in the lid regions was according to the number in the original amino acid sequences. The corresponding amino acids in S4-3M and S4-3N were also colored in the same way.
that Thr has only a weak hydrogen bonding interaction (Thr83 Oy–pNPL O2 = 2.98 Å) with the substrate pNPL in S4-3, while Ser showed a strong interaction (Ser83 Oy–pNPL O2 = 2.67 Å) with the substrate in S4-3M. S4-3M showed a 6.2-fold higher \( k^\text{cat} \) value compared with the parent, which was probably due to the stronger interaction by the mutation Thr83Ser. On the contrary, Joerger and Haas (12) reported that the same single mutation Thr83Ser in ROL caused 2- to 7-fold reductions in the activity toward triolein, tri-caprylin, and tributyrin. The single mutation might cause geometry disturbance of the substrate binding region, resulting in a large decrease in the lipase activity. However, in our case the replacement of the whole lid including the mutation Thr83Ser favored the interaction of the oxygen hole residue Ser with the substrate. Another important mutation was from Ala89 (S4-3) to Trp88 (RLM); it was postulated that this Trp might play a role in the interfacial activation process in RML (38). We observed a slightly broad substrate scope for the variant S4-3M (Fig. 5 and Table 1), which was probably attributable to the mutation of Ala89 to a more hydrophobic Trp in the substrate binding groove.

In order to verify that the hydrophobicity of the lid is involved in the catalytic process, a proof-of-concept site-directed mutagenesis was carried out to mutate a hydrophobic residue Phe94 (using RML numbering) into a polar residue Tyr in the S4-3M variant. Tyr was the corresponding residue in the lid of S4-3N. Thus, the mutation Phe94Tyr in the lid of S4-3M may contribute to an increased efficiency toward short-chain fatty acid esters. As expected, after mutation the variant exhibited an 8-fold higher relative activity toward the triacetin (C2)/trilaurin (C12) activity ratio. From this result, it clearly appeared that hydrophobic pattern of the lipase lid had drastic effects on its substrate specificity.

Lipases and esterases with diverse chain-length specificity are the most widely used classes of enzymes in industry. In the present study, for the first time a lipase has been converted into an esterase by modifying the lid region. The substrate specificity of the R. chinensis lipase was successfully modified toward water-soluble substrates by replacing the hydrophobic lid with a hydrophilic lid from AnFaeA. Homology-based modeling and site-directed mutagenesis reveals that not only the amphipathic nature of the lid but also its specific amino acid sequences are vitally important for the enzymatic properties of lipases. Our findings may provide an opportunity for better understanding of the structural basis of the substrate specificity of lipases and guide the design of novel lipases for application in industry.

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