Engineering of *Pseudomonas aeruginosa* lipase by directed evolution for enhanced amidase activity: mechanistic implication for amide hydrolysis by serine hydrolases

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A lipase from *Pseudomonas aeruginosa* was subjected to directed evolution for increased amidase activity to probe the catalytic mechanism of serine hydrolases for the hydrolysis of amides. Random mutagenesis combined with saturation mutagenesis for all the amino acid residues at the substrate-binding site successfully identified the mutation at the residue 252 next to the catalytic His as a hot spot for selectively increasing the amidase activity of the lipase. The saturation mutagenesis targeted for the oxyanion hole (M16 and H83) gave no positive results. The substitutions of Met or Phe for Leu252 significantly increased the amidase activity toward N-(2-naphthyl)oleamide (2), whereas the esterase activity toward structurally similar 2-naphthyl oleate (1) was not affected by the substitution. The triple mutant F207S/A213D/M252F (Sat252) exhibited amidase activity (*k*_cat/*K*_m) 28-fold higher than that of the wild-type lipase. Kinetic analysis of Sat252 and its parental clone 10F12 revealed that the amidase activity was increased by the increase in the catalytic efficiency (*k*_cat). The increase in *k*_cat suggested the importance of the leaving group prototiation by the catalytic His during the break down of the tetrahedral intermediate in the hydrolysis of amides.

**Keywords:** amidase activity/catalytic triad/directed evolution/leaving group prototiation/*Pseudomonas aeruginosa* lipase

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**Introduction**

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the serine hydrolases that catalyze the hydrolysis of fatty acid esters (triglycerides). In addition to their physiological substrates, lipases have extraordinarily broad substrate specificity, catalyzing the hydrolysis and transesterification of a wide range of structurally diverse esters, alcohols and carboxylic acids (Schmid and Verger, 1998). Furthermore, lipases are stable and active in organic solvents and show a high degree of enantio- and regio-selectivity for the conversion of various unnatural substrates in both aqueous and non-aqueous media (Zaks and Klibanov, 1984; Kazlauskas and Bornscheuer, 1998). Hence, lipases have found widespread applications as versatile acyl transfer catalysts for the preparation of optically active precursors for pharmaceuticals and fine chemicals (Kazlauskas and Bornscheuer, 1998; Schmid and Verger, 1998; Retz, 2002). The active site of lipases consists of a Ser-His-Asp/Glu catalytic triad that is topologically very similar to those found in serine proteases (Bradly et al., 1990; Winkler et al., 1990; Ollis et al., 1992). Accordingly, lipases catalyze the hydrolysis of esters by the same double displacement mechanism via an acyl enzyme intermediate as observed with serine proteases (Jaeger et al., 1999). Of particular interest is that lipases do not hydrolyze amides, whereas serine proteases such as chymotrypsin and subtilisin hydrolyze both amides and esters (Zerner et al., 1964; Abrahmsen et al., 1991; Bonneau et al., 1991). Although lipases serve as a very poor catalyst for the hydrolysis of amides (Smidt et al., 1996; Wagegg et al., 1998), lipases often exhibit a high degree of enantioselectivity toward amides and have been used successfully for the preparation of optically active amines (Quiroz et al., 1993; Jaeger et al., 1996; Balkenhol et al., 1997). However, most of the reactions catalyzed by lipases with respect to amines are the formation of amides (aminolysis of esters) that is chemically equivalent to the hydrolysis or alcoholysis of esters. Owing to the resonance-stabilized nature of amides, the hydrolysis of amides is energetically more demanding than that of esters and is not amenable to the catalysis by lipases (Henke and Bornscheuer, 2003). Consequently, it is of particular interest from both the mechanistic and practical points of view to understand why lipases do not hydrolyze amides, despite the similarities to serine proteases in the active site structure and the reaction mechanism. The function of the catalytic triad and the oxyanion hole of serine proteases has been probed by a number of protein engineering studies, but the catalytic roles of individual residues were interpreted by the reduction in the catalytic efficiency as a result of mutations (Bryan et al., 1986; Carter and Wells, 1988; Patricelli and Cravatt, 1999). As an alternate approach, we reported the directed evolution of a lipase from *Pseudomonas aeruginosa* for improved amide-hydrolyzing activities (Fuji et al., 2005). We showed that a single round of error-prone PCR could improve a low level of amidase activity toward N-(2-naphthyl)oleamide (2), and that the identified mutations (F207S, A213D and F265L) selectively increased the molecular activity toward amide 2, but not toward the corresponding naphthyl ester 1. However, the catalytic activities of the mutants were not high enough to deserve kinetic analysis. In this article, we combined another round of random mutagenesis and saturation mutagenesis at the substrate-binding site to expand the accessible sequence space to find mutant lipases with significantly improved amidase activities. The residue 252 next to the catalytic His was identified as a hot spot for selectively increasing the amidase activity. The triple mutant Sat252 containing...
F207S/A213D/M252F exhibited amidase activity 28-fold higher than that of the wild-type (WT) lipase. Kinetic analysis of Sat252 and its parental clone 10F12 indicated that the increase in the apparent amidase activity was ascribed to the increase in the catalytic efficiency ($k_{cat}$). The results were discussed in terms of the leaving group protonation by the catalytic His.

Scheme 1

**Materials and methods**

**Materials**

The commercial lipase (LPL-312) purified homogeneously from *P. aeruginosa* TE3285 and an expression host strain *P. aeruginosa* PAO1162 were kindly provided by TOYOBO Co., Ltd (Osaka, Japan). The ester substrate, 2-naphthyl oleate 1, and the amide substrate, *N*-(2-naphthyl)oleamide 2 (Scheme 1), were synthesized previously (Fujii et al., 2005). The expression vector, pLPLsmA, was constructed as described in our previous article (Fujii et al., 2005). *Escherichia coli* DH5α was used for DNA manipulation. DNA purification columns (QIAprep and QIAquick) were purchased from Qiagen. Restriction enzymes were purchased from TOYOBO. PrimeSTAR HS polymerase and DNA Ligation Kit were purchased from TAKARA BIO (Otsu, Japan). Nitrocellulose membrane was purchased from Bio-Rad. FastGarnet GBC sulfate salt (F8761, dye content 90%) was purchased from Sigma-Aldrich. Micro-scale assay was carried out on a Molecular Devices SPECTRAmax 190 96-well plate reader. Fluorescence was measured on a HITACHI F-2000 spectrofluorimeter. A TaKaRa PCR Thermal Cycler Dice Gradient was used for PCR. DNA sequencing was carried out on an Applied Biosystems PRISM 377 Genetic Analyzer using BigDye Terminators v1.1 Cycle Sequencing Kit.

**Random mutagenesis**

Random mutagenesis was performed by error-prone PCR using *Taq* polymerase and 0.2 mM MnCl$_2$ according to the same conditions as described in our previous article (Fujii et al., 2005). A plasmid pLPLsmA harboring a lipase gene with F207S/A213D double mutations was used as a template.

**Calculation of substrate-binding site**

The amino acid residues involved in the substrate-binding site were estimated by calculating the interior voids or concave regions of lipase using CASTp on-line service (http://sts-fw.bioengr.uci.edu) (Dundas et al., 2006). The RCSB Protein Data Bank entry 1EX9 for a lipase from *P. aeruginosa* PAO1 (Nardini et al., 2000) was used as a query.

**Saturation mutagenesis**

The plasmid pLPLsmA 10F12 harboring a lipase gene with triple mutations (F207S, A213D and L252M) was used as a template for thermal cycling reaction. The primers used for saturation mutagenesis at each site are listed in Supplementary Table 1. Thermal cycling reactions were performed in a total volume of 50 µL containing 100 ng primer, 150 ng template, 1.25 U PrimeSTAR HS polymerase, 0.2 mM dNTP and the buffer provided by the supplier. The following thermal cycling program was used: 30 cycles of 98°C for 10 s, 50°C for 5 s and 72°C for 10 min. The thermal cycling products were digested by 10 U *DpnI* at 37°C for 3 h after purification by QIAquick.

**Library construction, expression and activity staining**

The detailed procedures for library construction, expression and activity staining of mutant lipases were described in our previous article (Fujii et al., 2005). The outline is as follows: the ligation products or the thermal cycling products digested by *DpnI* were transformed into *E. coli* DH5α, and the cells were plated and grown on LB plates containing 20 µg/ml streptomycin (Sm). The plasmid DNA was extracted from the cells using QIAprep to give a plasmid library of mutated lipase genes. *Pseudomonas aeruginosa* PAO1162 was transformed with the plasmids by electroporation and was plated on a nitrocellulose membrane placed on LB agarose plates containing 200 µg/ml Sm. The cells were incubated overnight at 30°C. The colonies were replicated on to a nitrocellulose membrane. The expression of the lipases was induced by placing and incubating the replica membrane at 30°C for 24 h on a 1.5% agar plate containing 10% LB, 100 µg/ml Sm and 1 mM IPTG. The resulting membrane was placed face up on the staining gel containing 100 mM Tris−HCl (pH 7.0), 1% Nonidet P-40 (NP-40), 300 µM FastGarnet GBC, 100 µM amide 2, 1% DMSO and 1.5% agarose for activity staining.

**Expression and preparation of lipase for solution assay**

The positive mutants screened by the activity staining were inoculated into LB medium (200 µl) containing 100 µg/ml Sm, 100 µM CaCl$_2$, 0.1% bovine serum albumin and 1 mM IPTG in each well of 96-well plates. The plates were incubated at 30°C for 16 h with shaking at 160 r.p.m. to express the lipase. Cells were removed by centrifuging the plate at 3100g for 20 min, followed by transferring the 75 µl of supernatant to deep-well plates. The lipase was partially purified by precipitation by adding 675 µl of methanol (final 90%) at 0°C. After incubation at 0°C for 1.5 h, the precipitate was recovered by centrifugation (3100g for 20 min), dried *in vacuo* for 15 min and was dissolved in 100 mM Tris−HCl (pH 7.0) containing 1% NP-40 to give a lipase solution.

**Standard assay for amide- and ester-hydrolysis in solution**

Initial rates of hydrolysis of ester 1 and amide 2 were measured in 100 mM Tris−HCl (pH 7.0) containing 1% NP-40, 300 µM FastGarnet GBC, 100 µM substrates (1 or 2), 1% DMSO and an appropriate amount of lipase solution at 25°C on a 96-well plate. The absorbance at 530 nm was measured continuously using SPECTRAmax for 5 min at 12 s intervals for the hydrolysis of ester 1 and for 90 min at 15 min intervals for the hydrolysis of amide 2.

**Active-site titration for molecular activity**

Active-site titration of lipase was conducted using ethyl 4-methylumbelliferyl heptylphosphonate (4MUP) according
to the reported procedure (Fujii et al., 2003) with a slight modification. Lipase and 4MUP (final concentration of 10 μM) were incubated in 100 mM Tris–HCl (pH 7.0) containing 1% NP-40 and 1% DMSO for 30 min. The reaction was terminated by adding 1 M Na2CO3 (100 μl), and the fluorescence was measured (Ex. 363 nm, Em. 445 nm). Values were determined in three parallel assays. Molecular activities of lipases were calculated by dividing the initial rates of amide- and ester-hydrolysis by the molar concentration of lipase determined by the active-site titration. The initial rates of hydrolysis were measured under the standard assay conditions except that the hydrolysis of ester 1 was conducted at pH 8.

**Determination of kinetics parameters**

Initial rates for the hydrolysis of amide 2 were measured at nine substrate concentrations from 10 to 150 μM in 100 mM Tris–HCl (pH 7.0) under the standard assay conditions except that the reaction was conducted in the presence of 0.25 mM CaCl2 at 37°C. The initial rates were plotted against the substrate concentration to determine the $K_m$ and $V_{max}$ values by fitting the curve directly to the Michaelis–Menten equation using KaleidaGraph version 4.0 (Synergy Software). Values of $k_{cat}$ were calculated by dividing $V_{max}$ by the lipase concentrations determined by the active-site titration.

**Results**

**Random mutagenesis**

The gene encoding the lipase with double mutations (F207S/A213D) was mutated randomly by error-prone PCR using rTaq polymerase, 0.2 mM dNTP and 0.2 mM MnCl2. It was confirmed in our previous article that mutations were introduced uniformly over the 855 bp gene with a mutational frequency of one or two amino acid substitutions under these conditions (Fujii et al., 2005). The amplified lipase gene was ligated into the expression vector pLPLsmA. The plasmid was transferred into E. coli to generate a library of the mutated lipase genes covering a total of 20000 genetic diversity. The plasmids were recovered from the E. coli transformants and were transferred by electroporation into the expression host P. aeruginosa PAO1162 for expression and secretion of active lipases. A total of 20000 transformants of P. aeruginosa was prepared. The transformants grown on a nitrocellulose membrane were treated with IPTG to express the lipase, and the resulting colonies were subjected to activity staining using the amide substrate 2 and FastGarnet GBC as diazo coupling agent. By this method, the excreted lipase hydrolyzed the substrate 2 to release 2-naphthylamine that reacted immediately with FastGarnet GBC to form a red dye precipitate on the colonies to allow rapid and qualitative screening of the mutant lipases on a first-stained-first-selected basis. In contrast to the first generation mutants (Fujii et al., 2005), where the colonies were not stained within an hour, the development of the red color was apparent in 30 min. Deeply stained colonies (a total of 874 clones) were selected and were inoculated into a liquid LB medium for the expression of the lipase for a quantitative solution assay. The lipase secreted into the culture medium was recovered by precipitation with methanol, and the resulting partially purified lipase was subjected to the second screening for improved rates for the hydrolysis of amide 2 ($v_0$,amide). In our previous article (Fujii et al., 2005), the mutants were screened for improved ratios between the amide- and ester-hydrolyzing activities (A/E ratio) on the assumption that the esterase activity toward 1 was proportional to the expression level of the lipase. However, we found that the molecular activity for the ester was also affected by mutations (Fujii et al., 2005), and therefore the A/E ratio was not a reliable criterion at least for the initial screening of the mutants. Hence, we evaluated the 874 clones by directly measuring the apparent hydrolytic rates for amide 2 on the assumption that the expression level of each clone did not differ significantly and selected 64 clones with higher amidase activities.

The molecular concentration of each lipase was measured by the active-site titration using a fluorescent phosphonate inhibitor (Fujii et al., 2003) to calculate the molecular activity of each mutant for the hydrolysis of amide 2. We selected the best five mutants (10F12, 2C7, 3E2, 10G5 and 3B9 descending order of the activity) that exhibited the molecular activity 1.6- to 1.8-fold higher than that of the parental clone. The sequence analysis of the five mutants identified two mutations, V76A (for 3E2) and L252M (for 10F12, 2C7, 10G5 and 3B9), in addition to the original mutations F207S/A213D. Therefore, 10F12, 2C7, 10G5 and 3B9 were found to be the identical clones (F207S/A213D/L252M).

For characterization of the 64 mutant lipases, the initial rates for the hydrolysis of ester 1 ($v_0$,ester) were also measured. The amidase activities ($v_0$,amide) were plotted against the esterase activities ($v_0$,ester) to give an A/E plot (Fig. 1). Most of the mutants (dots) were scattered near the mean.
line for the parental clone (open circles), whereas the four identical mutants (10F12, 10G5, 3B9 and 2C7) stood in a new line that appeared well above the parental clone. Interestingly, the mutant 3E2 remained on the line for the parental clone, indicating that the A/E ratio of 3E2 was the same, although the molecular activity for amide 2 was higher than that of the parental clone. On the basis of the molecular activity, 10F12 was chosen as a parental clone for further experiment.

**Calculation of substrate-binding site**

The substrate-binding site of the mutant lipase 10F12 was calculated using CASTp (Dundas et al., 2006). This program locates and measures concave surface regions on a three-dimensional structure of proteins. For calculation, we used the X-ray crystal structure of a lipase from *P. aeruginosa* PAO1 (PDB entry 1EX9) (Nardini et al., 2000), because its amino acid sequence is 99% homologous to that of our lipase. This X-ray structure contains a phosphonate inhibitor covalently bound to the catalytic Ser (S82) (Fig. 2A), and a substrate-binding pocket can be defined as the amino acid residues that are in van der Waals contact with the inhibitor. However, we focused on the calculated concavity that should span a wide concave surface area containing the substrate-binding pocket estimated from the X-ray structure. The calculation identified a total of 38 concavities on the surface of 1EX9, and the largest concavity that included the phosphonate inhibitor was chosen as the substrate-binding site (Fig. 2A). This site was composed of 22 amino acids including the catalytic Ser and His (S82 and H251), the oxyanion hole (M16 and H83) and the residue at 252, where the mutation was introduced (L252M) in the random mutagenesis, whereas the catalytic Asp (D229) and the other mutational sites (F207S and A213D) were not included in the substrate-binding site (Fig. 2B). Among the 22 amino acids, the catalytically important S82 and H251 were excluded from the targets for saturation mutagenesis.

**Saturation mutagenesis**

Each of the 20 amino acids that comprise the substrate-binding site of the mutant 10F12 was subjected to saturation mutagenesis. We initially used QuikChange Multi kit for saturation mutagenesis, but few transformants were obtained. This was probably because of the large size of the plasmid pLPLsmA (ca. 10 kb) that hindered the extension reaction by the DNA polymerase supplied by the kit. Therefore, we optimized the conditions for the extension reaction by using the DNA polymerase PrimeSTAR and by changing the reaction conditions accordingly (see Materials and methods). In order to cover the possible number of variants (20 amino acids), a library size of 200 is usually recommended for saturation mutagenesis (Georgescu et al., 2003). To ensure the coverage of all possible mutations, the thermal cycling product was transferred to *E. coli* DH5a to prepare a library of 350 mutants at each site. Thirty-two clones were selected randomly from the library and were subjected to sequence analysis to find 16 amino acids at one site. Hence, these conditions were satisfactory to explore all the possible mutations at each site. For the expression of lipase, a library of 1000 of *P. aeruginosa* transformants was prepared for each site.
expression libraries were screened for the hydrolytic activity toward amide 2 by the activity staining to select 50–100 colonies in the order of time for coloration. The selected mutants were then subjected to the solution assay for increased molecular activities toward amide 2.

The saturation mutagenesis for the oxyanion hole was interesting, because the oxyanion hole plays an important role in catalysis by stabilizing the tetrahedral intermediate (TI) or the transition-state for the formation and the hydrolysis of the acyl-enzyme intermediate (Scheme 2) (Braxton and Wells, 1991; Nardini et al., 2000). Furthermore, the oxyanion hole of the P. aeruginosa lipase is formed by the main-chain amide NH, but not by the side chain(s) (Noble et al., 1993; Nardini et al., 2000). Hence, the oxyanion hole is still present by any amino acid substitutions, but a subtle difference in its geometries by the mutations in the side chain may significantly affect the catalytic activity. Therefore, we undertook the saturation mutagenesis at M16 and H83 that comprise the oxyanion hole. The libraries (350 clones for each site) were prepared for screening for the hydrolysis of amide 2 by the activity staining. Apparently, no colonies were stained significantly faster than the parental 10F12, but a total of 50 and 65 clones for M16 and H83, respectively, were selected in the order of time for coloration. The clones were subjected to the solution assay for increased molecular activities for the hydrolysis of amide 2. However, no mutants with improved amide-hydrolyzing activities were found. The sequence analysis of randomly selected 16 clones with molecular activities comparable with that of 10F12 revealed that all the clones sequenced were identical to 10F12: no mutations with improved or comparable activities were found among saturation mutants either at the residue 16 or at the residue 83 comprising the oxyanion hole. However, it was interesting to characterize the saturation mutants by measuring the A/E ratios to understand the effects of the amino acid substitutions on the amidase/esterase activities. Thus, the initial rates for the hydrolysis of amide 1 were measured, and the amidase activities (v_0,amide) of a total of the 115 clones were plotted against their esterase activities (v_0,ester) (Fig. 3). As expected, the sequenced 16 clones were located on or near the line for the parental lipase 10F12 (a dotted line in Fig. 3), but another group of clones exhibiting molecular activities toward amide 2 were plotted against those for the hydrolysis of ester 1 (v_0,ester). Dots, saturation mutants at 16; closed diamonds, saturation mutants at 83; open circle, parental lipase 10F12; closed triangle, expressed WT lipase; open triangles, varying concentrations of purified WT lipase (LPL-312). The dotted line represents the amide-ester-hydrolyzing activity (A/E) ratio for the parental lipase 10F12 (see text).

The residue M252 was a promising target for saturation mutagenesis, because this amino acid is next to the catalytic H251 and was the mutational site (L252M) found in the random mutagenesis for improving the molecular activity toward amide 2 (see Random mutagenesis). A library of the saturation mutants at 252 was screened for the hydrolysis of amide 2 by the activity staining. In this case, several clones were stained within 15 min that was half the time for the coloration of the parental lipase 10F12. Sixty-six mutants were selected in the order of coloration time and were subjected to the solution assay for molecular activities. Interestingly, several clones showed molecular activities notably higher (ca. 3-fold) than that of 10F12. The sequence analysis of the eight clones that exhibited the highest activities revealed that these clones were identical and contained the same mutation of M252F. For the characterization of the 66 saturation mutants, an A/E plot was constructed (Fig. 4). According to the distribution, the clones were categorized into three groups along the lines (b), (c) and (d). The line (a) was for the expressed and the purified WT lipase (LPL-312). The group along the line (b) was composed of the mutants with decreased molecular activities. The sequence analysis revealed that the mutants in this group carried Leu at 252, indicating that these mutants were identical to their grandparent (F207S/A213D) before random mutagenesis as a result of back mutation. The clones along the line (c) exhibited the same level of the A/E ratio as that of 10F12. The sequence analysis of two mutants in this group confirmed that these mutants were indeed identical to 10F12. The group along the line (d) is worth noting. This group contained the best eight clones that exhibited the highest molecular activities. Although the sequences of all clones in this group were not examined, the clones along the line (d) were probably identical and contained M252F. This mutant (F207S/A213D/M252F) was referred to Sat252. The observed codons encoding the residue at 252 were Leu (CUG), Met (AUG) and Phe (UUU) for the WT lipase, 10F12 and Sat252, respectively. The substitution of Met for Leu (WT to...
10F12) was caused by one-base substitution that was accessible by random mutagenesis, whereas the substitution of Phe for Met (10F12 to Sat252) involved two-base substitutions that were accessible solely by the saturation mutagenesis at the substrate-binding site. However, among a total of ca. 6000 clones generated, no mutants exhibited amide-hydrolyzing activities notably higher than that of the parental 10F12. Therefore, we stopped further examination of those clones and focused on the kinetic analysis of the mutants at 252.

**Enzyme kinetics**

The kinetic parameters for the hydrolysis of amide 2 by 10F12 and Sat252 were determined (Table I). Because of a low amidase activity and poor solubility of the substrate 2, the kinetic parameters for the WT lipase were not determined. However, we estimated the $K_m$ value for the WT lipase at least larger than those for 10F12 and Sat252, because a Michaelis–Menten plot for the WT lipase did not give a saturation curve until the highest possible substrate concentrations (10 or 25 $\mu$M).

<table>
<thead>
<tr>
<th>Lipase</th>
<th>$K_m$ [\muM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat}/K_m$ [s$^{-1}$ M$^{-1}$]</th>
<th>$k_{cat}/K_m$ [s$^{-1}$ M$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sat252</td>
<td>330 $\pm$ 20</td>
<td>0.59 $\pm$ 0.02</td>
<td>1800</td>
<td>1600</td>
</tr>
<tr>
<td>10F12</td>
<td>140 $\pm$ 2</td>
<td>0.086 $\pm$ 0.00</td>
<td>610</td>
<td>770</td>
</tr>
<tr>
<td>WT</td>
<td>$&gt;$330</td>
<td>ND$^{d}$</td>
<td>ND$^{d}$</td>
<td>58</td>
</tr>
</tbody>
</table>

*Initial rates for the hydrolysis of amide 2 were measured as described in Materials and methods. Kinetic parameters (± standard error) were determined by fitting the data directly to the Michaelis–Menten equation using KaleidaGraph 4.0.

The $k_{cat}$ values were calculated by dividing $V_{max}$ values by lipase concentration determined by the active-site titration (see Materials and methods).

*Specificity constants were calculated from initial rates at low substrate concentrations (10 or 25 $\mu$M).

*ND, not determined.

**Discussion**

The hydrolytic activities of serine hydrolases toward esters and amides are an interesting subject, because typical esterolytic enzymes such as lipases have a Ser-His-Asp/Glu catalytic triad that is highly homologous to that of serine proteases (Brady et al., 1990; Winkler et al., 1990; Ollis et al., 1992), but lipases usually do not hydrolyze amides (Kazlauskas and Bornscheuer, 1998; Duarte et al., 2000; Henke and Bornscheuer, 2003). In our previous article, we showed that a low level of amide-hydrolyzing activities of a lipase from *P. aeruginosa* could be improved by random mutagenesis and that the amidase and the esterase activities of lipases could vary independently (Fujii et al., 2005). However, the activities of the mutants toward amide 2 were not yet high enough to be analyzed kinetically to probe the mechanistic basis for the increase in the amidase activities. In this article, we further explored the sequence space by combining another round of random mutagenesis and saturation mutagenesis at the substrate-binding site to find the mutants with high amidase activities for kinetic analysis.

A library of 20 000 random mutants generated from F207S/A213D mutant (Fujii et al., 2005) by error-prone PCR was screened for improved activity toward amide 2 to identify five clones (10F12, 10G5, 3B9, 2C7 and 3E2) that exhibited the molecular activities 1.6- to 1.8-fold higher than that of the parental lipase. The sequence analysis identified a couple of new mutations, L252M (for 10F12, 2C7, 10G5 and 3B9) and V76A (for 3E2), but the AIE plot (Fig. 1) and the measurement of molecular activity found that the mutation of L252M, but not V76A, caused selective increase in the amidase activity (3.6-fold in molecular activity at 100 $\mu$M of 2).
Increased activity was still modest, probably because the accessible sequence space explored by random mutagenesis is limited. Saturation mutagenesis should overcome this limitation and was used successfully for the molecular evolution of enzymes for improved thermostability (Miyazaki and Arnold, 1999), enantioselectivity (Liebeton et al., 2000) and substrate specificity (Whittle and Shanklin, 2001). Recently, Morley and Kazlauskas (2005) argued that the mutations close to the active site appeared to be more effective than the distant ones to alter the catalytic activities of enzymes. Therefore, we focused on the substrate-binding site of the lipase for saturation mutagenesis and calculated the site by CASTp (Dundas et al., 2006) based on the X-ray crystal structure of a lipase from P. aeruginosa PAO1 (PDB entry 1EX9) (Nardini et al., 2000). The oxyanion hole was our prime target, but contrary to our initial expectations, the saturation mutagenesis at M16 and H83 gave no mutants with improved amidase activities. The AIE analysis revealed the substitution of Leu for Met at residue 16 significantly reduced the AIE ratio (Fig. 3). Interestingly, the lipases from *Barkholderia cepacia* and *P. fluorescens* (commercially available as Amano PS and Amano AK, respectively) carry Leu at the residue corresponding to M16 of our lipase (Arpigny and Jaeger, 1999). We measured the amidase activities of Amano PS and AK under the same assay conditions, but these lipases did not exhibit any detectable activities toward amide 2, whereas their activities toward ester 1 were comparable with that of our WT lipase (data not shown). These results cogently suggested that the stabilization of the TI by the oxyanion hole played a critical role in amide-hydrolysis by lipases, thereby limiting the amino acid residues allowed at this position. It was conceivable that the oxyanion hole (M16 and H83) of *P. aeruginosa* lipases was already optimized for amide hydrolysis, but could accept other amino acids such as Leu at residue 16 for the hydrolysis of esters that is chemically less demanding than that of amides.

Saturation mutagenesis found that the substitution of Phe at residue 252 significantly increased the molecular activity for the amide hydrolysis. This clone, referred to Sat252, exhibited the amidase activity 28-fold and 2-fold higher than those of the WT and the parental lipase 10F12, respectively (Table I). In contrast, the esterase activity of Sat252 still remained in the same level as that of 10F12, which underscored again that the mutation at 252 selectively increased the amidase activity. As a result, the ratio of the specific activity for the hydrolysis of ester 1 and amide 2 ([\(k_{\text{cat}}/K_m\)ester]/[\(k_{\text{cat}}/K_m\)amidine]) was improved from 22 000 (WT) to 1100 (Sat252), although the mutant lipase still exhibited much higher activity toward ester 1 than toward amide 2. Despite all possible variants at 252 were most likely generated, the clones with the highest amidase activities in the saturation mutants carried Phe at 252, and no other amino acids were found at this position. This observation strongly suggested that Phe was the best amino acid at 252 for the amide hydrolysis. This site was also identified as the mutational site (L252M) by the random mutagenesis. Consequently, the site 252 was probably a hotspot for selectively increasing the amidase activity and was worth analyzing kinetically to understand the mechanisms for increasing the amidase activity by this mutation. The kinetic analysis of the mutants at 252 (10F12 and Sat252) revealed several interesting facets (Table I). First, the increase in the amidase activity of Sat252 when compared with 10F12 was caused by the increase in the \(K_m\) value, rather than the increase in the affinity with the substrate 2. The significant increase in the catalytic efficiency (6.9-fold) more than offset the decreased affinity (2.4-fold increase in \(K_m\), accounting for the overall increase (3.0-fold) in the specific activity of Sat252 over 10F12. Second, the increase in the \(k_{\text{cat}}/K_m\) value of 10F12 (13-fold, Table I) when compared with that of the WT lipase was due at least to the increase in the affinity with the substrate 2, though the possibility of a concurrent increase in the \(k_{\text{cat}}\) value was included. Therefore, the mutation at 252 could affect the amidase activity either by improving the affinity with the substrate \((K_m)\) or by increasing the catalytic efficiency \((k_{\text{cat}})\), but the latter was more interesting from a mechanistic point of view. In serine proteases, either acylation or deacylation (Scheme 2) could be a rate-limiting step for amide-hydrolysis (Hedstrom, 2002), as well as for ester-hydrolysis (Zerner et al., 1964; Wang et al., 2006). However, it is naturally assumed that the rate-limiting step for the hydrolysis of amide 2 by the lipase is the formation of the acyl enzyme intermediate, because the evolved lipase Sat252 still hydrolyzed ester 1 ca. 1100-times faster than amide 2. Consequently, the increase in the catalytic efficiency for the hydrolysis of amide 2 can be attributed to the increase in the acylation rate. We also assumed the rate-limiting acylation for the hydrolysis of ester 1, because the structure of ester 1 is significantly different from that of triglycerides, and the apparent \(K_m\) values for the hydrolysis of ester 1 by 10F12 and Sat252 (ca. 180 \(\mu\)M, respectively) were almost the same as those for the hydrolysis of structurally analogous amide 2 (Table I) where the rate-limiting acylation was assumed (Walsh, 1979). The hydrolysis of esters by lipases was also reported to proceed with a rate-limiting acylation (Sugiura and Isebe, 1976). With these kinetic assumptions in mind, the selective increase in the amidase activity was considered. In the acylation step, the catalytic histidine (H251) abstracts a proton from the catalytic serine (S82) to increase its nucleophilicity and donates the proton to the leaving group oxygen (for ester 1) or the leaving group nitrogen (for amide 2) to facilitate the breakdown of the TI to form the acyl enzyme intermediate (Scheme 2). The selective increase in the amidase activity is indicative of improved proton-donating ability of the catalytic His, because the reinforcement of the general-acid catalysis has more impact on the hydrolysis of amide 2 than on the hydrolysis of ester 1. The relative importance of the general-acid-catalyzed leaving group protonation for the hydrolysis of amides and esters was emphasized by Fersht (1971). In the X-ray crystal structures of *Pseudomonas* lipases in complex with phosphate-type transition state analogue inhibitors, the Ne2 atom of the catalytic His is hydrogen bonded to the oxygen of the leaving group alcohol (Cygler et al., 1994; Lang et al., 1998) and the leaving group amine (Bocola et al., 2003). Furthermore, one of the subtilisin variants hydrolyzes an amide with a rate-limiting breakdown of the TI, thus highlighting the leaving group protonation as a critical step (Bott et al., 2003).

According to a model using the X-ray crystal structure of the *P. aeruginosa* PA01 lipase in complex with a phosphate inhibitor (Nardini et al., 2000), the Phe at 252 is located near the chain (2) of the triglyceride-based phosphate inhibitor (Fig. 5). This chain is connected to the carbon 2 of

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**Enhanced amidase activity of lipase by directed evolution**

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**Table I**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amidase Activity</th>
<th>Ester Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10F12</td>
<td>Sat252</td>
</tr>
<tr>
<td>Phe</td>
<td>10F12</td>
<td>Sat252</td>
</tr>
<tr>
<td>Leu</td>
<td>10F12</td>
<td>Sat252</td>
</tr>
</tbody>
</table>

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**Figure 5**

[Diagram showing the X-ray crystal structure of the P. aeruginosa PA01 lipase in complex with a phosphate inhibitor.]
the glycerol moiety, which corresponds to the leaving group 2-naphthylamine of the amide substrate. Therefore, the Phe at 252 is most likely to come close to the leaving group 2-naphthylamine, thereby possibly decreasing the affinity of the substrate by steric hindrance, but increasing the rate-limiting acylation by promoting the leaving group protonation by the adjacent catalytic His.

In conclusion, we explored the amide- and ester-hydrolyzing activities of serine hydrolases by directed evolution of a lipase for improved amide-hydrolyzing activities. The saturation mutagenesis at the substrate-binding site successfully identified the mutational site 252 as a hotspot for selectively increasing the amidase activity. The kinetic analysis of the mutants Sat252 and 10F12 revealed that the catalytic efficiency ($k_{cat}$) was improved. We propose therefore that the leaving group protonation is important for amide hydrolysis and that the mutations affecting the function of the catalytic His is essential for increased amidase activities of the lipase. These observations should broaden our understanding of the mechanisms for the hydrolysis of energetically demanding amides by serine hydrolases and provide a mechanistic basis for creating novel biocatalysts for amide hydrolysis.

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References
