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Studying the Active Site Pocket of *Staphylococcus hyicus* Lipase by Site-Directed Mutagenesis

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Site-directed mutagenesis of a previously constructed, recombinant *Staphylococcus hyicus* lipase (49 kDa) showed that Val363 played a role in catalysis and substrate-binding. In comparison with wild type enzyme, the 64% and 89% decrease in the catalytic efficiency (kcat/K_m) of the V363N and V363A enzymes, respectively, were largely caused by a 3.5- and 5.5-fold increase in the substrate-binding affinity (K_m), respectively. In comparison with wild type enzyme, a G371A enzyme showed a 40% decrease in the K_m, suggesting that G371 was important for substrate-binding specificity. Site-directed mutagenesis of the active site Asp559 revealed that in comparison with wild type enzyme, a D559E enzyme exhibited a 47% decrease in the kcat/K_m but a twofold increase in the K_m for *p*-nitrophenyl butyrate, suggesting that Asp-559, a component of the catalytic triad, was involved in substrate-specificity.

Lipases (Triacyl glycerol hydrolase, E.C. 3. 1. 1. 3) are widely distributed in nature and hydrolyze triglycerides to diglycerides, monoglycerides, glycerol, and fatty acids, and the principal biological function of lipases is the breakdown of lipids as an initial event in the utilization of fat as an energy source. Lipases are also commonly used as food and detergent additives, or in industry and medicine (1). A Ser-His-Asp triad is essential for substrate catalysis in lipases (2). The activity of lipases is dramatically enhanced at the lipid-water interface, and the phenomenon of interfacial activation involves a conformational change (opening of a lid) in the enzymes (3,4,5,6,7,8,9).

An extracellular lipase from bacterium *Staphylococcus hyicus* (the lipase) has been expressed in other bacteria, characterized, and the gene encoding the lipase has been cloned and sequenced (10,11,12,13). The lipase possesses broad substrate specificity for triacylglycerols, various esters and phospholipids. Trypsin-cleavage of the lipase (expressed from *Staphylococcus carnosus*) at Ala248 yields a 46 kDa protein with a three-fold increase in the enzymatic activity. Full lipase activity also requires Ca^{2+} or Sr^{2+} (11). However, only limited structure-function studies have been done and no tertitary structure information is available for the *S. hyicus* lipase.

In a previous report we showed that a slightly-modified, recombinant lipase (49 kDa) could be overexpressed in *E. coli* and affinity-purified. The purified lipase and the previously-characterized 46 kDa lipase possessed similar enzymatic properties (14). Roles of several amino acids of and around the active site of the recombinant lipase are investigated by site-directed mutagenesis, and the findings are reported here.

MATERIALS AND METHODS

Materials. Enzymes for the recombinant DNA experiments were purchased from either Promega Co. or B. M. Biochemicals. Oligonucleotide primers were made by Bio-Synthesis Co., and Taq DNA polymerase and DNA sequenc-

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TABLE 1
Oligonucleotides Used for the PCR-Site-Directed Mutagenesis of the *S. hyicus* ''Ala248'' Recombinant Lipase Gene

Primers	Oligonucleotides (5′ to 3′)
V363N	GAAATGGTTTGGATGACC
V363A	GAAATGGGCTGGATGACC
F365Y	ATGGCCTATGTAATGGAC
G371A	CGTTTGTCCTGCCATACT
V363, F365, I366T	ATGGCCTGTGTAATGGTTTGGATG
D559E	CCGAATGAAGGCTTGGTA

Note. Mutated nucleotides are underlined.

ing kit were obtained from HT Biotechnology Ltd. and US Biochemicals, respectively. Geneclean kit was purchased from Bio 101 Co. and plasmid DNA purification kit was purchased from Promega Co. Isopropyl thio-β-D-galactoside (IPTG) was obtained from B. M. Biochemicals, and *p*-nitrophenyl butyrate, Fast blue RR salt and α-naphthyl butyrate were purchased from Sigma Co. Protein molecular weight markers (200 kDa to 2.5 kDa) were obtained from Novel Experimental Technology Co. Other chemicals were reagent grades.

Bacterial strains, plasmid, and bacterial growth conditions. Escherichia coli HB101 (15) was provided by Promega Co., and *E. coli* BL21(DE3) and plasmid PET20b(+) were obtained from Novagene Co. The bacteria were grown at 37°C or 30°C in L-broth (LB) or on LB/1.5% bacto-agar plates. Ampicillin (50 mg/mL final) was added when needed.

Site-directed mutageneses. Mutant genes were synthesized from *S. hyicus* chromosomal DNA by two-step, three-primer PCRs (16) in which the N-terminal and C-terminal oligonucleotides were identical to those used for the synthesis of the ''Ala248'' gene (14), and the oligonucleotides used for the synthesis of mutant genes are listed in Table 1. The products of the PCRs were digested with *Nco*I and *Xho*I, and were ligated to a *Nco*I/*Xho*I-predigested PET20b(+) DNA fragment. The desired ligated products were cloned, first into HB101, then into BL21(DE3). The DNA sequences surrounding mutant codons were determined (17) from plasmids isolated from BL21(DE3). All recombinant DNA experiments followed standard protocols (18) or protocols recommended by manufacturers.

Protein work. BL21(DE3) cells harboring the desired wild type (14) or mutant recombinant lipase genes on plasmid pET20b(+) were grown at 30°C in LB/ampicillin to an optical (600 nm) density of 0.5. IPTG was added to the cultures (100 mL each) to a final concentration of 0.4 mM, and the cells were harvested three hours after the IPTG induction. For protein purifications of the recombinant lipase, the cells (suspended in a buffer of 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, and 0.05% Tween 20) were broken on ice in a Microson Ultrasonic Cell Disruptor (Heat System Inc., Farmingdale, N.Y.), and, after 10,000 xg centrifugation, the proteins in the supernatant fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). The separated proteins were stained with coomassie blue (20) and assayed for the esterase activity on gels (21) after the removal of SDS (22). Supernatant fractions with positive gel esterase results were loaded onto $NiSO_4$-charged His-bind resins (Novagene Co.). After binding, the resins were washed with a buffer (20 mM Tris-HCl (pH 7.9), 60 mM imidazole, 0.5 M NaCl). The lipase was eluted with another buffer (20 mM Tris-HCl (pH 7.9), 1 M imidazole, 0.5 M NaCl) and dialyzed against a 25 mM phosphate buffer (pH 6.8). Protein concentrations were determined according to a dye-binding procedure (23), using the Bio-Rad protein assay kit system.

Enzyme assay. The purified enzymes and substrate *p*-nitrophenyl butyrate (2.639 mM predissolved in 2.1% Triton X-100) were mixed in 52.6 mM acetate buffer (pH 6.0). The reactions were carried out at 37°C for 20 minutes and were terminated by the addition of acetone (24). The absorption values of the reaction product (*p*-nitrophenol) at 410 nm were determined and the hydrolase activity (a general indicator for the lipolytic activity) of the purified enzymes was obtained, after converting the absorption values into mmols of *p*-nitrophenol produced which were based on a standard optical density (at 410 nm) versus *p*-nitrophenol quantity (at pH 6.0) curve.

RESULTS AND DISCUSSION

Four hydrophobic residues (Val363-His364-Phe365-Ile366) located in front of the catalytic Ser369 have been identified (10), and similar hydrophobic tetrapeptide patterns exist in other lipases (25,2,26,27). This fact suggests that the tetrapeptides possess similar functions. To study the role of the *S. hyicus* tetrapeptide, site-directed mutants were constructed. For mutant cells (F365Y and the mutant (V363N,F365Y,I366T)) no general lipolytic activity were detected on gels. Conversely in comparison with wild type cells, the V363N and V363A mutant cells

TABLE 2
Kinetic Analyses for the Recombinant Wild Type and Mutant Lipases[a]

Enzyme	K_m	kcat	kcat/K_m
Wild type	2.07	0.53	0.257
V363N	7.05	0.65	0.092
V363A	11.22	0.33	0.029
G371A	1.26	0.34	0.271
D559E	4.11	0.56	0.137

[a] Purified enzymes were assayed for the lipolytic activity for substrate *p*-nitrophenyl butyrate as described under Materials and Methods. Kinetic data were calculated from the Lineweaver–Burk plots (28) and linear regression plots. Each data point is the average value of three independent measurements. K_m, kcat, and kcat/K_m have the units of mM, s^{-1}, and s^{-1} mM^{-1}, respectively.

showed a decrease in the general lipolytic activity on gels. Similarily for the V363N and V363A enzymes, a dramatic decrease in the specific activity for the hydrolysis of *p*-nitrophenyl butyrate (54.9 units/mg and 18.2 units/mg, respectively) was observed when compared to that of wild type enzyme (146.2 units/mg). Kinetic analyses using *p*-nitrophenyl butyrate as substrate (Table 2) showed that in comparison with wild type enzyme, the 64% and 89% decrease in the catalytic efficiency (kcat/K_m) of the V363N and V363A mutant enzymes, respectively, were largely caused by a 3.5 and 5.5 fold increase in the substrate-binding affinity (K_m), respectively. For the V363N mutant enzyme a 22% improvement of the rate of catalysis (kcat) was also observed.

The use of the hydrophobic residues in lipases suggests that these residues may be involved in substrate-binding. The kinetic results proved that Val363 played a dominant role in the substrate-binding, which were contrary to an earlier proposal for the role of the equivalent tetrapeptide in human pancreatic lipase (in that proposal it was suggested that its tetrapeptide was not involved in substrate-binding (3)).

Gly371 is the last residue of a conserved pentapeptide found in lipases (2). A G371A mutant enzyme showed a slight improvement in the specific activity for the hydrolysis of *p*-nitrophenyl butyrate (158.2 units/mg) over that of wild type enzyme (146.2 units/mg). The kinetic data for the wild type and the G371A enzymes are listed in Table 2. In comparison with wild type enzyme the G371A enzyme showed a 6% improvement in the kcat/K_m, but a 40% and a 36% decrease in the K_m and in the kcat, respectively, for substrate *p*-nitrophenyl butyrate. The kinetic results suggested that G371 is not an essential element for substrate (*p*-nitrophenyl butyrate)-binding and catalysis, instead, A371 is better suited for the above roles. This phenomenon is likely to be a consequence of a slight conformational change around the substrate-binding site, which includes G371 (glycine is a structurally flexible molecule and tends to be a part of a loop, while alanine is likely to be a part of an α-helix).

The serines of the lipase catalytic centers (equivalents of S369 of the *S. hyicus* enzyme) conform an unusual and strained ϵ-position (or the serines are located within β-ϵ Ser-α motifs if the neighboring amino acids are included) which is essential for catalysis (2). The improvement of the G371A mutant in the K_m and kcat/K_m suggested that Ala371 may extend the predicted α-helix behind the active-site serine and this change promoted catalysis and binding.

Asp559 is a component of the catalytic triad according to a previous report which was based

solely on a whole cell agar diffusion plate assay and a whole cell spectrophotometric assay (12). In our study a D559E mutant enzyme had specific activity, K_m, kcat and kcat/K_m values of 76.9 units/mg, 4.11 mM, 0.56 s^{-1} and 0.137 $s^{-1}mM^{-1}$, respectively, for the hydrolysis of *p*-nitrophenyl butyrate (Table 2). Therefore, in comparison with wild type enzyme, the D559E enzyme (a) showed a 50% reduction in the substrate-binding ability and a 47% decrease in the catalytic efficiency, and (b) possessed a similar kcat value.

The kinetic results of this study proved that Asp559 did play a role in catalysis, like several other lipases the enzyme could use a Glu (Glu559) instead. The addition of a -CH_2 could influence certain kinetic behaviors (mainly the Km) of the lipase, and Asp559 could play a role in the substrate-specificity, contrary to an earlier proposal which was based on a whole cell lipase activity assay for a D559N mutant enzyme that suggested Asp559 did not involve in substrate-specificity (12).

Overall amino acid sequence homology between the *S. hyicus* lipase and known bacterial lipases (29,30,31,32,33) is medium to low. This is consistent with the fact that the *S. hyicus* lipase possesses additional phospholipase activity and requires Ca^{2+} or Sr^{2+} for full activity, and further suggests that unidentical or dissimilar amino acids can play roles in substrate-binding. X-ray crystallographic study is in progress. Future protein engineering works are needed in order to modify substrate range, or improve catalytic efficiency, of this multi-functional enzyme.

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