THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1993 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 268, No. 35, Issue of December 15, pp. 26212-26219, 1993
Printed in U.S.A.

Cloning and Expression of *Geotrichum candidum* Lipase II Gene in Yeast

PROBING OF THE ENZYME ACTIVE SITE BY SITE-DIRECTED MUTAGENESIS*

(Received for publication, May 6, 1993, and in revised form, July 15, 1993)

Thierry Vernet‡, Edmund Ziomek, Achim Recktenwald, Joseph D. Schrag, Chantal de Montigny, Daniel C. Tessier, David Y. Thomas, and Miroslaw Cygler‡

From the Biotechnology Research Institute, National Research Council of Canada, 6100 Avenue Royalmount, Montréal, Québec H4P 2R2, Canada

The three-dimensional structure of lipase II of *Geotrichum candidum* strain ATCC34614 (GCL II) has provided insights with respect to the nature of the catalytic machinery of lipases. To support these structural observations, we have carried out an analysis of GCL II by mutagenesis. The gene encoding lipase II of *Geotrichum candidum* strain ATCC34614 (GCL II) was amplified using the polymerase chain reaction, cloned, and sequenced. The intronless lipase gene was expressed and secreted from *Saccharomyces cerevisiae* at approximately 5 mg/liter of culture. Recombinant GCL II was purified by immunoaffinity chromatography and characterized using a combination of substrates and independent analytical methods. The recombinant enzyme and the enzyme isolated from its natural source have comparable specific activities against triolein of about 1000 μmol of oleic acid released/min/mg of protein. The putative catalytic triad Ser^{217}-His^{463}-Glu^{354} was probed by site-directed mutagenesis. The substitution of Ser^{217} by either Cys or Thr and of His^{463} by Ala led to a complete elimination of the activity against both triolein and tributyrin. Substitution of Glu^{354} by either Ser, Ala or Gln renders the enzyme inactive and also perturbs the enzyme stability. However, the enzyme with the conservative replacement Glu^{354}Asp is stable and displays only a small decrease of triolein activity but a 10-fold decrease in activity against tributyrin. There was no appreciable difference in esterase activity between the native, recombinant wild type, and Glu^{354}Asp mutant. These results confirm that the triad formed by Ser^{217}-Glu^{354}-His^{463} is essential for catalytic activity. They also show that the active site of GCL II is more tolerant to a conservative change of the carboxylic side chain within the triad than are other hydrolases with similar catalytic triads.

Lipases are triacylglycerol hydrolases (EC 3.1.1.3) whose substrates are insoluble lipids. These enzymes act at the water-lipid interface and show rather low activity toward small, water-soluble esters. The hydrolytic activity of lipases is greatly enhanced by the presence of an interface, a phenomenon known as the interfacial activation (Desnuelle, 1972). This activation was long ago proposed to be caused by a conformational change (rearrangement) in the enzyme and that notion was confirmed when the first 3-D[1] structures of lipases were elucidated (Winkler *et al.*, 1990; Brady *et al.*, 1990; Schrag *et al.*, 1991). They showed that in the absence of the interface the active site is covered by parts of the polypeptide chain and is not accessible for the substrate. Rearrangement of some loops has to occur to open the access to the active site (Brzozowski *et al.*, 1991; Grochulski *et al.*, 1993).

The reaction mechanism of lipases has been suspected to be similar to that of serine proteases (Chapus and Sémériva, 1976), and the active site was thought to be a Ser-His-Asp catalytic triad. The 3-D structure of all lipases supported this notion as all three lipases which have been crystallized contain such a catalytic triad. Although the presence of Asp has been observed in the catalytic triad of HPL and Rhizomucor mieher lipase, the structure of GCL II revealed a Ser-His-Glu triad arrangement (Schrag *et al.*, 1991, 1993). This amino acid triad is common to a large family of esterases and lipases (Cygler *et al.*, 1993). The other members of this family for which the 3-D structure has been determined are AChE (Sussman *et al.*, 1991) and very recently *Candida cylindracea* lipase (Grochulski *et al.*, 1993). The 3-D structures confirmed the presence of the Ser-His-Glu configuration. Mutations of AChEs and butyrylcholinesterases from various organisms have been studied extensively (Gibney *et al.*, 1990; Neville *et al.*, 1992a, 1992b; Shafferman *et al.*, 1992; Fournier *et al.*, 1992) confirming the assignment of the active site and probing the substrate binding gorge. The mutation of the active site Ser and Glu in human AChE resulted in a total loss of activity toward acetylcholine (Schafferman *et al.*, 1992). A conservative replacement of Glu^{334} by Asp also led to a loss of biological activity, indicating the fine tuning of this enzyme structure for the glutamate containing triad. This is of special interest since some of the enzymes of this homologous family, notably cholesterol esterases, were predicted to have the traditional Ser-His-Asp catalytic triad (Cygler *et al.*, 1993). The identification by site-directed mutagenesis of Ser^{194} (DiPersio *et al.*,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was issued as National Research Council of Canada publication No. 36152.

‡ To whom correspondence should be addressed: Biotechnology Research Institute, National Research Council of Canada, 6100 Ave. Royalmount, Montréal, Québec, H4P 2R2, Canada. Tel.: 514-496-6154; Fax: 514-496-6213; e-mail: Vernet @BRI.NRC.CA.

[1] The abbreviations used are: 3-D, three-dimensional; AChE, acetylcholinesterase, DO, diolein; GCL, *Geotrichum candidum* lipase; HL, hepatic lipase; HPL, human pancreatic lipase; MO, monoolein; PNPA, *p*-nitrophenyl-acetate; PNPB, *p*-nitrophenyl-butyrate; OA, oleic acid; oligo, oligodeoxyribonucleotide; TB, tributyrin; TO, triolein; WT, wild type; MES, 4-morpholinepropanesulfonic acid; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.

1990), His^{435} (DiPersio *et al.*, 1991), and Asp^{320} (DiPersio and Hui, 1993) as catalytically important in rat pancreatic cholesterol esterase leaves no doubt as to the identity of the triad. Interestingly, the $Asp^{320}Glu$ mutant of cholesterol esterase retained approximately 70% of the WT activity toward PNPB.

Our recent determination of the 3-D structure of the lipase II from *Geotrichum candidum* (Schrag *et al.*, 1991, 1993) offers further insight into the nature of the catalytic machinery of lipases. This lipase is secreted from the fungus together with other lipase isoenzymes and has a high specific activity toward triglycerides with a *cis*-Δ9-octadecanoic acyl residue (Jensen *et al.* 1965; Baillargeon and McCarthy, 1991; Sidebottom *et al.*, 1991). Two cDNAs encoding lipase I (GCL I) (Shimada *et al.*, 1989) and lipase II (GCL II) (Shimada *et al.*, 1990) have been cloned from *G. candidum* ATCC 34614. The two proteins of 544 amino acids share approximately 85% amino acid sequence identity. Biochemical and enzymatic characterization of these lipases has been hampered by the difficulties in purifying these isoenzymes which have similar physical and biochemical properties. To overcome this problem and to allow us to manipulate the structure of the enzyme, we have cloned and expressed the gene encoding GCL II in yeast. Perturbation of the enzyme structure by *in vitro* site-directed mutagenesis was used to confirm the assignment of the active site triad.

EXPERIMENTAL PROCEDURES

Yeast and E. coli Strains—*Saccharomyces cerevisiae* strains BJ3501 (MATα, pep4::HIS3 prb1-Δ1.6R his3-Δ200 ura3-52 can1 gal2) (Jones, 1991) and YE410 (MATα, his4 ura3-52 leu2 mnn9) (generously provided by J. Ernst) were used in this study. *Escherichia coli* strains CJ236 (Bio-Rad) and MC1061 (Casadaban and Cohen, 1980) were used for the production of single-stranded DNA and for the selection of mutated plasmids, respectively.

Lipase Gene Cloning, Construction of Mutants, and DNA Sequencing—The amplification of the gene encoding GCL II was achieved by polymerase chain reaction using primers designed from the 5′ (5′-TTCTTAGCCGCTGCCGTAAA-3′) and 3′ (5′-TTAACCGTAGAGATTAACGTC-3′) coding regions of the gene. The 3′ primer includes the complement of a stop codon (underlined). The 1.6-kilobase pair DNA fragment was purified from an agarose gel and cloned into YpDC222 (Vernet *et al.*, 1993) as follows. The cohesive ends of YpDC222 digested with *Eco*RI were converted into blunt ends by treatment with the *E. coli* DNA polymerase I Klenow fragment. A single T was added to the 5′ ends using the template-independent terminal transferase activity of the Taq polymerase. The modified vector was then ligated with the polymerase chain reaction amplified fragment, which often includes an extra A residue at the extremities (Clark, 1988). This protocol increases the ligation efficiency over blunt-end ligation about one hundred-fold (Marchuk *et al.*, 1991). A recombinant plasmid (YpDC235) containing the lipase gene in the correct orientation with respect to the α-factor promoter was isolated. This plasmid derived from pVT100-U (Vernet *et al.*, 1987) contains the phage f1 origin of replication, a feature facilitating the production of single-stranded DNA for *in vitro* site-directed mutagenesis (Zoller and Smith, 1982). After the deletion of undesired sequences within YpDC235 by site-directed mutagenesis using a uracil-containing template (Kunkel, 1985), a series of lipase mutant genes were constructed by *in vitro* site-directed mutagenesis by using both the templates and mutagenic oligo sequences described in Table I. The mutations were confirmed by restriction digests and DNA sequencing of the region surrounding the site of mutation (Sanger *et al.*, 1977).

Yeast Transformation and Culture Conditions—Yeast cells were transformed according to Ito *et al.* (1983). For enzyme production an inoculum was prepared from freshly transformed cells grown at 30 °C in selective SD-ura medium (Sherman *et al.*, 1982) to ensure a high frequency of plasmid maintenance. When cultures reached a density of approximately 7×10^6 cells/ml, the cells were collected by centrifugation for 3 min at $800 \times g$ and resuspended in 5 volumes of optimal medium (0.67% yeast nitrogen base, 8% casamino acids (Ernst, 1986), 4% glucose, 0.25% ammonium sulfate in 100 mM phosphate buffer, pH 7.0). The cultures were grown for 3–7 days, and the culture broth was separated from the cells by centrifugation at $2000 \times g$ for 15 min at 4 °C. The supernatants were stored at −20 °C or used directly for the purification of lipase.

Assay of Lipase Activity on Agar Plates—The assay was adapted from Samad *et al.* (1989) and uses agar plates containing emulsions of either TB or TO as a substrate. TB-containing plates (137 mm diameter) are composed of a 25-ml lower layer containing 1.5% agar and 0.003% phenol red, and an upper 30-ml emulsion layer prepared by vortexing vigorously for 2 min a solution of 1.5% agar, 50 mM MES, pH 7.0, cooled from boiling to 65 °C and containing 0.45% TB. GCL II standard or samples (10 μl) were applied directly onto the emulsion. A clearing zone develops within 10–48 h of incubation at 30 °C. For the direct testing of lipase secreted from yeast colonies, the upper layer included optimal medium. TO-containing plates were prepared by vortexing for 2 min 30 ml of 1.5% agar, containing 0.005% Victoria Blue B which had been cooled from boiling to 65 °C and then 0.25% of TO were added. Acid released by the hydrolysis of TO is revealed by the color indicator after 2–24 h of incubation at 30 °C. Standards of purified GCL II were diluted into the culture medium of control strain BJ3501(YpDC219) (where ( ) designates the plasmid-carrier state) grown as described above.

A minimum of two measurements of the clearing or colored zone diameter were averaged. The log of the concentration of GCL II is directly related to the diameter of the TB clearing zone for concentrations ranging from 5 to 100 μg/ml with a detection limit of 2 μg/ml (2 units/ml), and to the diameter of the colored zone for the TO assay for concentrations ranging from 2 to 100 μg/ml with a detection limit of 1 μg/ml (1 unit/ml).

HPLC-based Assay of Lipase Activity—This assay is based upon the analysis of the TO hydrolysis products by HPLC (Ergan and André, 1989). Samples of 5–200 μl of enzyme solution were added to the reaction mixture containing 0.75 ml of TO dispersed in 10 ml of 100 mM sodium phosphate buffer, pH 7.0. The reaction mixture was placed in a 20-ml scintillation vial, incubated at room temperature, and stirred at 700 revolutions/min using a 12-mm magnetic stirring bar. Samples of 40 μl were withdrawn after 30, 60, 120, 180, 240, 360, 480, and 600 min, diluted with 800 μl of acetone, filtered through a 0.4-μm filter (HV Millipore), and analyzed by HPLC (Waters Millipore, San Francisco, CA). The reaction products were resolved on a C_{18} silica column equilibrated with acetone/acetonitrile (1:1) at 30 °C. To compensate for inaccuracies resulting from sampling of the emulsion, raw HPLC data were normalized as described by Ergan and André (1989). One unit of enzyme activity is defined as the quantity of enzyme necessary to produce 1 μmol of OA/min at 20 °C. The activity was calculated on the basis of the initial rate of the reaction using the linear portion of the reaction curve.

Titrimetric Assay—The specific activities of native or recombinant WT and mutants of GCL II for TO and TB were measured by titrimetry (Sidebottom *et al.*, 1991). GCL II was added to the emulsion prepared by 2 min of sonication (medium setting on Heat Systems Ultrasonics Inc. sonicator) of 0.5 ml of triglyceride, 9 ml of 2% (w/v) gum arabic (Sigma), and 0.5 ml of 0.7 M $CaCl_2$. The release of the OA was continuously monitored by titration with 50 mM NaOH on a pH-stat (RTS822 Recording Titration System, Radiometer, Copenhagen, Denmark).

Spectrophotometric Esterase Activity Assay—The esterase activity was determined spectrophotometrically using PNPA and PNPB as substrates according to Chapus *et al.* (1976).

Preparation of Immunoglobulins and Western Blot Analysis—Antibodies against native GCL II or GCL II treated with SDS and endoglycosidase H were raised in rabbits. The antibodies raised against the denatured and deglycosylated antigen were used for Western blot analysis without further purification. The IgG against native GCL II was partially purified by fractionation with ammonium sulfate and ion-exchange chromatography on DEAE-cellulose (Livingstone *et al.*, 1974). Horseradish peroxidase was conjugated with immunoglobulins (IgG) according to the protocol of Avrameas and Ternynck (1971) and the IgG-horseradish peroxidase conjugate was purified on ConA-Sepharose (Arends, 1979) before use in the ELISA assay (see below).

Recombinant GCL II was characterized by Western blot analysis. When required, protein samples were treated with endoglycosidase H (Boehringer Mannheim) according to the supplier's specifications. Proteins were transferred from 8% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970) to nitrocellulose filters and incubated with rabbit antiserum raised against denatured GCL II. GCL II-antibody complexes were visualized with alkaline phosphatase conjugated goat anti-rabbit IgGs (Bio-Rad).

Quantification of Lipase by ELISA—Purified antibodies raised against native GCL II were covalently attached to 96-well hydrazine modified polystyrene plates (AVIDPLATE Hz, BioProbe International Inc.) according to the manufacturer's instructions. Unreacted hydrazine sites were blocked with a "blocking buffer" containing 0.1% bovine serum albumin in 100 mM Tris-HCl, pH 7.4, 0.05% Tween 20. A volume of 50 μl of the GCL II solution (1–100 ng of GCL II/ml) was added as the second layer to the wells and the plate was incubated for 2 h at room temperature. The plate was then washed several times with the "blocking buffer." Each plate was incubated for 1 h at room temperature with 100 μl of 13 μg/ml IgG-horseradish peroxidase conjugate in blocking buffer as the third layer. The plate was exhaustively washed with 0.05% Tween 20 in 100 mM Tris-HCl, pH 7.4, to remove unbound conjugate. The peroxidase activity was revealed according to Ziomek *et al.* (1984), and absorbance at 405 nm was measured with a multichannel ELISA Plate Reader (Titertech). The calibration curve was prepared by plotting A_{405} against log[GCL], with a linear range (95% confidence range) of 0.05–1 ng of GCL/ml of standard solution.

Purification of Recombinant Lipase—Yeast culture supernatant was diluted with 2 volumes of deionized water and mixed for 2 h at room temperature with 500 ml of Q-Sepharose gel equilibrated with 20 mM phosphate buffer, pH 7.2. The gel was packed into a column and washed with 20 mM phosphate buffer, pH 7.2. GCL II was eluted with a 2-column volume gradient ranging from 0 to 0.5 M NaCl in the same buffer. An immunoaffinity gel was prepared by immobilizing anti-native GCL II polyclonal antibodies on cyanogen bromide-activated Sepharose 4B (Szewczuk and Prusak, 1985). Approximately 1 mg of IgG was bound per ml of gel. Q-Sepharose-purified GCL II (total 600 μg) was applied onto 4 ml of the immunoaffinity beads packed in a 1 cm diameter, 5 cm long column. The column was washed with 10 volumes of 1 M NaCl in 20 mM phosphate buffer, pH 7.0, then cooled to 4 °C prior to the elution of the lipase with 0.1 M glycine-HCl buffer, pH 2.7. Fractions containing GCL II were immediately neutralized with 1 M Tris-HCl, pH 7.4, pooled, and concentrated with a YM-5 Amicon membrane. Salts were removed by diafiltration against deionized water in the same Amicon cell. A separate affinity column was used for each mutant.

Following electrotransfer onto a PVDF membrane (Immobilon Millipore) according to the procedure described by Matsudaira (1987), amino-terminal sequencing of purified GCL II was performed with an Applied Biosystems model 470A gas-phase protein sequenator (Hewick *et al.*, 1983).

RESULTS

Cloning and Sequencing of GCL II Gene from G. candidum—Primers based on the GCL II cDNA sequence (Shimada *et al.*, 1990) were used to polymerase chain reaction amplify the complete corresponding gene from the genomic DNA of *G. candidum* ATCC 34614. A 1.6-kilobase pair DNA fragment having the size and restriction pattern expected for the GCL II gene was directly cloned into an expression vector derived from pVT100-U (Vernet *et al.*, 1987) in which the ADH promoter was replaced by the α-factor promoter. The sequence of the cloned fragment was found to be identical to the published GCL II cDNA sequence, except for a stretch of nucleotides encoding amino acid positions 4–6 (see "Discussion").

Secretion of Recombinant GCL II from Yeast—The yeast *S. cerevisiae* is a eukaryotic organism widely used for the expression of heterologous proteins (Romanos *et al.*, 1992). Our own experience in successfully expressing other hydrolases in yeast (Vernet *et al.*, 1993, Brömme *et al.*, 1993) in systems based upon the α-factor promoter and 5′ coding region (Brake, 1989) prompted us to select yeast as the host for lipase production. The absence of detectable lipase activity secreted from yeast facilitates characterization and purification of lipase mutants. The α-factor pro-region is sometimes required for efficient expression and secretion of foreign proteins (Vernet *et al.*, 1993). Consequently, two constructions for the expression of mature GCL II were prepared initially. First, plasmid YpDC240 containing the GCL II gene fused to the α-factor prepro-region was assembled (Table I and Fig. 1). In this construction, secretion of GCL II relies upon efficient cleavage at the protein junction by the dibasic specific protease Kex2 (Julius *et al.*, 1984). In a second plasmid, YpDC250, GCL II was fused directly to the α-factor signal sequence (Table I and Fig. 1).

Colonies of transformed yeast and the supernatant from liquid cultured BJ3501(YpDC240) and BJ3501(YpDC250), but not of the control BJ3501(YpDC219), produced a clearing zone on TB emulsion-containing plates (Fig. 2, *A* and *B*). The secretion of lipolytic activity was confirmed by analyzing the culture supernatants of transformed yeast grown in liquid medium. Supernatants of cells harboring either BJ3501(YpDC240) or BJ3501(YpDC250), but not BJ3501(YpDC219) gave a positive signal on plates containing emulsions of TB (Fig. 2*B*) or TO (Fig. 2*C*). The amount of lipase production, as evaluated by comparing the diameters of the clearing zones, is comparable for both plasmids.

The amount of secreted GCL II was measured more accurately by ELISA. Seven days after transfer into the "optimum medium" (see "Experimental Procedures") a maximum of 7.5 and 5.4 mg/liter of GCL II was detected in the culture medium of BJ3501(YpDC240) and BJ3501(YpDC250), respectively. This corresponds to less than 1% of the total protein present in the culture broth. Due to its slightly higher level of secreted lipase, the construction YpDC240 was used for the experiments described in this study.

Secretion of GCLII from yeast was confirmed by Western blot analysis. In *S. cerevisiae* secreted proteins are often modified by *N*-linked glycosyl structures that contain heterogeneous amounts of outer chain mannose added to an oligosacharide core (Kukuruzinska *et al.*, 1987). It is often desirable to limit the extent of mannose addition since it can affect the characteristics of the protein and interfere with purification of proteins. GCL II has two potential *N*-linked glycosylation consensus sequences (Asn^{283}-Asp-Thr and Asn^{364}-Ala-Thr) that are modified in *G. candidum*. We have compared the production of GCL II from wild type strain BJ3501 with that of YE410, a yeast host deficient in the processing steps involved in outer chain mannose extension (Tsai *et al.*, 1984). Strain YE410(YpDC240) secreted two major immunoreactive species: one 64 kDa and one larger than 150 kDa (Fig. 3, *lane 2*). The higher molecular weight species is also found in the negative control YE410(YpDC219) (Fig. 3, *lane 6*). This species disappears upon treatment with endoglycosidase H (Fig. 3, *lane 5*), whereas the size of the 64-kDa species is reduced to 60 kDa (Fig. 3, *lane 1*) and co-migrates with GCL II purified from *G. candidum* (Fig. 3, *lane 9*). BJ3501(YpDC240) also secreted the 64-kDa protein and weak immunoreactive species ranging from 64 to more than 200 kDa (Fig. 3, *lane 4*). All species are reduced to 60 kDa upon treatment with endoglycosidase H (Fig. 3, *lane 3*). No immunoreactive bands were detected in the culture medium of the control BJ3501(YpDC219), neither prior to (Fig. 3, *lanes 7*) nor following endoglycosidase H treatment (Fig. 3, *lanes 8*). Most of the GCL II appears to be secreted, since only weak immunoreactive signals were detected in yeast cell extracts harboring YpDC240 (data not shown).

Purification and Characterization of WT Recombinant GCL II—Purification of recombinant GCL II from YE410-(YpDC240) was performed by ion-exchange chromatography followed by an immunoaffinity chromatography step. Purification yields and specific activities for TO are presented in Table II. Most of the loss of enzyme occurs at the concentration step following the immunoaffinity chromatography. A typical elution profile of the immunoaffinity step is presented

TABLE I

Plasmid constructions and oligonucleotides used in this study

The abbreviation used is: bp, base pair.

Plasmid source	Oligo sequence (5′ → 3′)[a]	Mutation (diagnostic)	Modified plasmid
YpDC235	AGAGAGGCTGAAGCTCAGGCCCCCACGGCC	Fusion of α-factor prepro-region with mature lipase (deletion of 273 bp)	YpDC240
YpDC235	CCTCCGCATTAGCTCAGGCCCCCACGGCC	Fusion of α-factor pre-region with mature lipase (deletion of 483 bp)	YpDC250
YpDC240	CAGGAGGATGATGGTACCGCTTTTGCTCC	$Glu^{354}Asp$ (creates *Kpn*I)	YpDC252
YpDC240	TTTCGGTGAGTGCGCAGGTGCCATGAG	$Ser^{217}Cys$ (creates *Fsp*I)	YpDC255
YpDC240	TTTCGGTGAGACAGCTGGTGCCAT	$Ser^{217}Thr$ (creates *Puu*II)	YpDC247
YpDC240	CCAGGAGGATTCCGGAACTGCTTTTGC	$Glu^{354}Ser$ (creates *Bsp*EI)	YpDC336
YpDC240	CAGGAGGATGCAGGTACCGCTTTTGCTCC	$Glu^{354}Ala$ (creates *Kpn*I)	YpDC338
YpDC240	CAGGAGGATGAAGGTACCGCTTTTGCTCC	$Glu^{354}Gln$ (creates *Kpn*I)	YpDC364
YpDC240	GGGTACTTTCGCTGGCAACGAGC	$His^{463}Ala$ (destroys *Nco*I)	YpDC394

[a] Mutated nucleotides are underlined.

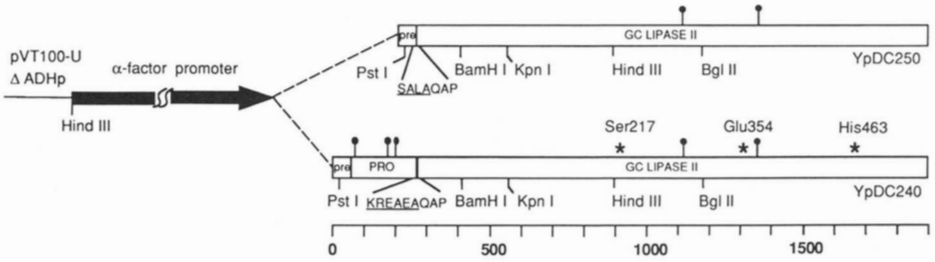

FIG. 1. **Physical and genetic map of the expression cassettes used in this study.** Regions originating from the α-factor coding sequence (*dotted boxes*) and from the mature lipase (*open boxes*) are represented. Putative *N*-linked glycosylation sites (⚲) are indicated. The amino acid sequence at the junction between the α-factor (*underlined*) and lipase is given. The amino acids that were subjected to mutagenesis in this study are indicated above *star symbols*. The locations of some restriction sites on the gene encoding GCL II are given. The scale indicates nucleotide size of the DNA fragments.

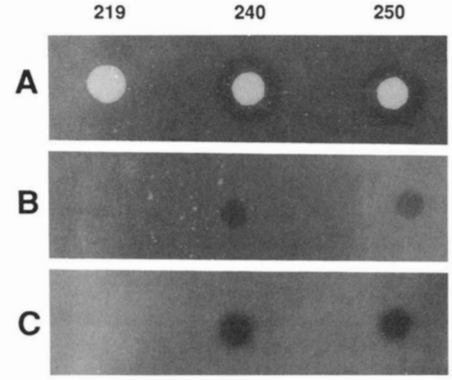

FIG. 2. **Clearing zone assay for recombinant lipase II activity produced by *S. cerevisiae*.** *A*, direct assay of transformed yeast cells. Agar plate containing an emulsion of TB 4 days after inoculation of yeast cultures. *B* and *C*, assay of culture supernatant (see "Experimental Procedures") on TB (*B*) and TO (*C*) emulsion containing plates. Sources of lipase are BJ3501(YpDC219) (219), BJ3501 (YpDC240) (240), and BJ3501(YpDC250) (250).

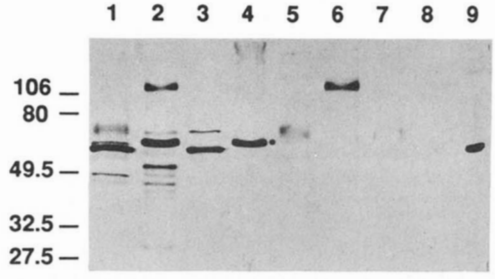

FIG. 3. **Secretion of glycosylated lipase from yeast.** Western blot analysis of culture supernatant of YE410(YpDC240) (*lanes 1* and *2*), BJ3501(YpDC240) (*lanes 3* and *4*), YE410(YpDC219) (*lanes 5* and *6*), BJ3501(YpDC219) (*lanes 7* and *8*) prior (*lanes 2, 4, 6,* and *8*) or following treatment with endoglycosidase H (*lanes 1, 3, 5,* and *7*). Purified lipase II from *G. candidum* (*lane 9*). Molecular masses of standards (kDa) are indicated in the *left margin*.

in Fig. 4. Pooled fractions from the major elution peak were analyzed by silver-stained SDS-polyacrylamide gel electrophoresis revealing a single 64 kDa band which comigrates with natural GCL II (Fig. 4, *inset*, *lanes 2* and *3*). The isoelectric point of the purified recombinant GCL II was measured by isoelectrofocusing gel electrophoresis. The measured pI value of 4.4 is about 0.2 pH units lower than for natural GCL II. The NH_2-terminal sequence of purified recombinant GCL II (Glu-Ala-Glu-Ala-Gln-Ala-Pro-Thr-Ala-Val) comprises a 4-amino-acid extension including 2 negatively charged glutamate residues that are probably responsible for the difference of pI.

The activity of natural and recombinant GCL II against various substrates were compared using three independent analytical methods. The profile of products released following hydrolysis of TO by GCL II was first determined by HPLC. Typical data sets for the natural and recombinant GCL II are very similar (Fig. 5). Specific activities were calculated from the initial rates of OA production where the data points were fitted to a straight line using a linear regression. These values (Table III) originate from two independent experiments and show a slightly lower specific activity for the natural enzyme. After 24 h of incubation under the same conditions, about 55% (w/w) of the TO is hydrolyzed and OA accumulates to approximately 40% (w/w), whereas DO drops from a maximum of 20% (w/w) to about 12% (w/w) of the initial TO concentration. For both natural and recombinant enzyme, traces of MO were also detected (data not shown).

More accurate measurements of the initial rates of release of OA were achieved with the titrimetric assay (Table III).

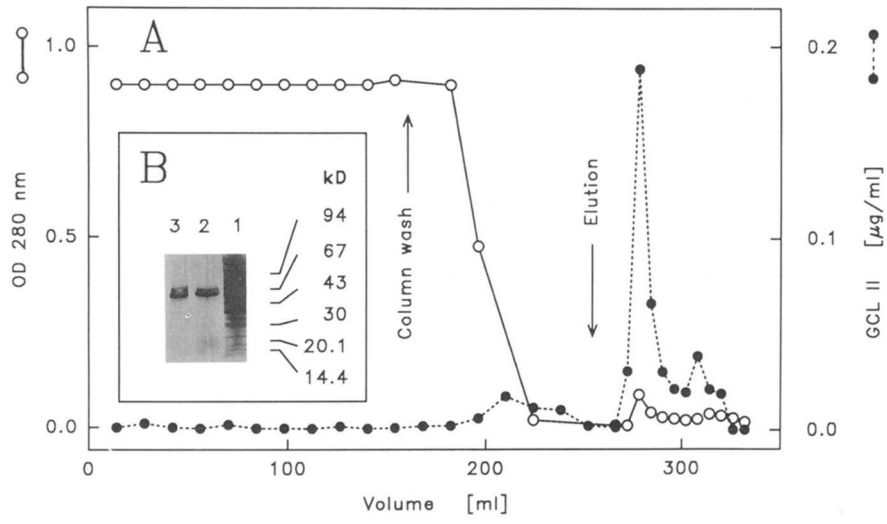

FIG. 4. **Purification of the WT recombinant lipase from YE410-(YpDC240) culture by immunoaffinity chromatography.** Elution profile of GCL II from the immunoaffinity column (*A*). The concentration of lipase was determined by ELISA (see "Experimental Procedures"). Silver-stained SDS-polyacrylamide gel electrophoresis (*B*). Concentrated culture medium from YE410(YpDC240) (1), purified recombinant GCL II (2), purified natural GCL II (3).

TABLE II

Purification of the recombinant wild type GCL II from S. cereviseae cultures

Purification step	Volume	Lipase activity[a]	Protein		Unit/mg[b]	%[c]
			Lowry	ELISA		
	ml	*unit/ml*	*mg/ml*			
Culture broth	1000	4.4	0.830	0.0028	5	100
Q-Sepharose	280	11.9	0.670	0.0078	18	72
Immunoaffinity	2.9	132.4	0.103	0.140	1285	15

[a] Lipase activity was determined titrimetrically with triolein as the substrate.

[b] Lipase-specific activity was calculated per milligram of protein determined with the modified Lowry method (Bensadoun *et al.*, 1976).

[c] Concentration of lipase was determined by ELISA and used to calculate the purification yield. Approximately 40% of the original amount of lipase was found before concentration of the pure enzyme.

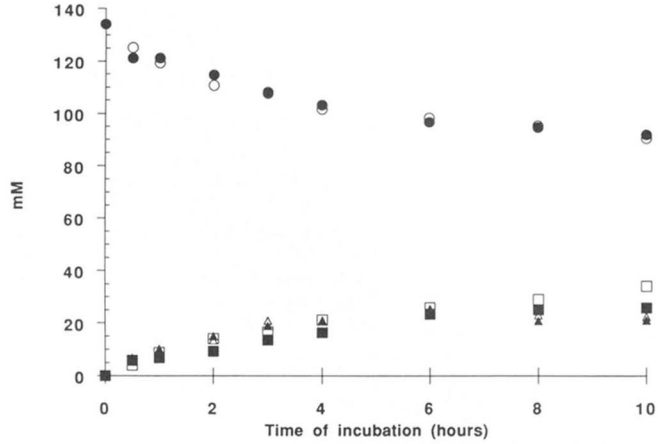

FIG. 5. **Typical curves for hydrolysis of TO by natural and recombinant GCL II monitored by HPLC.** Concentration of DO (*triangles*), OA (*squares*), and TO (*circles*) over time resulting from hydrolysis of TO using natural GCL II (*closed symbols*) or recombinant GCL II (*open symbols*).

Again, the specific activities for the recombinant and natural enzyme are not significantly different. Hydrolysis of TB was also monitored by the titrimetric assay. These results confirm the similar behavior of GCL II from the two sources and the validity of use of the yeast system for our further studies.

Since the lipase shows measurable activity toward small, soluble esters like PNPA or PNPB, the activity of natural and recombinant enzymes can be measured by spectrophotometric assay. No significant difference in the specific activities between the two sources of enzyme could be detected using either PNPA or PNPB (Table III).

Mutational Analysis of GCL II Putative Active Site—The determination of the 3-D structure of GCL II and analysis of the sequences of homologous proteins led to the identification of a putative Ser^{217}-His^{463}-Glu^{354} [2] catalytic triad (Schrag *et al.*, 1991; Cygler *et al.*, 1992). Further comparison of its 3-D structure with other hydrolases (Ollis *et al.*, 1991) provided additional support for this assignment. To supply more direct evidence for the presence and importance of the catalytic triad, we have constructed a series of mutants of GCL II. Preliminary characterization of the mutants was done using the plate assays (Fig. 6). The sensitivity of the assay was increased by concentrating the yeast culture supernatant 15-fold prior to the assays. No lipase activity against TO or TB could be detected when Ser^{217} was replaced by either Thr or Cys. Similarly, substitution of Glu^{354} by either Ala, Ser, or Gln inactivates the enzyme. The Glu^{354}Asp mutant retains a level of activity against TO that is slightly lower than that of the WT enzyme. However, the enzymatic activity of the mutant against TB is significantly reduced. No lipolytic activity was detected for the His^{463}Ala mutant enzyme.

The mutants were analyzed by Western blot and the level of GCL II secreted from yeast cells was measured by ELISA (Fig. 7). Each mutant is secreted from YE410 as a 64-kDa core-glycosylated form (compare equivalent lanes in Fig. 6, *A* and *B*). This molecular weight is slightly higher than natural GCL II indicating that the added sugars have a different structure in YE410 yeast and *G. candidum*. Following enzymatic removal of sugars all mutants co-migrate with deglycosylated GCL II from *G. candidum* with an apparent molecular mass of 60 kDa (Fig. 7*B*). GCL II mutants are secreted to levels comparable to the WT enzyme with the exception of Glu^{354}Ala, Glu^{354}Ser, and Glu^{354}Gln. Those mutants are secreted at about 7% of the WT level. Based upon the sensitivity of the plate assays, the activity of the mutants at position Ser^{217} and His^{463}Ala has been calculated to be 30-fold less than the WT activity against TO and 20-fold less than WT activity against TB. Due to the lower levels of secretion of the inactive Glu^{354} mutants, the limit values for enzymatic activities are about five times higher than those calculated for the Ser^{217} mutants.

Enzymatic Characterization of the Glu^{354}Asp Mutant—Specific activities of the Glu^{354}Asp mutant against TO and TB

[2] Numbering of amino acid positions is as defined by Shimada *et al.*, (1990) starting with Gln^{1} of mature lipase.

TABLE III

Comparison of specific activities of GCLII natural, recombinant and Glu^{354} Asp mutant

Substrate	Method[a]	GCLII-specific activity[b]			Mutant/WT
		Natural	Wild type recombinant	Glu^{354}Asp mutant	
			units/mg		
TO	HPLC[c]	491 ± 65	1017 ± 213	542 ± 598	0.53
TO	Titrimetric assay	958 ± 180	952 ± 190	422 ± 126	0.44
TB		54 ± 25	40 ± 14	5.3 ± 1.6	0.13
PNPA	Spectrophotometric assay	0.79 ± 0.2	0.83 ± 0.12	0.43 ± 0.19	0.52
PNPB		2.05 ± 0.36	2.95 ± 0.78	1.08 ± 0.25	0.37

[a] Lipase activity was measured as described under "Experimental Procedures."

[b] Protein concentration in recombinant lipase preparations was determined by ELISA using natural *G. candidum* as a standard. The values are averages from four independent experiments except for the HPLC assay (two measurements). Standard deviations are given.

[c] Activity of lipase was calculated from the initial rates of TO hydrolysis determined after plotting the amounts of OA released during the reaction using a first-order kinetic equation (Enzfitter).

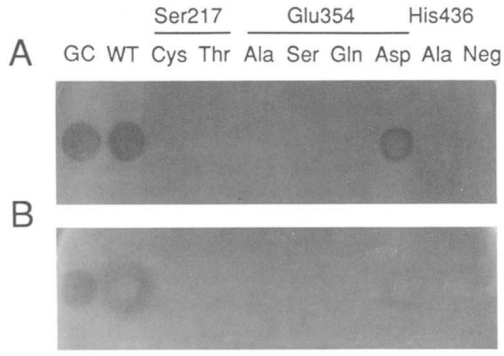

FIG. 6. **Clearing zone assay for GCL II mutants produced by *S cerevisiae*.** TO (*A*) and TB (*B*) emulsion-containing plates were used in the assays. Culture supernatants from YE410 transformants concentrated 15-fold were spotted onto the plates and incubated for 2 days (*A*) or 4 days (*B*) at 30 °C.

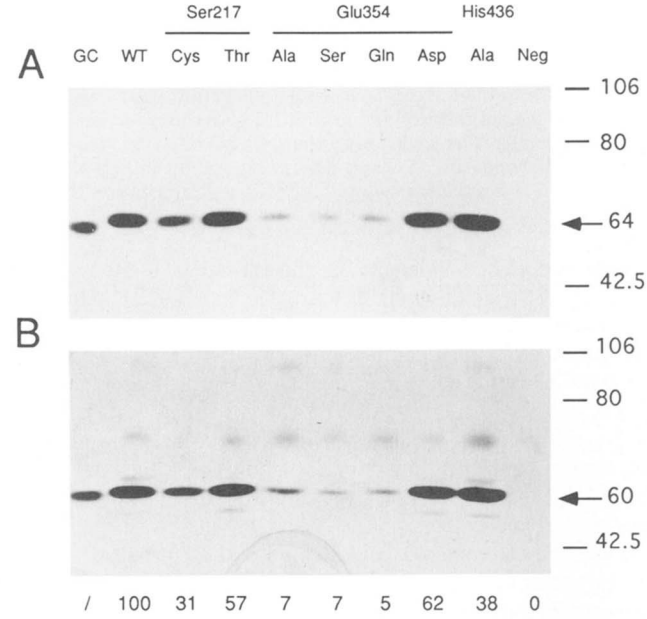

FIG. 7. **Western blot analysis of GCL II mutants.** Supernatant from YE410 cultures expressing GCL II mutants were analyzed by Western blot prior (*A*) or following (*B*) treatment with endoglycosidase H. Quantities of GCL II in the culture media were measured by ELISA (see "Experimental Procedures") and indicated below each column (% relative to WT). The nature of each mutant is indicated above the columns. Control GCL II from *G. candidum* (*GC*) prior (*A*) or after (*B*) treatment with endoglycosidase H. Supernatant from YE410(YpDC219) (*Neg*). Molecular masses of standards (kDa) are indicated in the *left margin*. The *arrows* in the *right margin* designate the molecular masses (kDa) of the detected proteins.

were determined by analysis of the product of hydrolysis using HPLC and by titrimetry. These values are about half and one-tenth that of the WT enzyme for TO and TB respectively (Table III). The esterolytic specific activity of the Glu^{354}Asp mutant is approximately half that of the WT enzyme when PNPA and PNPB are used as substrates (Table III).

Modeling of the Glu^{354}Asp Mutant—The lipase activity observed of the Glu^{354}Asp mutant indicates the ability of this shorter side chain to participate in the formation of a catalytic triad. To investigate whether the required H-bonds from Ser^{217} to His^{463} and from His^{463} to Asp^{354} can be formed in the mutant without significant structural changes, we have introduced such a mutation to the atomic model of GCL II. The Asp side chain could be brought within H-bonding distance of His^{463} (Oδ1. . .Nδ1 = 2.9 Å) by small adjustments of the χ1 and χ2 torsion angles (Fig. 8). An accompanying small movement of Asn^{350} would permit a H-bond from that side chain to Oδ2 of Asp^{354} to be maintained, similar to that observed in the native structure. Ser^{249}, which also H-bonds to the same carboxylic oxygen of Glu^{354}, as does the Asn^{350} side chain, could, after a small rotation around χ1, form a H-bond to Oδ1 of the Asp^{354} in the mutant. Although the conformation of the Asp^{354} side chain and the geometry of the other H-bonds are not ideal, small local movements of the neighboring residues could lead to a more favorable arrangement.

DISCUSSION

To study the molecular basis of GCL II enzymatic activity and substrate specificity, we have cloned and expressed the corresponding gene in *S. cerevisiae*. This strategy has three major advantages. First, recombinant expression provides the molecule in isolation from the complex mixture of isoenzymes found in the natural host, hence allowing for enzymatic characterization of homogenous enzyme preparations. Second, the structure and function of the molecule can be investigated by perturbation analysis using site-directed mutagenesis. Third, careful selection of the genetic background of the yeast host cell offers the possibility to "engineer" the heterologous protein by controlling post-translational modifications such as glycosylation and endoprotease processing.

Our sequence of the GCL II gene diverges slightly from the one reported by Shimada *et al.* (1990). Instead of the tripeptide Arg^4-Pro^5-Ser^6 we have found Thr^4-Ala^5-Val^6, which are identical to the corresponding residues in GCL I (Shimada *et al.*,

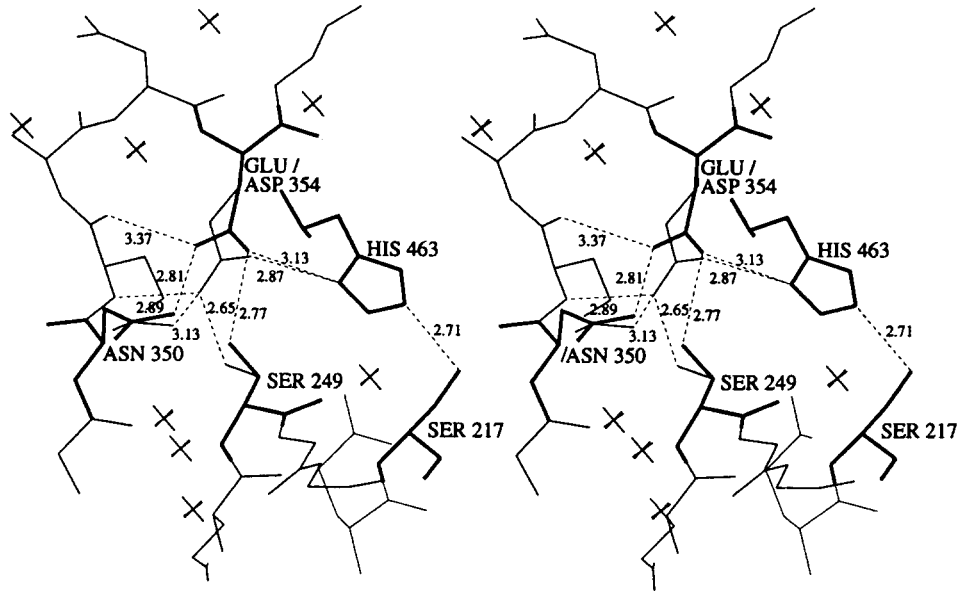

FIG. 8. **Model the Gln^{354}Asp catalytic triad.** Glu^{354} was mutated to Asp and the side chain was rotated around the $\chi 1$ and $\chi 2$ torsion angles to place the carboxyl group within H-bonding distance of His^{463}. Small rotations of the Ser^{249} and Asn^{350} side chains were also made to provide H-bonds. The positions of the residues of the model of the mutant are shown in *bold lines* and the native structure is shown in *thin lines*. The H-bonds are shown as *dashed lines* and the H-bond distances are shown. The positioning of the residues in the model was based primarily on H-bond distances and no energy minimization has been done.

1989). The electron density of the refined 3-D structure of GCL II (Schrag *et al.*, 1993) confirms the Thr^4-Ala^5-Val^6 tripeptide sequence. In fact, the 3-D structure also revealed the presence of a sugar residue attached to Thr^4, showing post-translational modification of this residue in *G. candidum*. Therefore, it is likely that the discrepancies between the cDNA and genomic sequences originate from a DNA sequencing error. Our sequencing data also show that the gene encoding GCL II is intron free, providing for direct expression of the gene in yeast.

The GCL II gene was placed under the control of the α-factor promoter and secreted from yeast. The absence of the α-factor pro-peptide NH_2-terminal to the GCL II did not markedly improve the level of lipase expression in contrast to our previous observations (Vernet *et al.*, 1993). This behavior of GCL II is more consistent with what is generally reported for a variety of recombinant proteins (Ernst, 1988; Brake, 1989; Brömme *et al.*, 1993). We have shown that GCL II is hyperglycosylated in wild type yeast. Mannose addition could be limited by the use of a yeast host deficient in the sugar processing enzyme encoded by the MNN9 gene (Tsai *et al.*, 1984). The resulting proteins contain core glycosylated *N*-linked residues that add 4 kDa to the protein molecular mass, a product having a structure closer to the one secreted from *G. candidum*. The mutant mnn9-recipient strain did not affect the amount of GCL II secreted or the enzyme function. Moreover, production of a more homogenous population of GCL II molecules should facilitate the growth of protein crystals for future 3-D structure determination of GCLII mutants.

GCL II was purified to apparent homogeneity by immunoaffinity chromatography. NH_2-terminal sequencing of the purified enzyme revealed the presence of the 4-amino-acid extension Glu-Ala-Glu-Ala. A similar extension was observed previously (Brake, 1989) and is the result of the relative level of expression of recombinant proteins compared to the dipeptidyl amino peptidase encoded by the STE13 gene (Julius *et al.*, 1983). The enzymatic properties of the recombinant GCL II were compared to natural GCL II. Hydrolysis of TO, TB, and esterolytic activity was followed using independent analytical methods. These experiments demonstrated that the recombinant GCL II has enzymatic properties that are not significantly different from those of the protein of natural origin despite the presence of the tetrapeptide NH_2-terminal extension. These results validate the exploitation of the expression system for mutational analysis of the enzyme.

Our site-directed mutagenesis experiments support the previous assignment of the catalytic triad based upon structural arguments. Mutants at position Ser^{217}, His^{463}, and Glu^{354} were screened for activity by the plate assay. Conservative replacement of Ser^{217} by Cys or Thr led to enzymes having no measurable activity, in agreement with the effect of similar mutations of the corresponding serine residue in AChE (Shafferman *et al.*, 1992) and ChE (DiPersio *et al.*, 1990). The active site nucleophile residue of other hydrolases has also been replaced to evaluate the role of the side chain in the catalytic process. For instance, the substitution of subtilisin Ser^{221} (Carter and Wells, 1988) and trypsin Ser^{195} (Corey and Craik, 1992) by Ala led to a decrease of enzymatic activity by 5×10^7 and 10^5, respectively. Similarly, the cysteine protease papain was shown to be completely inactivated by the replacement of the nucleophile Cys^{25} by Ser (Vernet *et al.*, 1991). Our results confirm the importance of the nucleophile Ser^{217} in the catalytic process of GCL II. Replacement of His^{463} by Ala also led to an inactive enzyme, supporting its role in enzyme activity.

The effect of replacement of the carboxyl group at position 354 is more complex as it combines structural and functional consequences. When the Glu^{354} is substituted by Ala, Ser, or Gln, the enzyme is inactivated and the expression-secretion from yeast is impaired. This latter observation suggests that Glu^{354} participates in the folding and/or stability of the protein. WT-like level of secretion is restored when position 354 is occupied by an Asp. This observation and the detrimental effect of the conservative Glu^{354}Gln mutation seems to indicate that the charge and/or the H-bonding pattern, and not

just the volume of the side chain at position 354 has a structural role. A similar effect on the expression levels of mutants of the acidic member of the catalytic triad has been observed for AChE (Shafferman *et al.*, 1992) and for a microbial lipase (Yamagushi *et al.*, 1992). Therefore, the structural role of the negatively charged side chain of the active site appears to be a general one.

We have shown that the size of the side chain carrying the carboxyl group could be shortened by one C atom ($Glu^{354}Asp$) without a major perturbation of the enzyme activity toward the substrate TO. The equivalent mutation in human AChE led to a complete inactivation of the enzyme (Shafferman *et al.*, 1992), whereas enzymatic activity is maintained after addition of one C atom to the active site Asp^{320} of pancreatic cholesterol esterase (DiPersio and Hui, 1993). It is possible that the greater tolerance for the type of acidic side chain of GCL II and cholesterol esterase has a kinetic basis. The substrate turnover for large molecules is much slower than the turnover of acetylcholine, a small substrate, by AChE. Since significant interactions between the hydrophobic parts of the substrate and a hydrophobic surface in the binding site are likely (Grochulski *et al.*, 1993), one might expect a slower off-rate for such substrates, allowing for catalysis with an active site which geometry is suboptimal. A complete understanding of the different behavior of the lipase mutants will require determination of their 3-D structure. Surprisingly, the $Glu^{354}Asp$ mutant of GCL II has a decreased ability to hydrolyze TB. In the absence of the detailed knowledge of the substrate binding site, the molecular basis for this altered substrate specificity cannot be easily explained.

Our data suggest that GCL II is more tolerant to change in the geometry of the active site triad than most other hydrolases. Modeling of the Glu^{354} to Asp replacement indicates that Asp can form a H-bond with His^{463}, although energetically less favorable due to a somewhat strained conformation of Asp and a loss of one H-bond which stabilizes the Glu^{354} in the correct orientation. The fact that the rate of hydrolysis of TO is affected only 2-fold may indicate that the substrate remains bound to the enzyme long enough for the proper H-bonding between the triad members to occur.

As in serine proteases, the acid member of the triad in GCL II is H-bonded to a serine (Ser^{249}). It has been suggested that the second serine may be involved more directly in the proton transfer chain and that the active site is in fact a tetrad (for a review, see Meyer, 1992). From the model building, it appears that Asp^{354} can also form a H-bond with Ser^{249}. However, this bond is likely to be formed to the Oδ1 oxygen (rather than Oδ2) of Asp^{354}. His^{463} is also with H-bonded to the Oδ1 oxygen.

We now plan to compare the 3-D structures of WT and mutants of GCL II in their native forms and complexed with substrate-like inhibitors in order to further improve our understanding of the enzymatic mechanism of GCL II.

Acknowledgments—We thank Francesco Lipari, Louise Laramée, Jean-Francois Jetté, Birgitte Sørensen, and France Dumas for excellent technical assistance and Andrew Storer for useful discussions throughout this work. The antibodies against SDS-denatured lipase were kindly provided by Dr. Shi-Hsiang Shen.

REFERENCES

Arends, J. (1979) *J. Immunol. Methods* **25,** 171–175
Avrameas, S., and Ternynck, T. (1971) *Immunochemistry* **8,** 1175–1179
Baillargeon, M. W., and McCarthy, S. G. (1991) *Lipids* **26,** 831–836
Bensadoun, A., and Weinstein, D. (1976) *Anal. Biochem.* **70,** 241–250
Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dobson, E., Dobson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Huge-Jensen, B, Norskow, L., Thim, L., and Menge, U. (1990) *Nature* **343,** 767–770
Brake, A. J. (1989) in *Yeast Genetic Engineering* (Davies, J. E., ed) pp. 269–278, Butterworths, Boston
Brömme, D., Bonneau, P. R., Wiederanders, B., Kirschke, H., Peters, C., Thomas, D. Y., Storer, A. C., and Vernet, T. (1993) *J. Biol. Chem.* **268,** 4832–4838
Brzozowski, A. M., Derewenda, U, Derewenda, Z. S., Dodson, G. G., Lawson, D. M. Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S., and Thim, L. (1991) *Nature* **351,** 491–494
Carter, P., and Wells, J. A. (1988) *Nature* **332,** 564–568
Casadaban, M., and Cohen, S. N. (1980) *J. Mol. Biol.* **138,** 179–207
Chapus, C., and Sémériva, M. (1976) *Biochemistry* **15,** 4988–4991
Clark, J. M. (1988) *Nucleic Acids Res.* **16,** 9677–9686
Corey, D. R., and Craik, C. S. (1992) *J. Am. Chem. Soc.* **114,** 1784–1790
Cygler, M., Schrag, J. D., and Ergan, F. (1992) *Biotech. Genet. Rev.* **10,** 141–181
Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K., and Doctor, B. (1993) *Protein* **2,** 366–382
Desnuelle, P. (1972) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed., Vol. 7, pp. 575–616, Academic Press Inc., New York
DiPersio, L. P., and Hui, D. Y. (1993) *J. Biol. Chem.* **268,** 300–304
DiPersio, L. P., Fontaine, R. N., and Hui, D. Y. (1990) *J. Biol. Chem.* **265,** 16801–16806
DiPersio, L. P., Fontaine, R. N., and Hui, D. Y. (1991) *J. Biol. Chem.* **266,** 4033–4036
Ergan, F., and André, G. (1989) *Lipids* **24,** 76–78
Ernst, J. F. (1986) *DNA* **5,** 8–14
Ernst, J. F.(1988) *DNA* **7,** 355–360
Fournier, D., Bride, J. M., Hoffman, F., and Karch, F. (1992) *J. Biol. Chem.* **267,** 14270–14274
Gibney, G., Camp, S., Dionne, M., MacPhee-Quiley, K., and Taylor, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7546–7550
Grochulski, P., Li, Y., Schrag, J. D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B., and Cygler, M. (1993) *J. Biol. Chem.* **268,** 12843–12847
Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1983) *J. Biol. Chem.* **256,** 7990–7997
Ito, H., Futuda, Y., Masata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
Jensen, R. G., Sampugna, J., Quinn, J. G., Carpenter, D. L., Marks, T. L., and Alford, J. A. (1965) *J. Am. Oil Chem. Soc.* **42,** 1029–1033
Jones, E. W. (1991) *Methods Enzymol.* **194,** 428–453
Julius, D., Blair, L., Brake, A., Sprague, G., and Thorner, J. (1983) *Cell* **32,** 837–852
Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984) *Cell* **37,** 1075–1089
Kukuruzinska, M. A., Bergh, M. L. E., and Jackson, B. J. (1987) *Annu. Rev. Biochem.* **56,** 915–944
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Livingstone, D. M. (1974) *Methods Enzymol.* **34B,** 723–731
Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1991) *Nucleic Acids. Res.* **19,** 1154
Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
Meyer, E. (1992) *Protein Sci.* **1,** 1543–1562
Neville, L. F., Gnatt, A., Padan, R., Seidman, S., and Soreq, H. (1992a) *J. Biol. Chem.* **265,** 20735–20738
Neville, L. F., Gnatt, A., Loewenstein, Y., Seidman, S., Ehrlich, G., and Soreq, H. (1992b) *EMBO J.* **11,** 1641–1649
Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B. Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J. D., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) *Protein Eng.* **5,** 197–211
Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992) *Yeast* **8,** 423–488
Samad, M. Y. A., Razac, C. N. A., Salleh, A. B., Yunus, W. M. Z. W., Ampon, K., and Basri, M. (1989) *J. Microbiol. Methods* **9,** 51–56
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
Schrag, J. D., and Cygler, M. (1993) *J. Mol. Biol.* **230,** 575–591
Schrag, J. D., Li, Y., Wu, S., and Cygler, M. (1991) *Nature* **351,** 761–764
Shafferman, A. Kronman, C. Flashner, Y., Leitner, M., Grosfeld, H., Ordentlich, A., Gozes, Y., Cohen, S., Ariel, N., Barak, D., Harel, M., Silman, I., Sussman, J. L., and Velan, B. (1992) *J. Biol. Chem.* **267,** 17640–17648
Sherman, F., Fink, G. R., and Hicks, J. (1982) in *Methods in Yeast Genetics*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY
Shimada, Y., Sugihara, A., Tominaga, Y., Iizumi, T., and Tsunasawa, S. (1989) *J. Biochem. (Tokyo)* **106,** 383–388
Shimada, Y., Sugihara, A., Iizumi, T., and Tominaga, Y. (1990) *J. Biochem. (Tokyo)* **107,** 703–707
Sidebottom, C. M., Charton, E., Dunn, P. P. J., Mycock, G., Davies, C., Sutton, J. L., Macrae, A. R., and Slabas, A. R. (1991) *Eur. J. Biochem.* **202,** 485–491
Sussman, J. L., Harel, M., Frolow, F. Oefner, C., Godman, A., Toker, L., and Silman, I. (1991) *Science* **253,** 872–879
Szewczuk, A., and Prusak, E. (1985) in *Affinity Chromatography: a Practical Approach* (Dean, P. D. G., Johnson, W. S., and Middle, F. A., eds) pp. 117–124, IRL Press, Oxford
Tsai, P. K., Frevert, J., and Ballou, C. E. (1984) *J. Biol. Chem.* **259,** 3805–3811
Vernet, T., Dignard, D., and Thomas, D. Y. (1987) *Gene (Amst.)* **52,** 225–233
Vernet, T. Khouri, H. E., Laflamme, P., Tessier, D. C., Gour-Salin, B., Storer, A. C., and Thomas, D. Y. (1991) *J. Biol. Chem.* **266,** 21451–21457
Vernet, T., Chatellier, J., Tessier, D. C., and Thomas, D. Y. (1993) *Protein Eng.* **6,** 213–219
Winkler, F. K., D'Arcy, A., and Hunziker, W. (1990) *Nature* **343,** 771–774
Yamagushi, S., Tamio, M., and Kazuyuki, T. (1992) *Biosci. Biotech. Biochem.* **56,** 315–319
Ziomek, E., Martin, W. G., and Williams, R. E. (1984) *Can. J. Microbiol.* **30,** 1197–1204
Zoller, M. J., and Smith, M. (1982) *Nucleic Acids Res.* **10,** 6487–6500