

Improvement of the optimum temperature of lipase activity for *Rhizopus niveus* by random mutagenesis and its structural interpretation

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Abstract

Random mutagenesis was used to improve the optimum temperature for *Rhizopus niveus* lipase (RNL) activity. The lipase gene was mutated using the error-prone PCR technique. One desirable mutant was isolated, and three amino acids were substituted in this mutant (P18H, A36T and E218V). The wild-type and this randomly mutated lipase were both purified and characterized. The specific activity of the mutant lipase was 80% that of the wild-type. The optimum temperature of the mutant lipase was higher by 15°C than that of the wild-type. To confirm which substitution contributed to enhancing the optimum temperature for enzymic activity, two chimeric lipases from the wild-type and randomly mutated gene were constructed: chimeric lipase 1 (CL-1; P18H and A36T) and chimeric lipase 2 (CL-2; E218V). Each of the chimeric enzymes was purified, and the optimum temperature for lipase activity was measured. CL-1 had a similar optimum temperature to that of the wild-type, and CL-2 had a higher temperature like the randomly mutated lipase. The mutational effect is interpreted in terms of a three-dimensional structure for the wild-type lipase. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Lipase; Random mutagenesis; Optimum temperature; Chimera; *Rhizopus niveus*

1. Introduction

Lipase [EC 3.1.1.3] has proved useful for a number of years in a variety of industrial applica-

tions. For example, the improvement of oil by transesterification is suitable to produce high-value oil from less-expensive plant oil. The transesterification reaction must be performed at a temperature higher than the melting point of the oil, so lipase that is able to react at a high temperature is required. In other words, the property which is required for this purpose is not a high residual activity after incubation at high

Abbreviations: CL-1, chimeric lipase 1; CL-2, chimeric lipase 2; RNL, *Rhizopus niveus* lipase.

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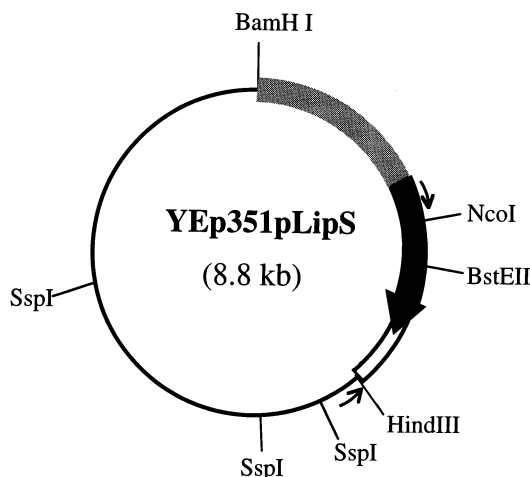


Fig. 1. Structure of plasmid YEp351pLipS. The solid thick arrow indicates the protein-coding regions of the *Rh. niveus* lipase gene. The *PGK* promoter and *Rh. niveus* lipase terminator are shown as a grey box and white box, respectively. The thin clockwise arrow indicates the forward primer (RPP101) site, and the thin anticlockwise arrow indicates the reverse primer (RPP201) site.

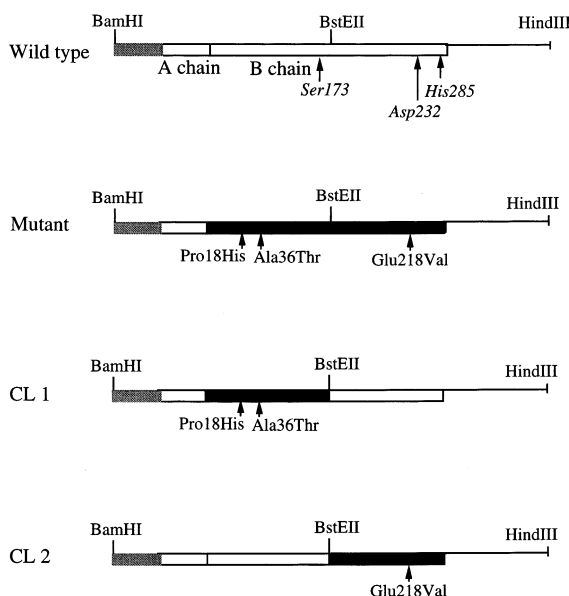


Fig. 2. Schematic illustration of the wild-type, mutant and chimeric lipase genes. The white box and black box, respectively, indicate the protein-coding regions of the wild-type lipase and the mutant lipase. The *PGK* promoter is shown as a grey box, and italic letters indicate amino acids at the catalytic center.

temperature, but a high optimum temperature for lipase activity.

The lipase from *Rhizopus niveus* is superior in its 1-3 positional specificity and has been used to produce a cacao butter substitute (Matsuo et al., 1980). Lipase I, which is a major component of *Rh. niveus* lipase (RNL), has a unique structure consisting of two polypeptides (A-chain, a small peptide with a sugar moiety; and B-chain, a large peptide with an active center) which bind non-covalently to each other (Kohno et al., 1994). We have applied protein engineering to increase the optimum temperature for RNL activity, and have already constructed an expression system for this lipase in the yeast, *Saccharomyces cerevisiae* (Kohno et al., 1998).

Many investigations have been made for improving the thermal stability of enzymes. The thermal stability of the lipase from *Pseudomonas aeruginosa* has been increased by the random mutagenesis technique, and the essential amino acid for thermal stability has been determined (Shinkai et al., 1996). Nagao et al. (1998) have revealed that 26 amino acids of the C-terminal peptide of *Fusarium heterosporum* lipase were necessary to increase its thermostability. The level for thermal stability of RNL has been increased by 15°C by non-cleavage of one peptide bond between the A-chain and B-chain (Kohno et al., 2000). However, the optimum temperatures of these lipases were similar to those of the respective native lipases.

We report in this paper a successful improvement of the optimum temperature for lipase activity by means of the in vitro random mutagenesis technique. One amino acid substitution that would contribute to improving this function is identified by a chimeric synthesis of the randomly mutated and wild-type lipase. The mutational effect will be interpreted using a structural model of the mutant based on the tertiary structure of the wild-type lipase (Kohno et al., 1996).

2. Materials and methods

2.1. Strain, plasmids and culture conditions

The host strain, *S. cerevisiae* AH22 (*MATa leu2-3 leu2-112 his4-519 can1*; ATCC 38626),

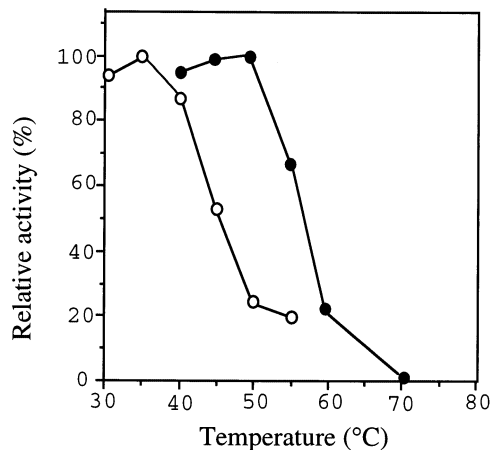


Fig. 3. Temperature dependence of lipase activity. Shown are the wild-type (open circles) and randomly mutant lipases (filled circles). The activity was assayed at the indicated temperature for 30 min, using a water jacket to maintain the temperature, as described in Section 2. Activity values are each given as a percentage of the most active sample. The most active samples of each lipases were 6100 U mg^{-1} of protein at 35°C for the wild-type and 5200 U mg^{-1} of protein at 50°C for the mutant.

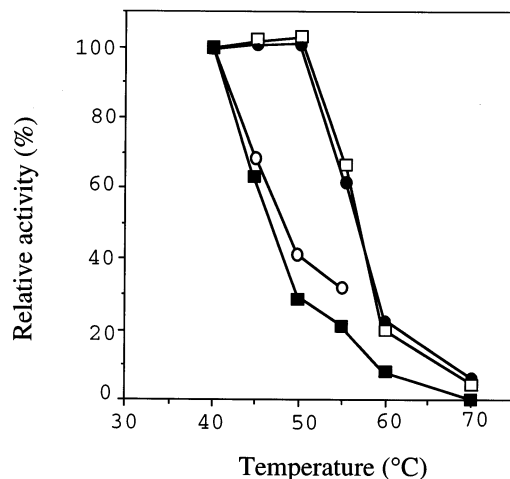


Fig. 5. Temperature dependence of the randomly mutant and chimeric lipase activities. Each activity is given as a percentage of the sample being incubated at 40°C for 30 min of the randomly mutant (filled circles), CL-1 (filled squares) CL-2 (open squares) and the wild-type (open circles) lipases.

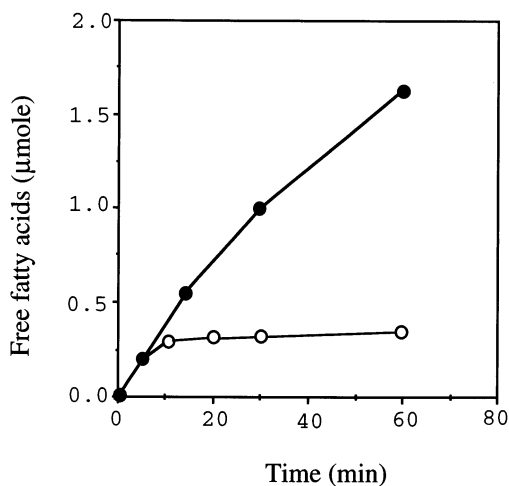


Fig. 4. Profile of the free fatty acids released by the lipases. The amounts of free fatty acids at 50°C at each defined time from the wild-type (open circles) and randomly mutant (filled circles) lipases were measured as described in Section 2.

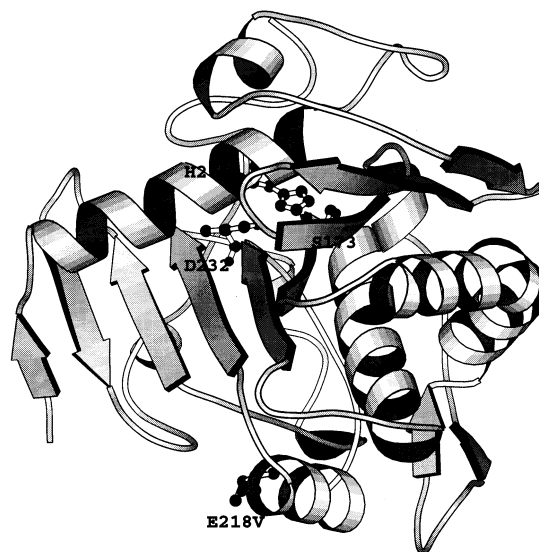


Fig. 6. Schematic illustration of the structure of the mutant lipase. Molscript (Kraulis, 1991) represents the mutant lipase (CL-2). This mutant structure was derived from the structure of the native lipase II. Presented by a ball and stick are the site of mutation (Glu218Val) and the catalytic center (Ser173, Asp232 and His285).

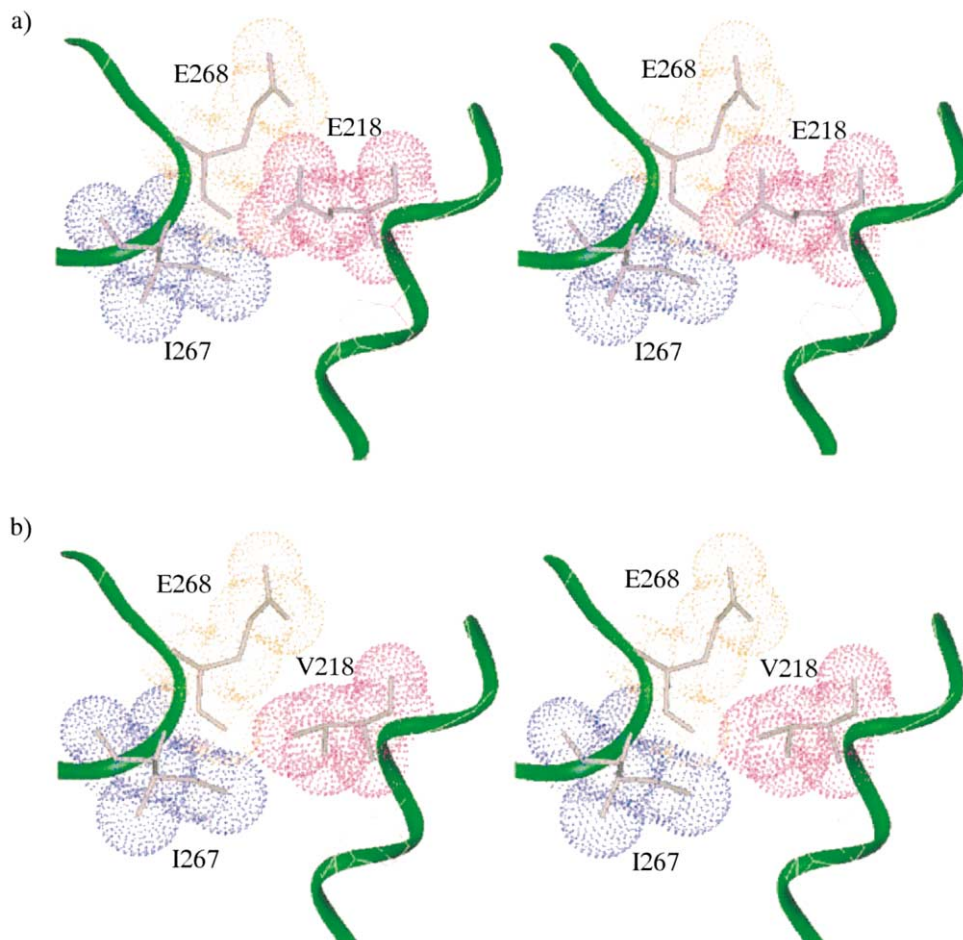


Fig. 7. Localized wild-type and mutant lipase structures. (a) A stereo ribbon diagram shows the interaction made by residues Glu218, Ile267 and Glu268 of the wild-type lipase. (b) The relationship between Val218, Ile267 and Glu268 of the wild-type lipase is shown. The green line indicates the main chain of the lipase structure, while the side chain structure of Val218 was modeled as described in Section 2. The van der Waals surface of each amino acid is represented by dots in a different color (E218 and V218, magenta; I267, blue; and E268, orange).

which was generously presented by Dr Yoshifumi Jigami of the National Institute of Bioscience and Human Technology, was used for expressing the lipase gene. Plasmid YEp351PLipS coding for RNL (Fig. 1) was constructed from YEp351, which was also provided by Dr Y. Jigami, by a similar method to that from YEp352PLipS in our previous study (Kohno et al., 1998) except for the marker amino acid. The recombinant strains were grown in a YPD medium of 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose.

2.2. Random mutagenesis

The error-prone PCR technique was applied for random mutagenesis, the error-prone PCR conditions being adopted from Leung et al. (1989). Template linear DNA was obtained as a 8.9-kbp fragment after *SspI* digestion of the YEp351PLipS plasmid. The forward primer (RPP101) was oligonucleotide 5'-AGCGATCTC-CAAGCTGAACCT-3' preceded by the *NcoI* restriction site in the RNL gene. The reverse primer

(RPP201) was oligonucleotide 3'-TGACCG-GCAGCAAAATGTT-5' followed by the *Hind*III restriction site as shown in Fig. 1. The reaction mixture (100 μ l) contained 67 mM Tris-HCl (pH 8.8), 6.1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 10 mM β -mercaptoethanol, 1 mM dGTP, 1 mM dATP, 1 mM dCTP, 0.2 mM dATP, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM EDTA, 0.017% bovine serum albumin, 4 U of *Taq* polymerase (Takara), and 3 μ l of each primer (0.4 mg ml⁻¹). The DNA was amplified in a DNA Thermal Cycler (Perkin Elmer Cetus) for 24 cycles at 94°C for 1 min, at 50°C for 1 min, and at 70°C for 4 min, after one cycle at 94°C for 7 min, 50°C for 1 min, and 70°C for 4 min.

2.3. Preparation and selection of the mutant library

The amplified DNA fragment was gel-purified and cloned back into vector YEp351PLipS using the *Nco*I and *Hind*III restriction sites. *E. coli* JM109 was used for transformation with these constructs, and the DNA was manipulated. The manipulated plasmids were extracted from the host *E. coli* and transformed to *S. cerevisiae* AH22 according to the electroporation method developed by Becker and Guarente (1991) using a gene pulsar with a pulse controller (Bio Rad). Electroporation was performed by a pulse of 1.5 kV, 25 μ F, and 200 Ω in a 0.2-cm electroporation cuvette (Bio Rad) per 10 ng of DNA.

The mutants having lipase activity at high temperature were selected by a halo assay, using tributyrin-containing plates according to Lawrence et al. (1967) with some modifications. For the first selection, the transformants were plated on YPD plates and cultured for 2 days at 30°C. The cultured samples were replicated on the YPD plates, and after incubation for 1 day at 30°C, with the agar diffusing homogeneously, 1.0% tributyrin, 1.0% bacto-peptone and 20 μ g ml⁻¹ streptomycin were poured on to the replicated YPD plates. These plates were cultured for 1 day at 55°C, and the colonies forming the halo were picked up from the master plates as positive clones. For the second selection, these positive colonies were cultivated in a YPD liquid medium while shaking at 300 rpm min⁻¹ and 30°C for 3

days. Each culture supernatant was collected by centrifugation at $6.5 \times 10^3 \times g$ for 10 min and then diluted by distilled water to 500 times the culture volume. The lipase activity of each solution was measured at 37 and 50°C, and those mutant strains which had higher activity at 50°C than at 37°C were selected for their lipase activity at high temperature.

2.4. DNA sequencing of the mutant lipase

Sequencing reactions were performed with a dye primer sequence kit (Perkin Elmer Cetus), using the reverse primer (RPP201) as a universal primer. Gel electrophoresis of the reaction products and base calling were carried out with an automated DNA sequencer (Perkin Elmer Cetus).

2.5. Enzyme purification

The purification procedure for the recombinant lipases was based on that used in our previous study (Kohno et al., 2000) with some modifications. The culture supernatant of each mutant was collected by centrifugation as already described, concentrated, and desalted by passing through an ultra-filtration membrane. Ten times the volume of a 10 mM acetate buffer (pH 5.0) was added to the concentrated solution, and the resulting precipitate was removed by centrifugation. The supernatant was transferred to an HPLC column of TSKgel CM-5PW (7.5 mm $\times$ 7.5 cm, Tosoh Co.) that had been previously equilibrated with a 10 mM acetate buffer (pH 5.0). After the column had been washed with the same buffer, the fractions with lipase activity were eluted with NaCl at a linearly increasing concentration (0–0.2 M).

2.6. Enzyme assay

Lipase activity was assayed by titrating against the free fatty acids liberated from olive oil (Wako Chem. Co.) as previously described (Kohno et al., 1994). One unit of lipase activity is defined as the amount of enzyme that liberated 1 μ mol of acid per min.

The optimum temperature for lipase activity was found by measuring the activity while incu-

bating over a temperature range of 30–70°C in a thermostatically controlled water bath for 30 min. The stability of the lipase activity at 50°C was measured as the free fatty acids liberated at defined times.

2.7. Construction of the chimeric enzymes

Chimeric lipases were constructed from the mutant plasmid (YEp351PLipM) with high optimum temperature for lipase activity and from wild-type plasmid YEp351PLipS. YEp351PLipM was digested with *Bst*EII, *Hind*III and *Bam*HI. The 2.4-kbp fragment (*Bst*EII–*Bam*HI fragment) was inserted into the *Bst*EII–*Bam*HI site (6.4-kbp) of YEp351PLipS, and the 0.6-kbp fragment (*Bst*EII–*Hind*III fragment) was inserted into the *Bst*HII–*Hind*III site (8.2-kbp) of YEp351PLipS. The former is named YEp351PLipC1 (a region from the N-terminal to Asn210 corresponding to the mutant gene), and the latter is named YEp351PLipC2 (a region from Pro211 to the C-terminal corresponding to the mutant gene) as shown in Fig. 2. Each chimeric plasmid was transformed to *S. cerevisiae* ND-12B (Kohno et al., 1998) according to the above-mentioned method, and each culture supernatant was purified and characterized.

2.8. Computer modeling

The mutant structure was derived from the structure of the native lipase II (PDB ID number 1LGY). The side chain structure of Val218 was modelled using the Insight II 95.0 (Molecular Simulations, San Diego, CA) program running on an Indy work station (Silicon Graphics, Palo Alto, CA) without changing the main chain torsion angle.

3. Results

3.1. Random mutagenesis and isolation of the mutant lipase

An improvement in the lipase activity at high temperature was achieved by means of phenotypic

selection from recombinant cells carrying a randomly mutated gene. Native RNL lipase I has a unique structure consisting of two polypeptides (A-chain and B-chain) which are non-covalently associated (Kohno et al., 1994). It is known that the lipase I A-chain would have, if anything, a slightly poor influence on the optimum temperature for lipase activity from the results of our previous study (Kohno et al., 1994). Consequently, the lipase I B-chain (297 amino acid residues) was chosen as the target DNA for random mutation.

The first selection, based on the halo assay at 55°C, identified 100 positive clones from among about 60 000 transformants. One positive clone was selected from the first 100 positive clones by a second selection based on the lipase activity at 50°C. The relative activity of this mutant at 50°C against that at 37°C was 110%, while that of the wild-type was only 22%.

The recombinant plasmid was extracted from this randomly mutated clone, and the DNA sequence of the inserted fragment was determined. Five base changes, resulting in the replacement of three amino acid residues, were found in this randomly mutated gene. The amino acid substitutions were located in the N-terminal region (P18H and A36T) and C-terminal region (E218V) near the active center of the primary structure as shown in Fig. 2.

3.2. Purification and characterization of the randomly mutated lipase

The recombinant strains producing the wild-type and randomly mutated lipases were cultured, and each lipase was purified. The specific activity at 37°C of the randomly mutated lipase was 4900 U mg⁻¹ of protein, this value being 80% that of the wild-type lipase (6100 U mg⁻¹ of protein). The optimum temperature for lipase activity of the randomly mutated enzyme was 50°C, this value being 15°C higher than that of the wild-type lipase (Fig. 3). The stability of the lipase activity at 50°C was then measured. The free fatty acids liberated by the hydrolysis of the randomly mutated lipase progressively increased, even after 1 h had passed, while the acids from the wild-type

lipase reached a maximum amount after 10 min of the reaction as shown in Fig. 4.

3.3. Construction and characterization of the chimeric enzymes

Chimeric enzymes were constructed to confirm the region in the randomly mutated lipase that was responsible for the activity at high temperature. The amino acid substitutions of one chimeric lipase (CL-1) were Pro18 to His and Ala36 to Thr in the N-terminal region of the lipase. The position of amino acid substitution in the other chimeric lipase (CL-2) was Glu218 to Val in the C-terminal region as shown in Fig. 2. Each of these chimeric lipases was purified, the optimum temperature for lipase activity was measured, and the free fatty acids liberated at 50°C were measured. The enzymatic characteristics for the reaction temperature of CL-2 were the same as those of the randomly mutated lipase, while those of CL-1 were the same as those of the wild-type lipase as shown in Fig. 5. Consequently, one amino acid substitution (E218V) was revealed to have enhanced the optimum temperature for lipase activity.

4. Discussion

We succeeded in creating a lipase that could maintain its activity at a high temperature by random mutagenesis with the error-prone PCR technique. One amino acid substitution (E218V) responsible for the higher optimum temperature of the enzyme activity was identified by the chimeric enzymes.

We have already resolved the three-dimensional structure of lipase II, a minor component of RNL, at a resolution of 2.2 Å (Kohno et al., 1996). This lipase II appeared due to the limited proteolysis of lipase I, and lacked the A-chain and 28 amino acid residues of the N-terminal region of the lipase I B-chain (Kohno et al., 1994). The active center of RNL lipase II was far from the N-terminal in its tertiary structure. Consequently, the structures of the active centers of lipase I and lipase II are regarded as being the same. In fact,

the enzymatic characteristics of each lipase were almost the same (Kohno et al., 1994). Consequently, the 3D structure of the mutant enzyme could be easily predicted by comparing it with that of lipase II. Lipase II has a typical α/β hydrolase fold containing the so-called nucleophilic elbow. In this study, the position (E218V) at which the mutation took place for improving the optimum temperature was far from the catalytic center of the enzyme in its tertiary structure (Fig. 6).

There are two assumptions that can be made to elucidate the improved optimum temperature for lipase activity by only an amino acid substitution. The first assumption is stabilization of the α -helix. Glu218, which was replaced with valine in the mutant, is on the α -helix (α -8) as shown in Fig. 6. According to Facchiano et al. (1998), there are 10 putative factors for the stabilization or destabilization of the α -helix. Indeed, two of these factors (the β -branched effect and hydrophobic interaction) fit this mutant. The β -branched effect is present to the most negative extent in all thermophilic proteins. Consequently, the substitution of Glu218 with valine acted negatively for stabilization by the β -branched effect. On the other hand, substitution of the hydrophilic amino acid, Glu, with the hydrophobic amino acid, Val, would be most likely to induce the interaction between hydrophobic side chains at intra-helical spacings of $(i, i + 3)$ and $(i, i + 4)$. Val substituting for Glu is regarded as having been at position $i + 3$ or $i + 4$ in this helix (α -8). The amino acids at the i position were the hydrophobic amino acids, Ala214 and Tyr215. However, the calculated free energy values for the interaction between Ala–Val and Tyr–Val were, respectively, zero and almost zero (Creamer and Rose, 1995). Therefore, intra-helical hydrophobic interaction was not enhanced by the substitution of Glu with Val. Consequently, the α -helix itself seems not to have been stabilized.

The second assumption to elucidate the high temperature stability of the mutant is strengthening the interaction between secondary structures. A conformational change near the mutated amino acid (E218V) responsible for improving the optimum temperature was compared with that of the native lipase. From this point of view, it is sug-

gested that a loop structure (from Ser265 to Thr269) adjacent to the α -helix (α -8) was stabilized. In native lipase II, the distance between the ϵ -oxygen of Glu218 and the ϵ -oxygen of Glu268 in this loop structure is only 2.91 Å. Since Glu is negatively charged and repels in the neutral pH range, the loop structure can be expected to become unstable at pH 7.0. The replacement of Glu218 with Val seemed to have resulted in the distance between the γ -carbon of valine and the β -carbon of Glu268 being too short to maintain a stable structure in respect of the unchanged main-chain torsion angles. The side-chain torsion angle of the replaced valine could be expected to have been changed to give greater stability. Consequently, a new mode of hydrophobic interaction between the γ -carbon of valine and the δ -carbon of Ile267 was created by the conformational change of side chain (Fig. 7). The result was that the exposed surface area was reduced by the replacement of Glu with Val. This value of side chain of Val218 of the mutant lipase is 23.1 Å² (20.2% for total side chain area) and 51.9 Å² (38.6%) of native Glu218.

There is a random structure from Lys270 to C-terminal Leu297. Only one disulfide bond between Cys57 and Cys296 is present to fix this region to the lipase structure. His285, which is located in this random structure, is one amino acid residue of the catalytic triad of an enzyme (Kugimiya et al., 1992). Stabilization of the loop structure (from Ser265 to Thr269) is therefore suggested to be important for the lipase activity at high temperature. However, a conformational change at the catalytic center of the enzyme is suggested to have resulted from a creation of the new hydrophobic interaction. In fact, the specific activity of the mutant lipase was only 80% that of the wild-type. We are attempting to resolve the tertiary structure of the mutant lipase to reveal in detail the conformational change near the catalytic center.

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