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# Improving tolerance of *Candida antarctica* lipase B towards irreversible thermal inactivation through directed evolution

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**To expand the functionality of lipase B from *Candida antarctica* (CALB) we have used directed evolution to create CALB mutants with improved resistance towards irreversible thermal inactivation. Two mutants, 23G5 and 195F1, were generated with over a 20-fold increase in half-life at 70°C compared with the wild-type CALB (WT-CALB). The increase in half-life was attributed to a lower propensity of the mutants to aggregate in the unfolded state and to an improved refolding. The first generation mutant, 23G5, obtained by error-prone PCR, had two amino acid mutations, V210I and A281E. The second generation mutant, 195F1, derived from 23G5 by error-prone PCR, had one additional mutation, V221D. Amino acid substitutions at positions 221 and 281 were determined to be critical for lipase stability, while the residue at position 210 had only a marginal effect. The catalytic efficiency of the mutants with *p*-nitrophenyl butyrate and 6,8-difluoro-4-methylumbelliferyl octanoate was also found to be superior to that of WT-CALB.**

*Keywords*: directed evolution/irreversible inactivation/lipase/protein aggregation/stability

## Introduction

Lipase B from *Candida antarctica* (CALB) is a highly versatile catalyst used successfully for resolution and desymmetrization of numerous compounds on the laboratory, pilot and commercial scales (Kirk *et al*., 1995; Anderson *et al*., 1998; Jaeger and Reetz, 1998; Homann *et al*., 2001; Jaeger and Eggert, 2002; Kirk and Christensen, 2002). The immobilized form of CALB is quite thermostable, particularly under non-aqueous conditions, where the catalyst remains active for many hours in the presence of high concentrations of reactants often with vigorous agitation (Morgan *et al*., 1997; Arroyo *et al*., 1998; Koops *et al*., 1999). In aqueous solutions, however, the lipase denatures relatively quickly at temperatures as low as 40°C (Homann *et al*., 2001). Prior attempts to improve the lipase stability via protein engineering have resulted in only a moderate improvement of its thermal properties with a concomitant decrease in activity (Patkar *et al*., 1997, 1998). For example, a mutant protein with a single amino acid substitution, T103G, had an increased half-life at 60°C, but only 50% of its original activity compared with the wild-type enzyme (Patkar *et al*., 1998).

Even though the CALB gene has been sequenced and the crystal structure of the protein determined (Uppenberg *et al*., 1994a) neither sequence homology searches nor protein alignment analysis have revealed any clues for improving the thermal stability of the enzyme without negatively affecting its activity. Since rational engineering approaches have been only marginally successful in improving the lipase stability, we decided to turn to directed evolution methodology which has been quite successful in improving functional properties of many enzymes including lipases (Arnold and Moore, 1997; Schmidt-Dannert and Arnold, 1999; Jaeger and Reetz, 2000; Petrounia and Arnold, 2000; Arnold *et al*., 2001; Farinas *et al*., 2001a,b; Wintrode *et al*., 2001; Jaeger and Eggert, 2002; May *et al*., 2002; Bulter *et al*., 2003).

This paper reports the application of error-prone PCR and saturation mutagenesis combined with high-temperature screening to identify CALB mutants with improved resistance towards irreversible inactivation at elevated temperatures.

## Materials and methods

### *Chemicals*

Glucose, glycerol, calcium chloride, sodium chloride, Tris-base, potassium phosphate, Tween-80, tributyrin and *p*-nitrophenyl butyrate (*p*-NB), were all from Fisher Scientific (Pittsburgh, PA). 6,8-Difluoro-4-methylumbelliferyl octanoate (DiFMU) was purchased from Molecular Probes, Inc. (Eugene, OR), SD-trp and other growth media were purchased from Bio101 (Carlsbad, CA) and Difco (Detroit, MI), respectively.

### *Cloning of CALB*

The mature lipase B gene was cloned from *C.antarctica* ATCC 32657 by PCR. Forward (5′-AGTA*GAATTC*CTACCTTC-CGGTTCGGAC-3′) and reverse (5′-AGTAC*GTCGAC*TC-AGGGGGTGAACGATGCC-3′) primers were derived from the beginning and the end of lipase B gene from *C.antarctica* LF 058, respectively (Uppenberg *et al*., 1994a). Restriction enzyme recognition sites, *Eco*RI in forward primer and *Sal*I in reverse primer, are shown as italics. Total DNA from *C.antarctica* ATCC 32657 was isolated by using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA). PCR was conducted on a PE Applied Biosystems 9700 thermal cycler (Foster City, CA) under the following conditions: 95°C for 1 min followed by 25 cycles of (95°C for 45 s, 55°C for 45 s, 72°C for 120 s), and finally 72°C for 10 min. The reaction mixture (100 µl) contained 2 µl of Pfu polymerase (2.5 units/µl; Stratagene, La Jolla, CA), 250 ng of each primer, 348 ng of genomic DNA of *C.antarctica* ATCC 32657, 0.2 mM dNTP mix and 10 µl of 10× Pfu buffer. The PCR-amplified fragment was purified by a Qiagen QIAqucik PCR purification kit and was cloned into the *Eco*RI/*Sal*I site of YEpFLAG-1 vector (Sigma, St Louis, MO). The recombinant plasmid was designated pWS52.

### *Gene mutagenesis and library construction*

Error-prone PCR mutagenesis was carried out using a Diversify™ PCR random mutagenesis kit from Clontech

(Palo Alto, CA). The same set of PCR primers was used for CALB gene cloning and mutagenesis. Plasmid pWS52 and 23G5 mutant gene were used as templates for the first and second generation mutagenesis, respectively.

PCR-mutated genes and vector YEpFLAG-1 were digested with *Eco*RI and *Sal*I and the fragments were separated on a 0.7% agarose gel. The desired DNA bands were excised and isolated from the gel using a Freeze-Squeeze kit from Bio-Rad (Hercules, CA). The digested vector backbone and PCR-mutated CALB genes were then ligated by T4 DNA ligase (New England Biolabs, Beverly, MA). The ligated genes were used to transform *Escherichia coli* ElectroMAX DH10B competent cells from Invitrogen Life Technology (Carlsbad, CA), and plated on LB agar plate containing 100 μg/ml ampicillin (Sigma). Transformants of *E.coli* were pooled and the plasmid DNA was isolated using a plasmid DNA Maxi preparation kit from Bio-Rad. The plasmid DNA library was then transformed into *Saccharomyces cerevisiae* BJ3505 according to protocols provided in the Yeast Maker Kit from Clontech. The BJ3505 yeast transformants were used for expressing CALB mutants.

Site-directed mutagenesis and saturation mutagenesis at amino acid positions 210, 221 and 281 of CALB were conducted by PCR with appropriate primers or by using the QuikChange™ site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

*Cell culture and enzyme expression*

*Saccharomyces cerevisiae* transformants were inoculated into 96-well deep plates (Corning Inc., Acton, MA) containing 0.6 ml of SD-trp selective medium and grown at 30°C for 2 days on an orbital shaker (model ISF-4-V; Adolf Kuhner, Switzerland) at 200 r.p.m. The seed (0.1 ml) was transferred into 0.5 ml of fresh expression medium (1% yeast extract, 8% peptone, 1% glucose, 3% glycerol and 20 mM $CaCl_2$) and grown at 20°C for 7 days at 200 r.p.m. The supernatant containing the enzyme was obtained by removing the cells by centrifugation at 2500 *g* (Eppendorf 5810R) for 10 min.

*Enzyme screening*

Culture supernatants (20 μl) containing the CALB enzyme were transferred from expression plates into 96-well assay plates (Corning Inc., Corning, NY), followed by the addition of 0.15 ml of 50 mM potassium phosphate (in the case of DiFMU) or Tris–HCl (in the case of *p*-NB) buffer, pH 7.0 into each well. One set of plates was kept at room temperature while the duplicate set was sealed with an aluminum sealing tape (USA Scientific, Inc., Ocala, FL) and heated at 60 (for G1) or 70°C (for G2) for 1 h. The actual pH of the phosphate buffer at 60°C and Tris buffer at 70°C was 7.0 and 6.5, respectively. Following incubation, the heated plates were placed on ice for 15 min and equilibrated at room temperature for an additional 15 min. The first generation library was assayed with DiFMU as substrate. The reactions were initiated by introducing 30 μl of 0.8 M DiFMU solution in acetonitrile. The fluorescent response was measured every 20 s for a duration of 2 min at room temperature (Perkin-Elmer HTS7000 Bioassay plate reader; 360/465 nm excitation/emission). To maximize the signal-to-noise ratio the second generation mutant library was screened using a colorimetric assay with *p*-NB. The reaction was initiated by the addition of the enzyme supernatant (10 μl) into 50 mM Tris–HCl buffer, pH 7.0 (160 μl) and 12 mM *p*-NB substrate in DMSO (30 μl). The residual activity (RA) was determined by measuring the change in absorbance at 405 nm for 1 min at room temperature with a 96-well plate reader (Model 384 plus; Molecular Devices, Sunnyvale, CA). The percentage of RA for mutants was calculated by dividing the activity after heat inactivation by the initial activity multiplied by 100. The standard deviation (SD) of RA calculated for every screen using 40 randomly picked WT-CALB clones and expressed as a percentage of the mean was $\leqslant$30%. The mutants with $RA_m > RA_{wt} + 2.5$ SD were selected for further confirmation. They were re-grown on a 50 ml scale and assayed at various dilutions in duplicate.

*Nucleotide sequencing and analysis*

Plasmid DNA from *S.cerevisiae* and *E.coli* were isolated using the Bio101 RPM yeast plasmid isolation kit and Bio-Rad Quantum isolation kit, respectively. DNA sequencing reactions were performed with the ABI Prism BigDye™ v3.0 reaction kit according to the manufacturer's instructions. Sequencing was conducted using an automatic capillary-sequencer (3700 DNA Analyzer; ABI Prism, Foster City, CA). Sequence analysis was conducted using Lasergene software (DNAStar Inc., Madison, WI).

*Enzyme purification and protein assay*

Lipase B was purified using agarose-bound Anti-Flag$^R$ M1 monoclonal antibodies (Sigma) on a BioCAD SPRINT perfusion chromatography system (Applied Biosystems). The enzyme solution was applied to a column containing 10 ml of Anti-Flag$^R$ M1 affinity resin equilibrated in 50 mM Tris–HCl pH 7.4, 150 mM NaCl and 2 mM $CaCl_2$. Enzyme fractions were eluted with 100 mM glycine–HCl, pH 3.0 at 1 ml/min. Following the elution, the fractions containing CALB were adjusted to pH 7.0 with 1 M Tris–HCl buffer, pH 8.0.

The enzyme concentration was determined using protein assay reagents from Bio-Rad with bovine serum albumin (BSA) as a standard.

*Determination of kinetic constants*

The initial rates of *p*-NB hydrolysis were determined at room temperature by following the formation of *p*-nitrophenol at $A_{405}$ ($\varepsilon$18 200 $M^{-1}$ $cm^{-1}$) in a Shimadzu spectrophotometer (model 2401PC UV-VIS; Columbia, MA). The reaction was initiated by adding 50 μl of the purified CALB enzyme solution to the reaction mixture containing 950 μl of 50 mM Tris–HCl, pH 7.0, 1% DMSO and varying the concentrations of *p*-NB. The initial rates of DiFMU hydrolysis at room temperature were determined by following the formation of fluorescent 6,8-difluorescent-7-hydroxy-4-methylcoumarin on a Perkin-Elmer plate reader (model HTS7000; Beaconsfield, UK). A CALB solution (20 μl) was added to the reaction mixture containing 180 μl of 50 mM potassium phosphate buffer, pH 7.0, 5% acetonitrile and varying concentrations of DiFMU. To determine $k_{cat}$ and $K_M$, initial rates were determined at five different substrate concentrations over the range 0.1–1.6 mM for *p*-NB and 1.9–30 μM for DiFMU. For each initial rate, the average of three (for *p*-NB) and five (for DiFMU) measurements was used (SD was determined to be <10%). The kinetic constants were calculated by fitting the initial rate data into the Michaelis–Menten equation using the GraFit software (version 5; Erithacus Software Ltd, Horley, UK).

*Thermal denaturation of purified CALB*

Approximately 0.8 ml of each purified enzyme preparation (0.06 mg/ml protein in 50 mM Tris–HCl, pH 7.0) was placed into a 1.6 ml microcentrifuge tube and incubated at 70°C in a water bath. Periodically, 0.1 ml aliquots were removed, placed

at room temperature for 1 h and then stored at –20°C for further analysis.

The protein aggregation at elevated temperature was determined by heating the purified enzyme preparations (0.1 mg/ml) under the above conditions followed by removal of protein aggregates by centrifugation at $14\times10^3$ *g* for 10 min. The concentration of the remaining soluble protein was determined with Bio-Rad protein assay reagent as described earlier in the text.

*Circular dichroism studies*

The far-UV circular dichroism (CD) spectra of WT-CALB and mutants were determined in a JASCO 810 spectropolarimeter with protein concentrations between 0.15 and 0.2 mg/ml in 50 mM Tris–HCl, pH 7.0. Spectra were recorded using a 2 nm band width, 8 s response rate, scan rate of 50 nm/min and a 0.1 nm pitch. Four accumulations were recorded and averaged for each spectrum using a 0.1 cm path length cell. Temperature control was performed using a Jasco Peltier-type temperature controller (model PTC-424S/L).

The temperature-dependent far-UV CD spectra in Figure 2 were obtained using a heating rate of 1°C/min from 20 to 70°C with the spectrum at each temperature recorded during the increase in temperature. The temperature-dependent unfolding profiles in Figure 3 were obtained by heating at a rate of 1°C/min from 20 to 80°C and measuring the change in ellipticity at 226 nm. The spectra of refolded samples shown in Figure 4 were obtained by heating the sample from 25 to 70°C in the cuvette at a rate of 6°C/min. The sample was then equilibrated for 10 min at 70°C and then cooled to 25°C at a rate of 9°C/min. The spectrum of the refolded sample was recorded after a 10 min equilibration at 25°C.

## Results and discussion

*Sequence analysis and expression of CALB in S.cerevisiae*

*Aspergillus* and *Pichia* were successfully used for expressing active CALB (Hoegh *et al*., 1995; Rotticci-Mulder *et al*., 2001). Both systems randomly integrated the lipase gene into the genomic DNA resulting in an undesirable clone to clone variation of protein expression. To circumvent this problem and to develop an expression system suitable for high-throughput screening we cloned CALB from strain ATCC 32657 into an *S.cerevisiae* Yeast FLAG expression system. The expression vector, YEpFLAG-1, contained a yeast alcohol dehydrogenase promoter, ADH2, regulated by glucose repression, and an α-factor leader sequence followed by an eight amino acid FLAG peptide. The presence of FLAG peptide had no effect on α-factor cleavage.

The amino acid sequence of the cloned CALB from ATCC 32657 was 99.4% identical to the lipase B from LF 058 (Uppenberg *et al*., 1994a) with only two differences, Thr57Ala and Ala89Thr. The N-terminus of CALB described in this study also contained an eight amino acid-long FLAG peptide followed by a Glu–Phe sequence which was derived from the *Eco*RI cloning site. The expression of active CALB was temperature dependent with maximum activity (~40 μmol of tributyrin hydrolyzed/min/ml of growth culture) obtained at 20°C. The activity level of WT-CALB expressed at 25 and 30°C was approximately half of that obtained at 20°C. Consequently, experiments described in this study were conducted using the lipase expressed at 20°C. No significant differences in the expression levels of the WT-CALB and the mutants were observed.

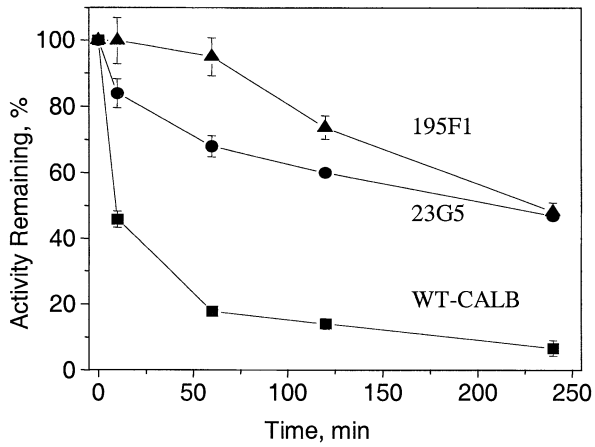


**Fig. 1.** The thermal inactivation profiles of WT-CALB and the mutants at 70°C (for experimental details see Materials and methods). The values are averages of three replicates with SD bars indicated.

*Screening of mutant libraries*

Two rounds of error-prone PCR mutagenesis were used for generating libraries of CALB mutants. The first generation (G1) mutant library was constructed using the WT-CALB gene as template. The mutation conditions were selected to achieve a nucleotide mutational rate of ~0.15% (determined by sequencing 12 randomly selected clones) resulting in a library consisting of ~80% active mutants. The selection process consisted of incubating the mutant libraries for 1 h at an elevated temperature as described in Materials and methods, and then assaying for the RA at 20°C. It should be emphasized that this approach does not directly select for mutants that are active at elevated temperatures and consequently have higher $T_m$, but rather identifies variants with increased propensity for refolding (as compared with the wild-type enzyme) or/and those that are able to retain their native structure at the elevated temperature. After screening 10 000 colonies from the G1 library, only one mutant (23G5) was selected that satisfied both established selection criteria ($RA_{mutant} > RA_{parent} + 2.5$ SD and the activity prior to inactivation was at least 75% that of the parent). The sequence analysis of 23G5 revealed two amino acid substitutions, V210I and A281E. The gene encoding 23G5 was used as a template for generating the second generation (G2) error-prone PCR library. The nucleotide mutational rate of this library was made similar to that of the G1. Eight thousand four hundred colonies of the G2 library were screened, revealing a highly active mutant, 195F1, with RA exceeding that of the 23G5 by at least 2.5 SD. The new mutant acquired three mutations compared with the WT-CALB: it retained both amino acid substitutions of its parent, V210I and A281E, and acquired one additional mutation, V221D. In an attempt to improve the thermal stability even further, three additional saturation mutagenesis libraries (G3) were constructed by utilizing the 195F1 gene as a template and substituting each of the three mutated sites with each of the 19 remaining amino acids. The screening of 1800 colonies (600 from each of the three G3 libraries) revealed no new mutants that satisfied the selection criteria.

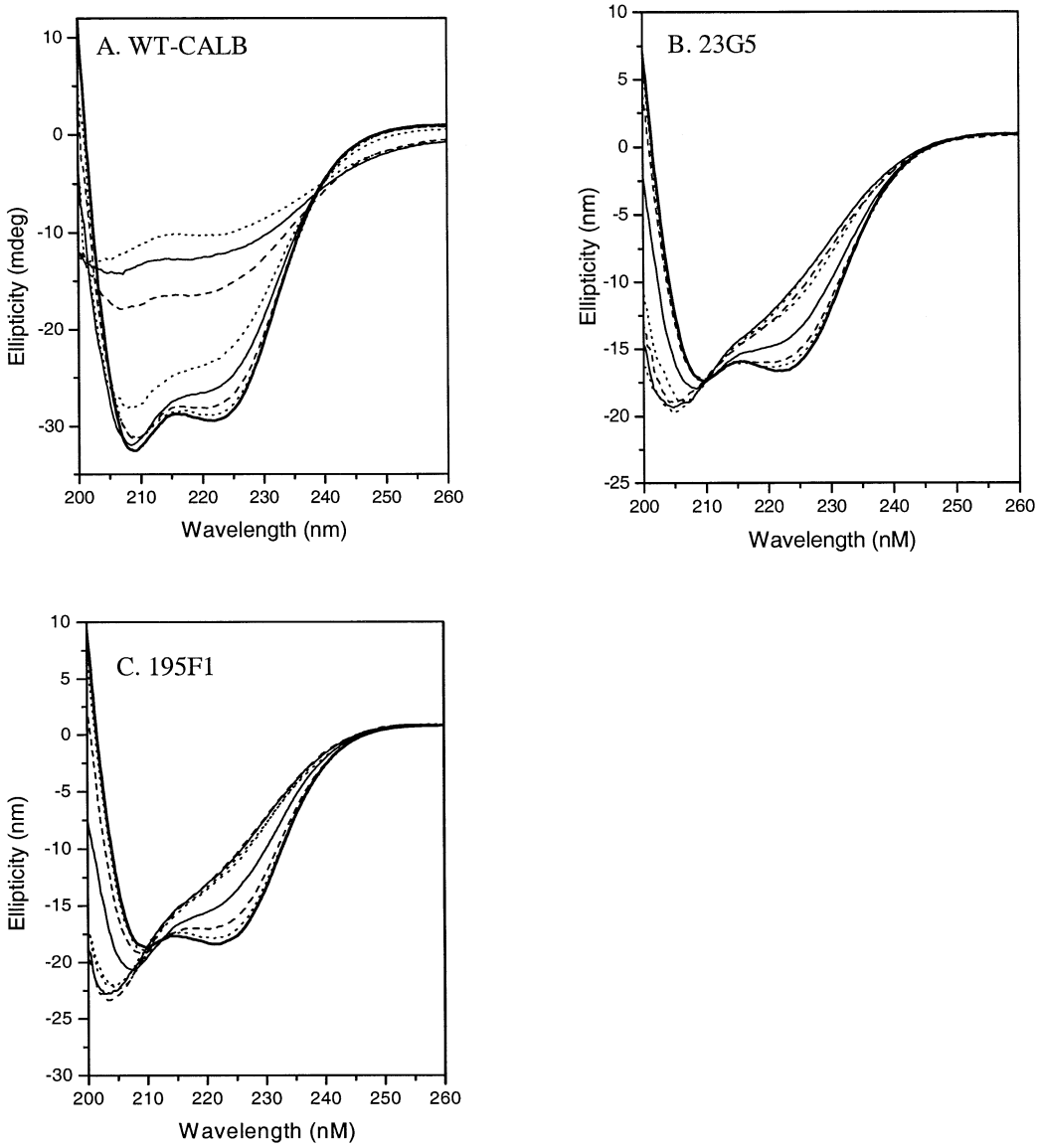


**Fig. 2.** Temperature-dependent far-UV CD spectra of WT-CALB and the mutants. The spectra represented by thick solid lines in each figure (having the highest absolute ellipticity value at 230 nm) represent the initial scan recorded at 25°C. For each protein the absolute value of ellipticity at 230 nm decreases with increasing temperature. (**A**) WT-CALB at 25, 44, 48, 52, 56, 60, 64 and 70°C. (**B**) Mutant 23G5 at 25, 40, 44, 48, 52, 56, 60 and 70°C. (**C**) Mutant 195F1 at 25, 40, 44, 48, 52, 56, 60 and 70°C.

*Thermal stability of 23G5 and 195F1 CALB mutants*

*Inactivation profiles.* Irreversible thermal inactivation of WT-CALB and the two mutants was characterized by incubating each enzyme in an aqueous buffer at 70°C followed by cooling and assessing the RA at room temperature. The inactivation profiles of the WT-CALB, 23G5 and 195F1 mutants are presented in Figure 1. The inactivation of all three recombinant enzymes followed neither first- nor second-order kinetics indicating the contribution of multiple factors to the inactivation process. As illustrated in Figure 1, stability of both mutants against irreversible inactivation was significantly higher than that of the WT-CALB. The approximated $T_{1/2}$ at 70°C of the WT-CALB, 23G5 and 195F1 mutants were 8, 211 and 232 min, respectively (Table I).

*Circular dichroism studies.* The CD spectra for WT-CALB, 23G5 and 195F1 (Figure 2A–C) measured at 25°C show minima at 208 and 222 nm. This profile is consistent with an α-helical protein which, according to the crystal structure of WT-CALB, contains 34% helical structure (Uppenberg *et al.*, 1994a). The thermal unfolding of each protein was evaluated by measuring the temperature-dependent CD response at 226 nm from 20 to 80°C. The midpoint of unfolding, $T_m$, was determined by fitting the ellipticity versus temperature transition to a two-state unfolding reaction mechanism (Figure 3). The $T_m$ for WT-CALB, 23G5 and 195F1 mutants was 57.6, 52.1 and 50.8°C, respectively (Table I). The lower $T_m$ value for the mutants compared with WT-CALB was somewhat unexpected, but not very surprising considering that the selection process was based on an assay carried out at 20°C.

Additional studies evaluating the temperature-dependent spectra for each protein at eight discrete temperatures provided an explanation for these findings (Figure 2). The spectra for WT-CALB recorded at several temperatures using a temperature ramp of 1°C/min (Figure 2A) indicated that the protein undergoes a large, 50%, spectral change by the time the

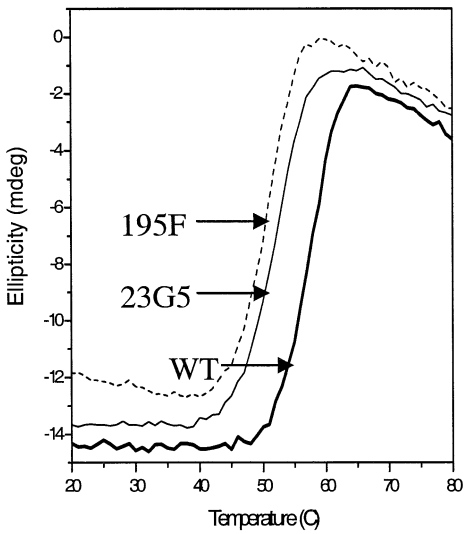


**Fig. 3.** Unfolding profiles of WT-CALB, 23G5 and 195F1. The samples were heated at 1°C/min from 20 to 80°C and the ellipticity recorded at 226 nm. The $T_m$ = 57.7 (WT-CALB), 52.1 (23G5) and 50.8°C (195F1) were calculated by a non-linear least-squares fit of the transition temperatures.

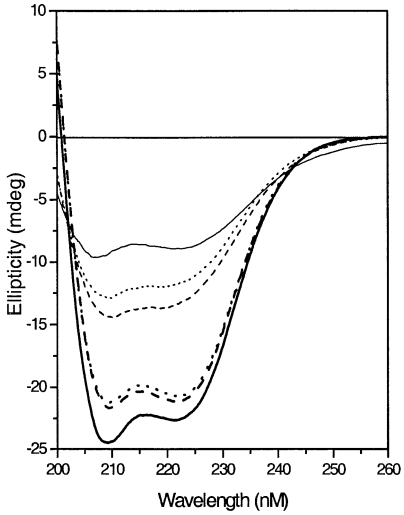


**Fig. 4.** Far-UV CD spectra of WT-CALB and the mutants before and after incubation at 70°C. (Solid line) WT-CALB, (dashed line) 23G5, (dotted line) 195F1. Thick and thin lines correspond to the spectra before and after the incubation, respectively (for experimental details see Materials and methods).

temperature reaches 52–56°C. This is consistent with the observed $T_m$ of 57.7°C (Figure 3). Analysis of the spectrum in Figure 2A indicated that the ellipticity decreased along the entire spectrum and that there was no clear isosbestic point which is a signature for a two-state (folded versus unfolded) model (Kelly and Price, 2000). The decrease in ellipticity signal was consistent with a loss of protein due to temperature-induced protein precipitation. Inspection of the cuvette following heating to 70°C indicated that the sample contained a large amount of precipitate. The quantitative analysis of the soluble protein (see Materials and methods) indicated that <50% of WT-CALB remained in solution following a 10 min incubation at 70°C versus 70 and 95% remaining in solution for 23G5 and 195F1, respectively.

On the contrary, the temperature-dependent spectral profiles for the mutant proteins (Figure 2B and C) showed a classical isosbestic point near ~211 nm which suggested protein unfolding of the mutants involved a two-state mechanism and was reversible. In fact, the samples were clear in the cuvette at 70°C, consistent with the fact that the samples did not undergo temperature-dependent precipitation like WT-CALB. Based on the temperature-dependent spectra (Figure 2B and C) mutants 23G5 and 195F1 appear to have $T_m$ values between 48 and 52°C. These values correlate with that observed in Figure 3 (52.1 and 50.8°C, Table I). The observation that the mutant proteins unfolded reversibly suggested that the improved activity of the lipase mutants following heating to 70°C was mainly due to a higher recovery of active protein compared with the wild-type protein which underwent an irreversible denaturation at elevated temperatures to a much higher degree.

To further characterize the folding–refolding profile for each enzyme the CD spectra were determined for samples equilibrated at 20°C (native) and compared with that of samples equilibrated at 70°C for 10 min (unfolded) followed by cooling to 20°C (refolded). The spectra for each protein at 20°C (native) and the ones following the refolding are shown in Figure 4. The refolded WT-CALB sample, cooled to 20°C after being heated to 70°C, had an ellipticity value that was only 40% at 226 nm compared with the 20°C native control. Conversely, the refolded ellipticity values for 23G5 and 195F1 were ~61 and 55%, respectively, compared with their 20°C native controls. These results agree with the previously described temperature-dependent CD data which suggested that the mutant proteins retained a higher percentage of activity following heating to 70°C compared with the WT-CALB because the mutations introduced in 23G5 (V210I, A281E) and 195F1 (V210I, A281E, V221D) facilitated protein refolding perhaps by inhibiting protein aggregation in the unfolded state. These CD results also agree with the protein-recovery experiments where WT-CALB produced a larger amount of insoluble protein compared with the 23G5 mutant (>50 versus <20%).

So why do the amino acid substitutions in 23G5 and 195F1 mutants have such a profound effect on the protein aggregation? It is well accepted that protein aggregation at elevated temperatures is attributed to favorable interactions between hydrophobic regions of partly unfolded protein molecules (De Bernardez Clark, 1998; Broglia and Tiana, 2001). The onset of aggregation occurs at prolonged sequences of hydrophobic amino acid residues, so-called nucleation sites. The amino acid sequence analysis revealed that the position 281 was in the middle of a long hydrophobic amino acid sequence

**Table I.** The $T_{1/2}$ and $T_m$ of WT-CALB and some mutants

| Lipase | Amino acid changes | $T_{1/2}$ (min)[a] | $T_m$ (°C)[b] |
|---|---|---|---|
| WT-CALB | – | 8 | 57.7 |
| 23G5 | V210I, A281E | 211 | 52.1 |
| 195F1 | V210I, A281E, V221D | 232 | 50.8 |
| Sdm210 | V210I | 8 | 58.5 |
| Sdm221 | V221D | 33 | 55.7 |
| Sdm281 | A281E | 180 | 50.9 |

[a]$T_{1/2}$ values are based on the inactivation profiles depicted in Figure 1.
[b]$T_m$ values are based on the CD spectra depicted in Figure 3.

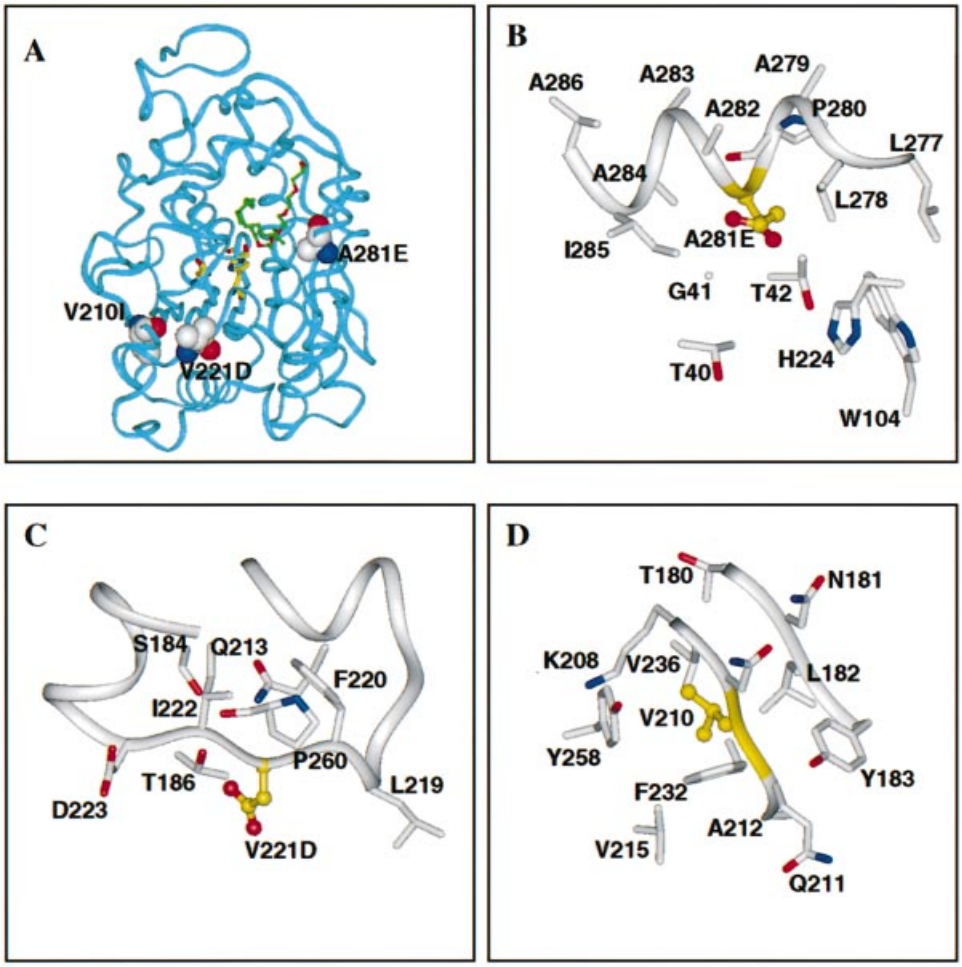


**Fig. 5.** Structure modeling based on the published crystal structure of CALB by Uppenberg *et al.* (Uppenberg *et al*., 1994b). (**A**) The global structure of CALB shown in CPK with the carbon atoms in yellow, oxygen atoms in red and nitrogen atoms in blue. The catalytic triad and the ligand are in stick with carbon atoms colored in green. (**B**–**D**) The local environment of the three single amino acid mutations, A281E, V221D and V210I, respectively; each mutation is marked in ball and stick with carbon atoms colored in yellow.

**Table II.** Kinetic characterization of WT-CALB and the mutants

| Lipase | Specific activity (nmol/min/μg protein) | | $K_m$ (μM) | | $k_{cat}$ ($min^{-1}$) | | $k_{cat}/K_m$ ($min^{-1}$ $\mu M^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | DiFMU | *p*-NB | DiFMU | *p*-NB | DiFMU | *p*-NB | DiFMU | *p*-NB |
| WT-CALB | 2.4 | 21 | 5.7 | 170 | 84 | 730 | 15 | 4.3 |
| 23G5 | 54 | 86 | 13 | 150 | 1900 | 2900 | 150 | 19 |
| 195F1 | 10 | 72 | 7.5 | 180 | 360 | 2500 | 48 | 14 |

($V_{272}$AAAALLAP<u>A</u>AAAIV$_{286}$). The A281E substitution in the 23G5 mutant appears to disrupt the hydrophobic stretch which may in turn inhibit the nucleation site responsible for inducing protein precipitation. Similarly, the V221D substitution (195F1 mutant) decreases the hydrophobicity of the sequence $P_{218}$LF<u>V</u>IDH$_{224}$, although to a lesser extent than that seen at the 281 position. In contrast, position 210 is in the middle of a rather polar $G_{207}$KN<u>V</u>QAQ$_{213}$ sequence. Since its hydrophobicity is not altered significantly by the conservative V210I substitution, the effect of mutation on stability of the mutant was expected to be minimal.

To confirm the above hypothesis, the effect of a single amino acid substitution on thermal stability of CALB was investigated. The three CALB mutants with a single amino acid substitution were constructed using site-directed mutagenesis and their thermal inactivation at 70°C was analyzed (Table I). As expected, the A281E mutation had the most significant effect on stability increasing $T_{1/2}$ 22-fold compared with the WT-CALB. A single V221D mutation had a more moderate effect on stability (a 4-fold improvement). Consistent with these findings was the fact that the two single-site mutants showed a lower tendency to aggregate at elevated temperature

than the WT-CALB (0, 32 and 52% of protein aggregated for A281E, V221D and WT-CALB, respectively, after incubation at 70°C for 10 min). As expected, the stability of the V210I mutant was similar to the wild-type enzyme.

Both the A281E and V221D mutations decreased the $T_m$ value of the lipase. The decrease in stability may be due to mutation-induced structural perturbations. Analysis of a three-dimensional model of CALB (Figure 5A) based on the crystal structure (Uppenberg *et al*., 1994b) clearly shows that residue A281 is a part of an α-helix (α10) located at the top of the substrate-binding pocket in a highly hydrophobic environment (Figure 5B illustrates the residues within a 5 Å sphere with A281 at the center). The Glu side chain of A281E projects into the binding pocket. Due to a close contact with the neighboring hydrophobic residues, the carboxylate would have to be partially dehydrated which may destabilize the native conformation. Alternatively, the hydrophilic property of the side chain may induce a conformational change to facilitate solvent exposure of the carboxylic acid. This argument was supported experimentally when the $T_m$ of the single A281E mutant was determined and found to be 7°C lower than that of the WT-CALB (Table I). Residue 221 is located on an extended strand on the surface of the protein with the Val side chain (or Asp of the mutant) exposed to the solvent (Figure 5C). Since the environment of the solvent-exposed residue is not likely to change upon unfolding, the substitution of Val for Asp should have only a small effect on $T_m$. This result is consistent with the experimental results (56°C for the mutant versus 58°C for the WT-CALB, Table I). Finally, if one assumes that the V210I mutant has the same side chain conformation as WT-CALB, i.e. projecting into the largely hydrophobic interior of the protein, the extra methyl group of Ile must contact the OH group of Y258 (Figure 5D). It would appear that the extra hydrophobicity of Ile over Val realized upon unfolding is counterbalanced by an unfavorable contact with the polar OH group of the Tyr (in the folded state) resulting in a minimal change in $T_m$ which is also consistent with the experimental data.

*Kinetic characterization.* Both *p*-NB and DiFMU were used as substrates to screen the lipase libraries and to characterize the selected mutants. The specific activity and kinetic constants of the mutants and WT-CALB are presented in Table II. It is apparent that the 23G5 mutant had the highest specific activity demonstrating a 21- and 3-fold increase with DiFMU and *p*-NB, respectively, over the WT-CALB. The specific activity of the 195F1 mutant was also significantly higher than that of the WT-CALB (see Table II). While the $K_m$ value of the mutants for *p*-NB was similar to that of the WT-CALB, the $K_m$ for DiFMU increased for both mutants (Table II). Based on the X-ray crystal structure of the WT-CALB, it appears that of the three mutated amino acid residues, only one, A281, was located in the proximity of the enzyme's active site. Docking calculations performed with the GOLD 1.2 program (CCDC, Cambridge, UK) (Jones *et al*., 1997) for DiFMU and *p*-NB revealed that while the smaller substrate, *p*-NB, fits tightly into the active site of the WT-CALB and both mutant enzymes, the significantly larger substrate, DiFMU, did not bind nearly as well due to steric hindrance interacting with the Glu side chain of the A281E mutant. This structural observation was consistent with the observed increase in $K_m$ value for DiFMU of the A281E mutant. Surprisingly, this interaction did not have a detrimental effect on the activity; in fact the catalytic efficiency of the mutants expressed as $k_{cat}/K_m$ for both substrates exceeded that of the wild-type enzyme.

In summary, two mutants with a significantly higher activity and resistance towards irreversible thermal inactivation were created via error-prone PCR mutagenesis. The improved half-life of the mutants at 70°C was brought about by a cumulative effect of A281E and V221D mutations that inhibited protein aggregation and facilitated refolding of the catalytically active conformation of the protein.

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