Crystal Structure of the Open Form of Dog Gastric Lipase in Complex with a Phosphonate Inhibitor*

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Fat digestion in humans and some mammals such as dogs requires the successive intervention of two lipases: gastric lipase, which is stable and active despite the highly acidic stomach environment, followed by the classical pancreatic lipase secreted into the duodenum. We previously solved the structure of recombinant human gastric lipase (HGL) at 3.0-Å resolution in its closed form; this was the first structure to be described within the mammalian acid lipase family. Here we report on the open structure of the recombinant dog gastric lipase (r-DGL) at 2.7-Å resolution in complex with the undecylbutyl (C11Y4) phosphonate inhibitor. HGL and r-DGL show 85.7% amino acid sequence identity, which makes it relevant to compare the forms from two different species. The open r-DGL structure confirms the previous description of the HGL catalytic triad (Ser153, His353, and Asp324) with the catalytic serine buried and an oxyanion hole (NH groups of Gln154 and Leu67). In r-DGL, the binding of the C11Y4 phosphate inhibitor induces part of the cap domain, the lid, to roll over the enzyme surface and to expose a catalytic crevice measuring ~20 × 20 × 7 Å3. The C11Y4 phosphate fits into this crevice, and a molecule of β-octyl glucoside fills up the crevice. The C11Y4 phosphate inhibitor and the detergent molecule suggest a possible binding mode for the natural substrates, the triglyceride molecules.

Among the mammalian lipases, the preduodenal and lysosomal lipases belong to a family of enzymes that are able to withstand acidic conditions, under which they continue to be active. This family shows no sequence homology with any other known lipase families. The preduodenal lipases form a group of closely related enzymes originating from the stomach, the tongue, or the pharynx, and none of them requires any specific protein cofactor for its activity (1–3).

The state of malnutrition of patients suffering from pancreatic insufficiency, which is often associated with cystic fibrosis, can be improved by prescribing dietary supplements prepared from pancreatic extracts of animal origin. The use of gastric lipases may lead to considerable progress in the treatment of exocrine pancreatic insufficiency (4) because of their resistance to the gastric medium and their high lipolytic activity at the low pH values observed in the duodenum A screening program (1) and biochemical studies (5–7) led to the conclusion that dog gastric lipase (DGL), a glycoprotein with a molecular mass of 49 kDa, was the most active enzyme when tested on long-chain triacylglycerols. These lipids are the main components of human dietary fats. The first attempts to produce an active recombinant DGL (r-DGL) in procaryotes (4) were unsuccessful. Joliff et al. (8) described the expression and secretion of an active r-DGL in the baculovirus insect cell system. However, with a view to therapeutic applications, r-DGL production by a safe eukaryotic system of expression such as transgenic plants provides an attractive alternative. Transgenic plants offer considerable advantages, such as large production yields and the fact that they entail no risk of contamination by pathogens infectious to humans, whereas they contain the cell machinery mediating eukaryotic protein modifications. The first transgenic plants producing r-DGL were constructed in tobacco (9) and then in maize by Meristem therapeutics in collaboration with the Institut de Recherche Jouveauil/Parke-Davis during the last decade.

All the triglyceride lipases for which the three-dimensional structures have been determined so far belong to the serine esterase class (10–14). The active serine is part of a catalytic triad, which is similar to that observed in the case of serine proteases. The three-dimensional structure of the Candida rugosa lipase, which is inhibited by two enantiomers of a phosphonate inhibitor, provided the structural basis for establishing the chiral preferences of lipases (15). Covalent adducts of Rhizomucor miehei lipase (16, 17), C. rugosa lipase (18), and human pancreatic lipase (HPL; Ref. 19) with organophosphate or organophosphonate inhibitors have been structurally characterized. The structural differences observed between the native (closed) and inhibited (open) lipase forms range from a relatively simple rigid hinge-type motion of a single helix in R. miehei lipase (16, 17) to much more complex reorganization of multiple loops, involving profound changes in their secondary structures (18, 19). Various lipase inhibitors were synthesized by Marguet et al. (20) and Cavalier et al. (21) by replacing the

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The atomic coordinates and structure factors (code 1K8Q) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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†††† The abbreviations used are: DGL, dog gastric lipase; r-DGL, recombinant DGL; HPL, human pancreatic lipase; HGL, human gastric lipase; β-OG, β-octyl glucoside; AMA, 1-aminanthracene; T1, first generation; T2, second generation.
carbonyl of the hydrolyzable ester bonds by a phosphate group with a suitable leaving group and were investigated as potential HPL and human gastric lipase (HGL) inhibitors. The crystal structure of the pancreatic lipase-colipase complex inhibited by C11-carboxymethyl phosphate was determined at a resolution of 2.46 Å (19). It has been confirmed that the covalently inhibited lipases are in the so-called open conformation, i.e. that the lid has moved away, giving free access to the active site serine (22).

After suitable crystals of r-HGL were obtained, Roussel et al. (23) solved its three-dimensional structure at 3.0-Å resolution. This was the first structure of a mammalian acid lipase to be elucidated. Both the connectivity and the structure of HGL are similar to those of the serine carboxypeptidases: the protein is globular and composed of three superimposed structural elements, namely a core domain, a cap domain, which was assumed to be the lipid binding site, and a putative lid covering the catalytic serine. Thus Roussel et al. (23) concluded that the HGL was crystallized in its closed conformation and that the lid-opening mechanism might be more complex than a simple rigid body movement. Because HGL and DGL show 85.7% amino acid sequence identity, the crystal structure at 2.7-Å resolution of r-DGL inhibited by a C11Y4 phosphonate was determined in the present study.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The DGL cDNA used was obtained from plasmid pDGL5.303 (24) on a 1-kb BglII-XbaI fragment. The DGL coding sequence was fused by performing restriction enzyme-based cloning to the polymerase chain reaction-amplified N-terminal signal sequence of the rabbit gastric lipase precursor composed of its first 22 amino acids (6, 25). The resulting sequence was placed under the control of the γ-zein promoter of maize (26), which confers a seed expression, and the nopaline synthase terminator of Agrobacterium tumefaciens (27) in the pUC18 plasmid (Invitrogen). The resulting plasmid is called pDGLm. The plasmid pbar carries the Streptomyces hygroscopicus bar gene (28) used to select the transformed plants. The bar gene is under the control of the rice actin 1 promoter (29) and the nopaline synthase terminator of maize (26), which confers a seed expression, and the rice actin 1 promoter (29) and the nopaline synthase terminator of maize (26), which confers a seed expression.

**Maize Transformation**—An established callus line derived from a single immature embryo of the Hi-II maize germplasm (30) was transformed using particle bombardment-mediated transformation procedures with a helium-powered particle acceleration device, PDS 1000 (Bio-Rad). Tissue showing friable type II embryogenic morphology was sieved and cotransformed with the two plasmids, pDGLm and pbar, as described by Tomes et al. (31). Transformants expressing the bar gene were selected. Multiple callus-derived plants were generated, transferred to the greenhouse, and crossed with an untransformed elite inbred to produce T1 seeds. The T1 seeds were assayed to determine the lipase expression levels, and the highest expressors were selected. Several T1 plants derived from a selected event were self-pollinated; the resulting T2 seeds and their progeny were subjected to analysis; and the r-DGL was extracted and purified.

**r-DGL Extraction and Purification**—Dried maize seeds were ground, giving a yellowish white powder, which was subsequently washed twice with 5 volumes of hexane. The dried filtration cake was then extracted (10 liters of buffer/kg of flour) by 50 mM glycine-HCl, pH 3, containing 250 mM NaCl, 1 mM EDTA, and 1 mM Trisodium 100 under stirring at room temperature for 16 h. The macerate was filtered through 25- and 5-μm filters and diluted with 50 mM glycine-HCl, pH 3, until the conductivity was <20 mS at 20 °C. This solution was then loaded onto an S Ceramic Hyper D column (cationic exchanger; Biosepra) using a linear flow rate of 300 cm/h. The r-DGL was eluted using a 50 mM acetate buffer, pH 4, containing 500 mM NaCl. To perform immobilized metal ion affinity chromatography, the eluate was loaded onto a Cu²⁺-Fractogel EMD chelate 650M (Merck). The purified r-DGL was then eluted with a 10 mM glycine buffer, pH 3, containing 500 mM acetate buffer, pH 3. The enzyme was freeze-dried in the presence of mannitol and kept at a low temperature (~20 °C). Under these conditions, no loss of enzyme activity occurs for

**TABLE I**

| Statistic | Value |
|----------|-------|
| Data collection | |
| Resolution limit (Å) | 40–2.7 |
| Completion (%) | 97.9 (97.5) |
| Redundancy | 3.0 (2.2) |
| Rsym* | 5.8 (21.2) |
| I/σ(I) | 7.0 (1.8) |
| Refinement | |
| Resolution limits (Å) | 15–2.7 |
| No. of reflections (test set) | 24389 (1174) |
| No. of protein atoms | 6064 |
| No. of sugar atoms | 112 |
| No. of inhibitor atoms | 36 |
| No. of β-OG atoms | 40 |
| No. of water molecules | 420 |
| Final R-factor/R-free (%) | 19.2/25.8 |
| B-Factors (Å²) | |
| Protein | 38.5 |
| Sugar | 53.6 |
| Inhibitor | 35.7 |
| B-OG | 61.3 |
| Water | 35.5 |
| Root mean square deviations | |
| Bond (Å) | 0.0086 |
| Angles (°) | 1.50 |
| Improper/dihedral angles (°) | 0.75/26.1 |

* R-sym = σ₁σ₂ | Iₙₒₜ – Iₙₒₜ | / σ₁σ₂Iₙₒₜ,

where F₁, F₂, and Fₗₖ are the observed and calculated structure factor amplitudes, respectively.

* R-free is calculated with 5% of the diffraction data, which were not used during the refinement.
The lyophilized powder used in this study contained 70% (w/w) protein, 20% (w/w) mannitol, used as a stabilizer, and 10% various salts and buffer. At this stage, the rDGL was ~97% pure. For crystallization purposes, further purification was needed, and 2 mg of r-DGL were dissolved in 500 ml of water and applied to a MonoQ fast protein liquid chromatography column equilibrated with 10 mM 4-morpholineethanesulfonic acid, pH 7. The active fractions were eluted with a linear NaCl gradient (0–500 mM) and concentrated using a Speed Vacuum apparatus. The SDS gels displayed a single protein band (Fig. 1A) and its isoelectric point, as analyzed with isoelectric focusing gels, was ~6.7 (Fig. 1B).

**Protein Assay and Gastric Lipase Activity Measurement**—Proteins were quantified using the bicinchoninic acid protein assay method (32). The r-DGL activity was measured potentiometrically using a pH-stat set. The substrates used were either a defined mixture of long-chain triacylglycerols (Intralipid 30%; Amersham Biosciences, Inc.) or a short-chain triacylglycerol (tributyrin). With both substrates, the lipolytic activity was measured at 37 °C under the conditions described by Gargouri et al. (5). One unit of r-DGL activity corresponds to the release of 1 μmol of fatty acid/min.

**Lipase Inhibition for Crystallization Purposes**—Inhibition of r-DGL was carried out in the presence of β-octyl glucoside (β-OG, 30 mM final concentration) and a molar excess of C11Y4 phosphonate to r-DGL of 50-fold. After the evaporation of the chloroform, which dissolves the C11Y4 phosphonate, the inhibitor was dissolved in isopropyl alcohol (4% w/w final concentration). After adding β-OG to the inhibitor, r-DGL was also added (final volume, 50 μl). The residual activity measured after 1 h of incubation was 10%.

**Fluorescence Assays**—Spectra were recorded with a Kontron spectrofluorimeter using a front face fluorescence accessory. The spectra were computed with Hyperterminal software program. All fluorescence experiments were carried out in 10 mM sodium acetate and 0.1 M NaCl, pH 5, at 20 °C (excitation 297 nm; emission, 400–575 nm). A slit width of 1 nm was used for the fluorescence emission recordings. The fluorescence of 1-amino-anthracene (AMA) was measured by adding to the lipase samples (0.5 mM, final concentration) aliquots of a stock solution of AMA (5 mM) solubilized in 100% isopropyl alcohol. The final concentration of AMA was 10 μM. The fluorescence emission was recorded after the spectroscopic signal had been left to stabilize for 5 min. Control experiments were performed in the absence of lipase.

**Crystallization and X-ray Diffraction Studies**—Crystallization experiments were performed using the hanging drop vapor diffusion method. Crystals of the inhibited r-DGL were obtained by mixing 1 μl of a well solution (30% polyethylene glycol monomethyl ether 2000, 0.1 M sodium acetate, 0.2 M ammonium sulfate, pH 4.6) with 1 μl of protein solution at 5 mg/ml.

**FIG. 3. Stereo ribbon view of r-DGL superimposed on HGL.** A, the cores of both molecules are colored according the secondary structures (helix, red; strand, blue; rest, yellow). The lid of the closed HGL is brown, and that of the open r-DGL is green. The missing parts in HGL (residues 1–9 and 53–56) are identified in the structure. The nucleophilic active residue, Ser153, is represented by green sticks. B, view of the superimposed lids at 90° from A. C, view of the GRASP surfaces of the closed HGL with the open lid of r-DGL superimposed in ribbon representation (blue). D, view of the GRASP surfaces of the open r-DGL with the closed lid of HGL superimposed in ribbon representation (red). The active site crevice is indicated by the green arrow.
X-ray diffraction data were collected to 2.7-Å resolution on a Mar Research CDD camera with a = 0.979 Å on beam line BM14 at ESRF (European Synchrotron Radiation Facility, Grenoble, France). The data were processed using the DENZO software package (33). r-DGL phosphonate inhibitor complex crystallized in the space group C222\textsubscript{1} with cell dimensions \(a = 61.2\ \text{Å}, b = 164.5\ \text{Å},\) and \(c = 177\ \text{Å}.\) Specific volume calculations yielded two molecules per asymmetric unit, with a solvent content of 40%. A total of 24,389 unique reflections were indexed using the SCALA software program (CCP4 suite program, 1997). The data collection statistics are given in Table I.

**Structure Determination**

The structure of the r-DGL phosphonate inhibitor complex was solved with the molecular replacement method using the AMoRe program (34). The HGL structure (Protein Data Bank code 1HLG) was used as the search model. A zone containing the putative lid (residues 208–264) was removed from the model. The rotation function gave a single solution, leading to two positions in the translation function. Before the fitting procedure, the correlation coefficient and \(R\)-factor were 38.7 and 47.5%, respectively, which refined to 45.0 and 44.2%, respectively. The lid in the open form and the C11Y4 phosphonate inhibitor were built unambiguously in the Fourier difference map with the Turbo-Frodo software program (35). Refinement with CNS (36) was carried out between 20- and 2.7-Å resolution. The final model has a satisfactory geometry, with an \(R\)-free factor of 25.8% and an \(R\)-factor of 19.2%. The refinement statistics are given in Table I. The coordinates have been deposited in the Protein Data Bank with accession number 1K8Q.

**RESULTS AND DISCUSSION**

**DGL Production and Purification**

A program of large-scale r-DGL production has been carried out by the Institut de Recherche Joueival/Parke-Davis in view of using it as a treatment for patients suffering from exocrine pancreatic insufficiency. Production of enzymes by transgenic plants makes it...
possible to obtain large production yields without risk of contamination by pathogens infectious to humans but containing the cell machinery mediating eukaryotic protein modifications, such as glycosylation. Indeed, the initial investment is considerable, and this procedure is limited to industrial projects. This kind of production in plants, although very efficient, is not likely to be used on the laboratory scale. However, we took advantage of the availability of gram quantities of DGL for setting up crystallization trials after further purification (see "Experimental Procedures"). The glycosylation pattern in plants, an essential point for the heavily glycosylated gastric lipases, is close to that of eukaryotic systems and does not present the hyperglycosylation features found in yeast.

The influence of pH on r-DGL specific activity was tested using an emulsion of short-chain (tributylglycerol) and long-chain (30% Intralipid, purified soybean oil emulsified with egg phosphatidylcholine) triglycerides as substrates. The maximal specific activity on long-chain triglycerides was 1000 units/mg.
at pH 4.0. With tributylglycerol, the activity follows a Gaussian curve centered at pH 5.4, with a maximum value of 420 units/mg and an S.D. of 1.4 pH units.

Crystallographic Procedure—After numerous unsuccessful attempts at obtaining crystals of gastric lipase from human and other species in its open form, we obtained dog gastric lipase crystals in complex with the C11Y4 phosphonate inhibitor. The high level of amino acid sequence identity between human and dog lipases (85.7%) made it possible to solve the structure using molecular replacement procedures with the core of the protein. The root mean square deviation between the two helices becomes buried, whereas the hydrophobic part constitutes a nonmobile extra domain. The lid and this extra domain have been called the cap in the closed HGL structure (23). Similar cap domains have also been observed in wheat serine carboxypeptidase II and in human protective protein, (23).

The core domain is located between residues 1–183 and 309–377 and contains a central β sheet composed of eight strands, seven of which are parallel and one anti-parallel (strand 2), with 1(–2)435678 connectivity and six helices, 3 on each side of the β sheet (Fig. 3A). The lid, the conformation of which differs between the closed (HGL) and open (r-DGL) forms (Fig. 3, B–D), starts abruptly at residue 212 and finishes at residue 251 and is formed of two helices, two elongated structures, and one turn (Fig. 3B). The movement of the lid cannot be described as a simple rigid body rotation. Two events occur during the lid opening: a conformational reorganization and a large movement. Although both closed and open lid conformations are mostly helical, their numbers of helices are different. The closed lid is made of three short helices (named α3, α4, and α5; Ref. 23) instead of two helices in the open form (Table II and Fig. 3B). Helix H1 (residues 217–228) of the open form is an extension of helix α3, and helix H2 (residues 231–244) originates from helices α4 and α5. The two helices, H1 and H2, are linked together by a disulfide bridge (Cys227–Cys236) in a similar way as helices α3 and α5 were in the closed form (23). The reorganization of the secondary structural elements leads to a structure that looks more compact and rigid. The opening of the lid is achieved by a 180° rotation of helices H1 and H2 around the axis of helix H2. As a result, the polar face of the two helices becomes buried, whereas the hydrophobic part becomes exposed, as previously described for other lipases. Whereas helix H2 rotates around itself, cysteine 244, at its terminal end, remains in the vicinity of the active site residues, which explains the inhibitory effect of mercurial compounds.

The domain comprising residues 184–211 and 252–308 is formed of four helices and does not belong to the core but constitutes a nonmobile extra domain. The lid and this extra domain have been called the cap in the closed HGL structure (23). Similar cap domains have also been observed in wheat serine carboxypeptidase II and in human protective protein, two protease members of the α/β hydrolase fold family. The HGL and r-DGL sequences contain four N-glycosylation consensus sites at Asn15, Asn80, Asn252, and Asn308. Electron density patches compatible with the first GlcNAc sugar attached to Asn were observed and introduced at all four sites. The sequence also contains three cysteine residues, one of which is free (Cys244), whereas the other two form a disulfide bridge (Cys227–Cys236), as previously described in the case of HGL (23).

Catalytic Machinery—Like other lipases and serine pro-
teases, r-DGL possesses catalytic machinery consisting of a Ser-His-Asp triad and an oxyanion hole (38). The catalytic serine (Ser153) is in a characteristic \( \text{H}_{9280} \) conformation (39) and is located in a tight turn with the G-H-S-Q-G sequence belonging to the usual consensus sequence of the \( \text{H}_{9251} \) \( \text{H}_{9252} \) hydrolase fold family. The r-DGL catalytic triad, which is composed of residues Ser153, His353, and Asp324, superimposes well on those of all the other lipases. The nucleophilic serine 153, which was covered by the lid in the closed form of HGL, becomes freely accessible to the solvent and binds to the inhibitor (Figs. 4 and 5). This finding definitively showed that Ser153 and not Cys244 is the nucleophilic residue involved in the catalytic event. On the basis of the present structure, the powerful inhibition observed with sulphydryl reagents (40, 41) can therefore be assigned to steric hindrance occurring at the level of the active site.

An oxygen atom originating from the inhibitor is at hydrogen bonding distance from the backbone nitrogens of Leu67 and Gln154 (2.60 and 2.92 Å, respectively). We previously suggested that these two residues might be oxyanion hole residues in the closed HGL form (Ref. 23 and Fig. 4). Apart from the lid, very few differences were observed between the open and closed structures. The loops bearing the catalytic serine and the oxyanion hole residue Leu67 are among the most mobile, however. The former residue moves 0.6 Å and the latter residue moves 1.6 Å between the open and closed forms. These movements result from the steric changes in the active site which occur on lid opening and ligand binding. They indicate only a slight adaptation of the oxyanion hole of gastric lipases, which is thus
mainly preformed, as occurs in other esterases such as cutinase (42) and C. rugosa lipase (43) but not in HPL (22).

The environment of the r-DGL catalytic triad does not seem to display any particularly original features, because one might expect His$_{195}^{308}$ to show an apparent $pK_a$ decrease to explain the acidic pH optimum of r-DGL. As in the closed HGL structure, however, there are no charged residues within a 10-A sphere centered on the Ser$_{153}^{157}$ O$_{\gamma}$ atom, which makes it impossible to suggest what mechanism may possibly be responsible for the apparent $pK_a$ modulation.

**Catalytic Crevice and Bound Inhibitor**—The catalytic crevice consists of a deep canyon 20 A long, 7 A wide, and 20 A deep (Fig. 5). This canyon is almost completely filled by a molecule of inhibitor bound to Ser$_{153}^{157}$ and by a molecule of $\beta$-OG, the detergent present in the crystallization medium (Fig. 5). Most of the canyon wall residues are hydrophobic, the majority being leucines, isoleucines, and valines (Fig. 4). The phosphonate inhibitor molecule covers 287 A$^2$ of the canyon water-accessible surface, and 86% of it consists of hydrophobic residues. Another canyon surface area of 274 A$^2$ is covered by the detergent molecule with the same proportion of hydrophobic residues. The $\beta$-OG molecule is stacked against the open lid, with which most of its interactions occur. Consequently, the lid area covered by the $\beta$-OG molecule is 156 A$^2$, whereas the latter detergent molecule covers only 73 and 45 A$^2$ of the cap and the core, respectively. The inhibitor is located opposite the lid and covers only 32 A$^2$ of the lid in a single interaction with Leu$_{143}^{243}$, whereas the inhibitor covers 103 and 152 A$^2$ of the cap and the core, respectively.

When the structures of the open HPL (19) and open r-DGL are superimposed using the core $\beta$ sheet as the reference, the phosphonate bound in HPL and the ligands in r-DGL fall on top of each other (Fig. 6A). The C4 chain of the r-DGL phosphonate inhibitor coincides with one of the HPL phosphonate enantiomers, whereas the other HPL phosphonate enantiomer superimposes with the alkyl moiety of the $\beta$-OG. The C11 chain of the r-DGL phosphonate enantiomer occupies a median position relative to the HPL inhibitor. However, after carbon 6, the C11 branch of the phosphonate inhibitor passes over the C4 branch and points sidewise toward one of the crevice side walls. The last carbon residues superimpose with the last carbon atoms of the HPL C11 inhibitor (Fig. 6A). This change of direction might be attributable to the steric hindrance of the $\beta$-OG polar head, contrary to what occurs with an alkyl chain. It seems likely that with a triglyceride, the sn1 chain may occupy the C4 chain of the r-DGL inhibitor and then continue up to and beyond the level of the last carbon atoms of the C11 chain (Fig. 6B). The median chain may follow the first five or six carbon atoms of the C11 chain and then continue toward the outside of the crevice (Fig. 6B). The sn3 triglyceride chain may follow the C8 alkyl chain of the detergent and continue up to the glycosyl head (Fig. 6B). The triglyceride chains may be embedded in the active site crevice up to carbons 12–14, and beyond this level they may interact with the lipid matrix. This binding pattern would also be in complete agreement with what has been observed in the active site crevice of HPL in complex with either a phospholipid or the C11-carboxymethyl phosphonate inhibitor (19, 22).

On the basis of the HPL phosphonate enantiomer conformations observed, we have suggested a triglyceride conformation bound to HPL (19). The same model could apply to the r-DGL active site, with the extra feature consisting of the median chain, which was not present in the case of the HPL phosphonate structure. Each branch of the triglyceride might then follow either the chain of the inhibitor or the alkyl chain of the detergent.

**Crystal Packing**—In all lipase systems, the opening of the lid buries the hydrophilic part of the lid and uncovers the hydrophobic part, thus setting up more favorable surface conditions for interactions to occur with the lipids. In the absence of a true lipidic aggregate, as in the crystallization medium, some lipase molecules can mimic the lipidic interface for each other, as observed in the case of pancreatic lipase (11, 44). In the r-DGL crystal packing arrangement, two symmetry-related molecules are placed with their lids facing each other in an interaction involving residues 201–208 from the cap and 218–225 from the lid. This interaction is less pronounced, however, than in the case of the human pancreatic lipase-porcine colipase complex (19). In particular, no electron density regions attributable to a detergent molecule have been detected in the intermolecular spaces.

**Fluorescence Study**—AMA has been found to be an excellent marker for hydrophobic binding studies (45, 46), because it gives considerably enhanced fluorescence emission levels, increasing from an aqueous to an apolar environment. We have thus recorded the spectra of r-DGL in the presence of increasing amounts of AMA, with and without the presence of phosphonate inhibitor (Fig. 7A). In the absence of the inhibitor, the closed lipase does not accommodate AMA, because no increase in the fluorescence emission was observed, whereas with the inhibited enzyme, both an increase in the fluorescence emission and a blue shift of the maximum emission wavelength of AMA were observed, indicating its binding to r-DGL and a drastic change in its environment. The binding of AMA can be described by a hyperbolic binding curve (Fig. 7B), giving a $K_d$ value of 7.5 $\mu$m. Indeed, part of the active site of inhibited r-DGL is occupied by the phosphonate inhibitor (Fig. 5). However, the x-ray structure indicates that there is room for an extra hydrophobic moiety in the active site crevice, which may be either $\beta$-OG (crystal structure) or AMA (fluorescence study). Modeling experiments indicate that an AMA molecule can replace the $\beta$-OG molecule and fit together with the inhibitor into the active site crevice of r-DGL (data not shown).

**Conclusion**—The structure of the complex between r-DGL and an alkyl phosphonate inhibitor makes it possible to confirm all the hypotheses put forward so far, based on the structure of the closed form of HGL as to the catalytic machinery and the lid position. The present data show for the first time the shape and the nature of the gastric lipase catalytic site, which turns out to form a long, deep, and narrow canyon. This canyon is about the size of a triglyceride molecule, with the three tips of the lipid chains pointing into the lipid phase. This completely new structural information will now make it possible to model the interactions between the enzyme and its inhibitors and substrates and might help explain the data obtained in comparative studies on the substrate specificities of gastric and pancreatic lipases.

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**REFERENCES**

1. Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L., and Verger, R. (1988) Biochim. Biophys. Acta 959, 247–252
2. Carrière, F., Gargouri, Y., Moreau, H., Ransac, S., Rogalska, E., and Verger, R. (1994) in Lipases: Their Structure, Biochemistry and Application (Wooley, P., and Petersen, S. B., eds), pp. 181–205, Cambridge University Press, Cambridge, United Kingdom
3. Hamosh, M. (1994) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed.), Vol. 1 and 2, 3rd Ed., pp. 1239–1253, Raven Press, New York
4. Bénicourt, C., Blanchard, C., Carrière, F., Verger, R., and Junien, J.-L. (1993) in *Clinical Ecology of Cystic Fibrosis* (Escobar, H., Baquero, C. F., and Suárez, L., eds), pp. 291–295, Elsevier Science Publishers, Amsterdam
5. Gargouri, Y., Piéroni, G., Rivière, C., Saumière, J.-F., Lowe, P. A., Sardà, L., and Verger, R. (1996) *Gastroenterology* 91, 819–925
6. Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L., and Verger, R. (1988) *Biochim. Biophys. Acta* 960, 288–293
7. Carrière, F., Moreau, H., Baghel, V., Laugier, R., Bénicourt, C., Junien, J.-L., and Verger, R. (1991) *EuJ. Biochem.* 202, 75–83
8. Joliff, G., Vaganay, S., Legay, C., and Bénicourt, C. (1998) *Biotechnol. Lett.* 7, 697–702
9. Gruber, V., Berna, P., Arnaud, T., Bournat, P., Clément, C., Mison, D., Olagnier, B., Philippe, L., Theisen, M., Baudino, S., Bénicourt, C., Cudrey, C., Blots, C., Duchateau, N., Dufour, S., Guescu, C., Jacquet, S., Olivo, C., Poncetta, C., Zorn, N., Ludévid, D., Van Dorselaer, A., Verger, R., Dubert, A., Mérat, B., and Danzin, C. (2001) *Mol. Breeding* 4, 329–340
10. Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, A., Arcya, A., and Hunziker, W. (1990) *Nature* 347, 814–815
11. Winkler, F. K., d'Arcy, A., and Hunziker, W. (1990) *Nature* 343, 771–774
12. Schrag, J. D., Li, Y., Wu, S., and Cygler, M. (1991) *Nature* 351, 761–764
13. Martinez, C., de Geus, P., Lauwereyns, M., Matthysse, G., and Cambillau, C. (1992) *Nature* 356, 615–618
14. Noble, M. E. M., Clesa, A., Johnson, L. N., Egmond, M. R., and Frenken, L. G. J. (1995) *FEBS Lett.* 331, 125–128
15. Cygler, M., Grochulski, P., Kazlauska, R. J., Schrag, J. D., Bouthillier, F., Rubin, B., Serreqi, A. N., and Gupta, A. K. (1993) *J. Am. Chem. Soc.* 115, 6017–6018
16. Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Borkjoling, F., Hug-Jensen, B., Patkar, S. A., and Thim, L. (1991) *Nature* 351, 491–494
17. Derewenda, U., Brzozowski, A. M., Lawson, D. M., and Derewenda, Z. S. (1992) *Biochemistry* 31, 1532–1541
18. Grochulski, P., Bouthillier, F., Kazlauska, R. J., Serreqi, A. N., Schrag, J. D., Ziomek, E., and Cygler, M. (1994) *Biochemistry* 33, 3494–3500
19. Eglof, M.-P., Marguet, F., Buono, G., Verger, R., Cambillau, C., and van Tilbeurgh, H. (1995) *Biochemistry* 34, 2751–2762
20. Marguet, F., Cydery, C., Verger, R., and Buono, G. (1994) *Biochim. Biophys. Acta* 1210, 157–166
21. Cavalier, J. F., Ransac, S., Verger, R., and Buono, G. (1999) *Chem. Phys. Lipids* 100, 3–11
22. van Tilbeurgh, H., Eglof, M.-P., Martinez, C., Bugani, N., Verger, R., and Cambillau, C. (1993) *Nature* 362, 814–820
23. Roussel, A., Canaan, S., Eglof, M. P., Rivière, M., Dupuis, L., Verger, R., and Cambillau, C. (1999) *J. Biol. Chem.* 274, 16995–17002
24. Blanchard, C., Bénicourt, C., and Junien, J. L. (June 23, 1994) Patent Coope- ration Treaty Patent WO94/13816
25. Junien, J. L., Verger, R., Lecat, D., and Moreau, H. (March 10, 1993) U.S. Patent 5075231 (December 24, 1991), EP Patent 0261016
26. Reina, M., Ponte, I., Guillen, B., Borrat, A., and Palau, J. (1990) *Nucleic Acids Res.* 18, 6496
27. Depecker, C., Stachel, S., Dhaese, P., Zambryski, P., and Goodman, H. M. (1982) *J. Mol. Appl. Genet.* 1, 561–573
28. Thompson, C. J., Novoa, N. R., Tizard, R., Cramerli, R., Davies, J. E., Lauwereyns, M., and Böttger, J. (1987) *EMBO J.* 6, 2519–2523
29. McElroy, D., Blowers, A. D., Jenes, B., and Wu, R. (1991) *Mol. Gen. Genet.* 221, 150–160
30. Armstrong, C. L., Green, C. E., and Phillips, R. L. (1991) *Molecular Genet. Coop. News* 65, 92–93
31. Tomes, D. T., Ross, M. C., and Songstad, D. D. (1996) in *Plant Cell and Organ Culture: Fundamental Methods* (Gamborg, O. L., and Philpis, C. G., eds), pp. 197–213, Springer-Verlag, Berlin
32. Smith, P. K., Krehl, B., Hermansg, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goek, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anat. Biochem.* 150, 76–85
33. Otwinovksy, Z., and Minor, W. (1997) *Methods Enzymol.* 276, 307–326
34. Navaza, J. (1994) *Acta Crystalllogr.* A50, 157–163
35. Roussel, A., and Cambillau, C. (1991) in *Silicon Graphics Geometry Geometry Partners Directory*, pp. 86, Silicon Graphics, Mountain View, CA
36. Brungar, A. T. (1996) *X-plor Version 3.843 Manual*, Yale University Press, New Haven, CT
37. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussexman, J. L., Verger, R., and Cambillau, C. (1992) *Protein Eng.* 5, 197–211
38. Kraut, J. (1977) *Ann. Rev. Biochem.* 46, 331–358
39. Brenner, S. (1988) *Nature* 334, 528–530
40. Gargouri, Y., Moreau, H., Piéroni, G., and Verger, R. (1988) *J. Biol. Chem.* 263, 2159–2162
41. Moreau, H., Gargouri, Y., Piéroni, G., and Verger, R. (1988) *FEBS Lett.* 236, 383–387
42. Martinez, C., Nicolais, A., van Tilbeurgh, H., Eglof, M.-P., Cydery, C., Verger, R., and Cambillau, C. (1994) *Biochemistry* 33, 83–89
43. 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
44. van Tilbeurgh, H., Sardà, L., and Verger, R. (1992) *Nature* 359, 159–162
45. Paoletti, S., Tanfani, F., Fino, C., Bertoli, E., Pelosi, P., and Breer, H. (1999) *Biochim. Biophys. Acta* 1431, 179–188
46. Campanacci, V., Krieger, J., Bette, S., Sturgis, J. N., Artigilie, C., Cambillau, C., Breer, H., and Tegoni, M. (2001) *J. Biol. Chem.* 276, 20678–20684
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