A Structural Domain (the Lid) Found in Pancreatic Lipases Is Absent in the Guinea Pig (Phospho)lipase†

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ABSTRACT: Typically pancreatic lipases are characterized by the following properties: (1) they are activated by lipid/water interfaces (interfacial activation), (2) they are inhibited by bile salts but reactivated by colipase (a small activator protein), and (3) they do not hydrolyze significantly phospholipids. A cDNA clone encoding a guinea pig pancreatic (phospho)lipase (GPL) has been sequenced and expressed. The enzyme (recombinant as well as native) differs from other pancreatic lipases in that (1) it is not interfacially activated, (2) its activity is unaffected by the presence of bile salts and/or colipase using tributyrin as substrate, and (3) it exhibits equally phospholipase A_1 and lipase activities. The amino acid sequence of GPL is highly homologous to that of other known pancreatic lipases, with the exception of a deletion in the so-called lid domain that regulates access to the active centers of other lipases. We propose that this deletion is directly responsible for the anomalous behavior of this enzyme. Thus GPL challenges the classical distinction between lipases, esterases, and phospholipases.

One characteristic and intriguing feature of lipolytic enzymes is their high activity in the presence of interfaces. In 1910, Rosenheim and Shaw-MacKenzie wrote "The physical condition of the zymolyte was found to be of the greatest importance for the action of lipase, a factor which has been neglected by many of the earlier observers". More recently, it has been shown that whereas the rate of breakdown of a dilute solution of triacylglycerol by pancreatic lipase is very slow, the enzymic activity increases dramatically once the substrate solubility is exceeded (Holwerda et al., 1936; Schonheyder & Volqvartz, 1945). In 1958, when highly purified preparations of enzyme became available, this aspect (interfacial activation) was reinvestigated in a more quantitative way, demonstrating a fundamental difference between esterase and pancreatic lipase based upon the presence or absence of interfacial activation (Sarda & Desnuelle, 1958). In contrast to esterases, which show normal Michaelis–Menten kinetics with respect to substrate concentration, lipases display almost no activity with the substrate present in its monomeric state. Once the solubility limit of triacetylglycerol is exceeded, there is, however, a sharp increase in lipase activity. On the basis of these observations, lipases were defined as a special class of esterases capable of hydrolyzing multimolecular aggregates at a high rate.

The X-ray structural studies on fungal (Brady et al., 1990; Brzozowski et al., 1991; Derewenda et al., 1990) and human (Winkler et al., 1990) pancreatic lipases suggest a structural basis for interfacial activation. Under aqueous conditions, the enzyme is inactive, as the catalytic apparatus is buried beneath a short amphipathic α-helix (the "lid"). However, in the presence of water-insoluble substances, the lid is displaced to one side, exposing both the active site and a larger hydrophobic surface, which is stabilized by adsorption to a nonpolar interface. This is the active conformation of the enzyme.

In addition to interfacial activation, pancreatic lipases are characterized by two other properties. First, they can be considered to be specific triacylglycerol hydrolases since they exhibit essentially no hydrolytic activity on phospholipid substrates such as lecithin and their enzymatic activity toward negatively charged phospholipids is very low compared to their activity upon triacylglycerols. Second, purified pancreatic lipases are inactive on an emulsified triacylglycerol substrate in the presence of a micellar concentration of bile salt. The bile salt coating of triglyceride globules present a negatively charged surface to the surrounding medium, thus inhibiting pancreatic lipase adsorption and activation. However, a specific lipase-anchoring protein present in the exocrine secretion of pancreas, colipase, counteracts this effect (Maylié et al., 1971; Borgström & Erlanson, 1971) through the formation of a specific 1:1 complex with lipase that facilitates adsorption at bile salt-covered lipid/water interfaces (van Tilbeurgh et al., 1992).

The guinea pig pancreatic (phospho)lipase (GPL) has been previously demonstrated to differ significantly from other known mammalian pancreatic lipases. It was first observed that phospholipase A activity was detectable in guinea pig pancreatic juice without trypsin activation (White & Hawthorne, 1970; Meldolesi et al., 1971), this being in direct contrast to all other reports on mammalian pancreatic phospholipase A_2 (Verheij & de Haas, 1991). Partial purification of this enzyme from guinea pig pancreas furthermore revealed that the phospholipase activity remained associated with lipase activity (Durand et al., 1978). Fauvel et al. (1981a) have purified two cationic lipases with high phospholipase A_1 activity from guinea pig pancreas (500 units/mg using the classical egg yolk assay (De Haas et al., 1968)) and demonstrated an unusually high phospholipase/lipase

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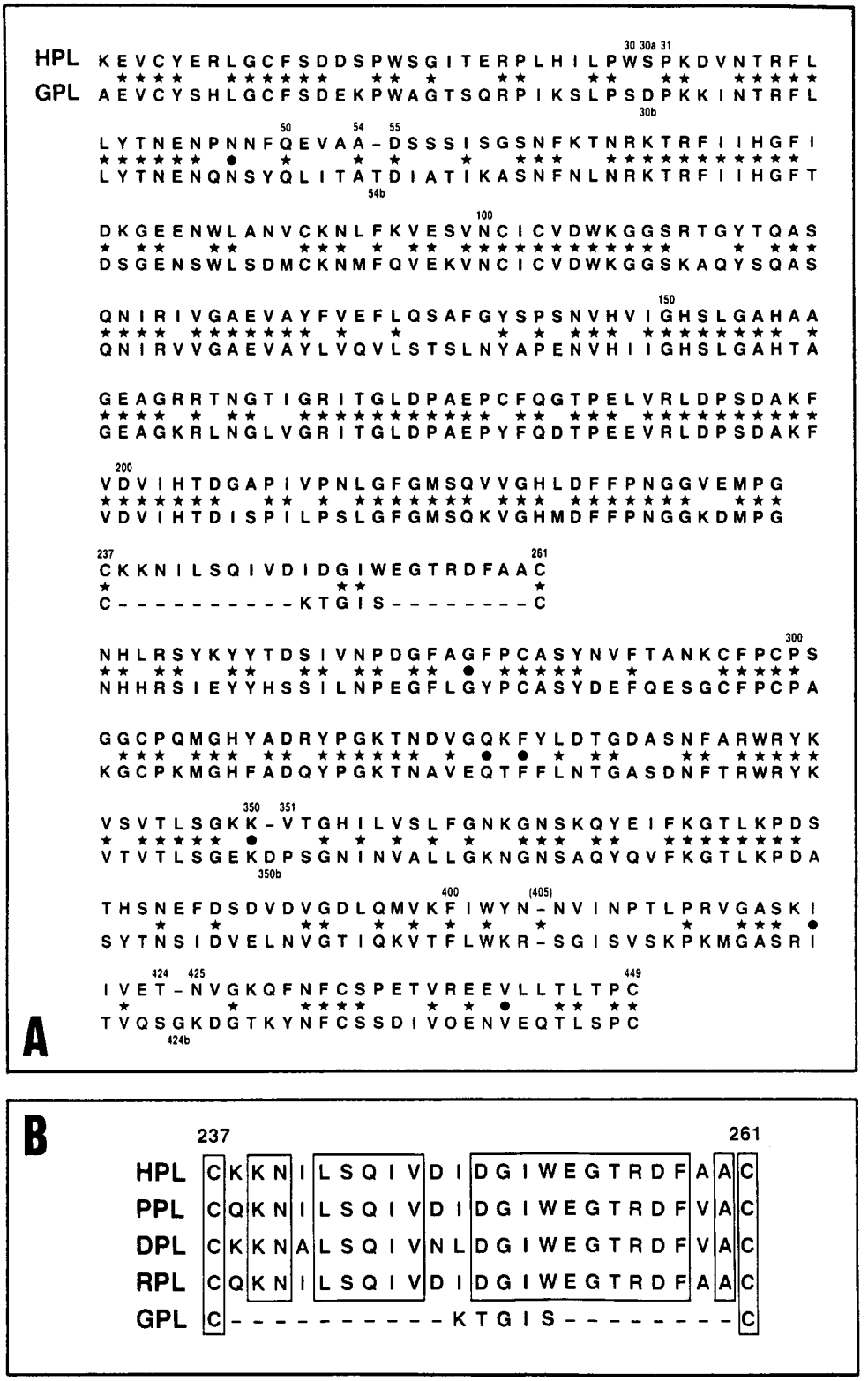

FIGURE 1: Sequence comparison of the guinea pig pancreatic (phospho)lipase (GPL) and the other known mammalian pancreatic lipases. (A) Sequence comparison of the GPL and the human pancreatic lipase. Identical amino acid residues are indicated by a star. (B) Sequence alignment of the lid domain of mammalian pancreatic lipases. The highly conserved nature of the lid domain is indicated by boxes of residues found at fully conserved positions. Abbrevations: HPL, human pancreatic lipase (Winkler et al., 1990); RPL, rat pancreatic lipase (H. F. Sims, GenBank Accession Number M58369); DPL, canine pancreatic lipase (Kerfelec et al., 1986); PPL, porcine pancreatic lipase (De Caro et al., 1981); GPL, guinea pig pancreatic lipase.

activity ratio of 1, higher by 3–5 orders of magnitude compared to lipases from other sources (Verger, 1984). Finally, no evidence suggesting the existence of a classical secretory phospholipase A_2 in guinea pig pancreas was found (Fauvel et al., 1981b). The high phospholipase A_1 activity of GPL might thus be of physiological significance for the degradation of dietary phospholipids, and a detailed study of this enzyme seemed pertinent.

EXPERIMENTAL PROCEDURES

Purification of Lipases from Guinea Pig Pancreas (GPL) and Dog Pancreatic Juice (DPL). GPL was purified from

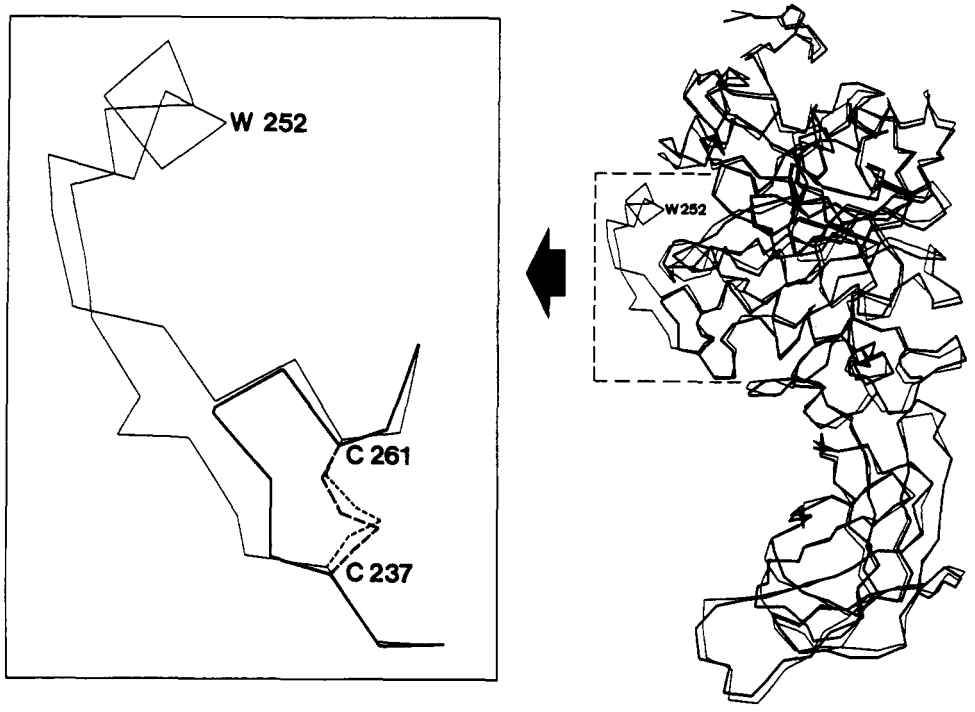

FIGURE 2: View of the superposition of GPL model (thick lines) and HPL (thin line) Cα backbones. GPL modeling was based on the HPL structure (Winkler et al., 1990) and the sequence comparison shown in Figure 1A. The closeup view shows the major difference between HPL and GPL: the lid domain in HPL between Cys 237 and Cys 261 being substituted by the GPL mini-lid.

guinea pig pancreatic powder (Sigma) and DPL from dog pancreatic juice using fast protein liquid chromatography on Mono Q and Mono S ionic exchangers and a Superose 12 gel-filtration column (Pharmacia, Les Ullys, France). GPL homogeneity was controlled by SDS–PAGE. Its isoelectric point was found at 9.4 using IEF. DPL homogeneity was also controlled by SDS–PAGE and N-terminal sequence analysis by comparison with the primary sequence derived from DPL cDNA (Kerfelec et al., 1986).

Lipase Activity Measurements. Using a pH-stat (TTT 80 radiometer, Copenhagen), lipase activities were measured potentiometrically in a thermostated reaction vessel (37 °C) containing a mechanically stirred emulsion of tributyrin (Fluka, Paris, France) in the assay buffers 0.3 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 1.4 mM $CaCl_2$ for interfacial activation measurements and 1 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 2 mM $CaCl_2$ for measurements of lipase sensitivity to bile salts (sodium taurodeoxycholate; Fluka, Paris, France). In the latter experiments, colipase (Boehringer Mannheim, Germany) was added at a molar excess of about 2.

Phospholipase Activity Measurements. Phospholipase activities were measured by the monolayer technique (Ransac et al., 1991) using a monomolecular films of various phospholipids as substrate (1,2-didodecanoylphosphatidylcholine, 1,2-didodecanoylphosphatidylethanolamine, 1,2-didodecanoylphosphatidylglycerol; Fluka, Paris, France). The monolayer was spread over 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA, contained in a zero-order trough. In all cases lipases were injected into the reaction compartment (volume, 51 mL; surface, 31 cm^2). For each phospholipid substrate, the enzymatic activity was measured at different surface pressures and compared to that obtained with diglyceride as substrate (1,2-didecanoylglycerol; Fluka, Paris, France).

Cloning and Expression of GPL. A homogeneous preparation of GPL (approximately 1 nmol) was reduced with dithiothreitol, and cysteine residues were derivatized with 4-vinylpyridine (Andrews & Dixon, 1981). The pyridylethylated GPL was isolated by chromatography on a Vydac 218TP54 column and digested with trypsin, and several tryptic peptides including the N- and C-terminal fragments were purified and sequenced on an Applied Biosystems Model 470A gas-phase sequencer (Thim et al., 1987). The N- and C-terminal peptides were of the following sequence: AEVCYSHLGCFSDEKPWAGTSQR and YNFCSSDIVQENVEQTLSPC, respectively. Poly(A+) RNA was prepared by the guanidine isothiocyanate/CsCl method (Chirgwin et al., 1979), followed by chromatography on oligo(dT) cellulose (Aviv & Leder, 1972). The guinea pig pancreatic cDNA library was constructed by the vector-primer method in plasmid vector pcDV1 (Okayama & Berg, 1983). The cDNA library was screened by hybridization (4 h at 45 °C in 6 × SSC) using ^{32}P-labeled oligonucleotides designed on the basis of sequences derived from the tryptic N- and C-terminal fragments. The sequences of the oligonucleotides (and the corresponding amino acids) were

N-terminal: 5′ GAY GAR AAR CCN TGG GC
D E K P W (A)

C-terminal: 5′ CAR GAR AAY GTN GAR CA
Q E N V E (Q)

Four-independent isolates that hybridized to both oligonucleotides were identified and subjected to DNA sequence analysis (Tabor & Richardson, 1987) (Sequenase; U.S. Biochemicals). The mini-lid structure was common to all four isolates, one of which corresponded to a full-length clone (1.4-kb insert).

Recombinant GPL was obtained by expression in *Aspergillus oryzae* (Christensen et al., 1988).

Modeling of the GPL Three-Dimensional Structure. GPL modeling was based on the HPL structure (Winkler et al., 1990) and the sequence comparison shown in Figure 1A. The GPL model was built using the Turbo-FRODO program

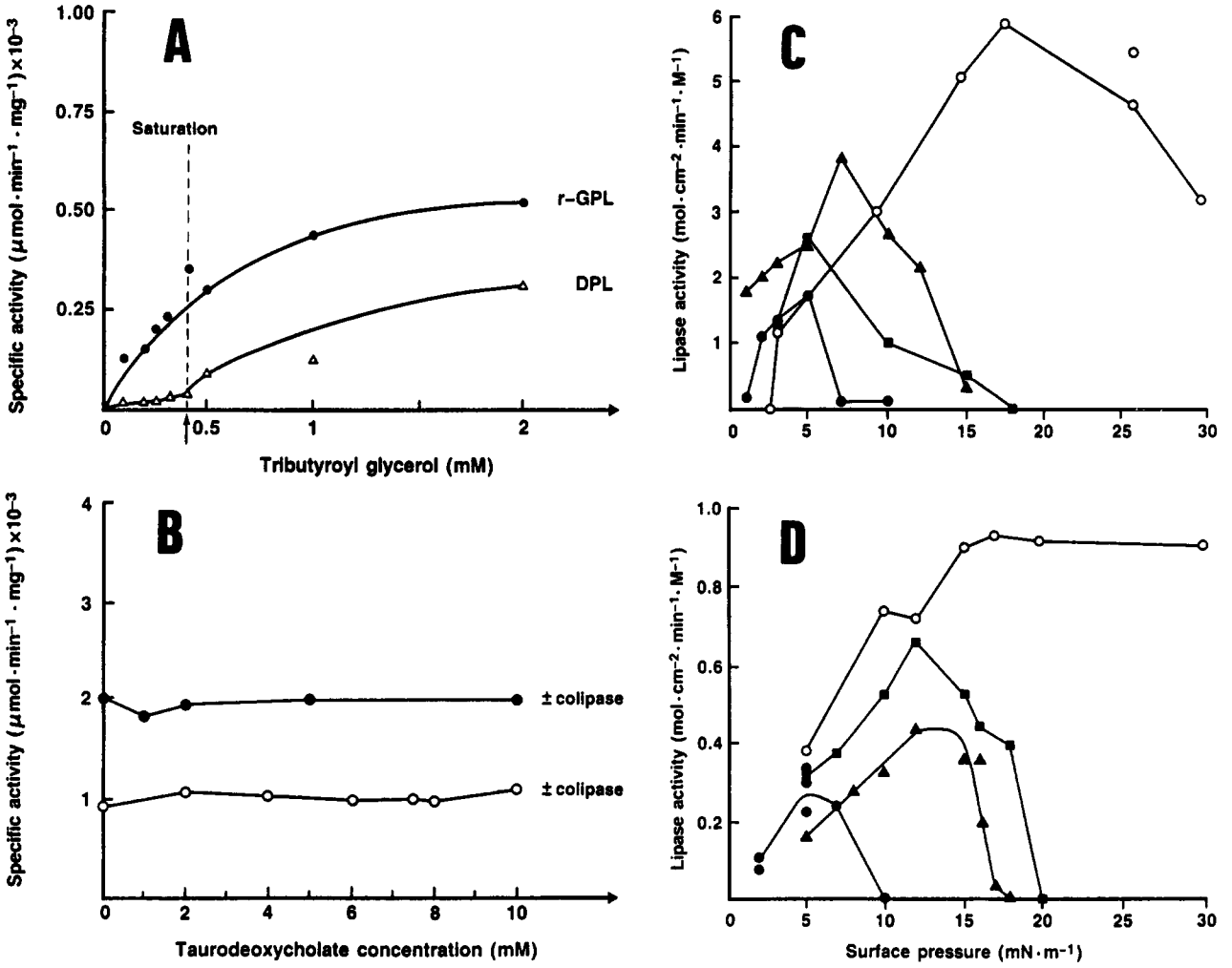

FIGURE 3: Kinetic properties of GPL. (A) Interfacial activation: (△) DPL (dog pancreatic lipase); (●) rGPL (recombinant GPL). The influence of tributyrin cncentration on lipase specific activity was measured by the pH-stat technique. As indicated by the stippled line, tributyrin saturation under the present experimental conditions was found at 0.4 mM by both turbidimetry and interfacial tension measurements. The assay (15 mL) contained 3.5–7.0 μg of rGPL and 2.5–5.0 μg of DPL, respectively, in 15 mL of assay buffer and various concentrations of tributyrin. (B) Sensitivity to bile salts: (●) recombinant GPL (average values of two determinations obtained with 2.2 μg) or (○) native GPL (average values of two determinations obtained with 2.8 μg). The lipase specific activity was measured by the pH-stat technique. The final assay volume consisted of 15 mL, containing 0.5 mL of tributyrin, 14.5 mL of assay buffer, and various concentrations of sodium taurodeoxycholate. (C and D) Comparison of GPL activity versus diglyceride and phospholipid substrates. Using the monolayer technique (Ransac et al., 1991), we measured the variation in lipase activity with surface pressure of 1,2-didecanoylglycerol (○), 1,2-didodecanoylphosphatidylcholine (●), 1,2-didodecanoylphosphatidylethanolamine (▲), and 1,2-didodecanoylphosphatidylglycerol (■) monolayers. Hydrolysis was catalyzed by native GPL (57 ng, panel C) or recombinant GPL (1 μg, panel D).

(Roussel & Cambillau, 1990) for residue replacement, deletions (18 residues in the lid domain), and insertions (Thr 54b, Asp 350b, Gly 424b). This model was subsequently subjected to molecular dynamics using the X-PLOR program (Brünger et al., 1989). HPL was numbered according to Winkler et al. (1990) and GPL according to Figure 1A.

RESULTS AND DISCUSSION

A full-length cDNA clone encoding guinea pig pancreatic lipase (GPL) was isolated and sequenced, demonstrating a high degree of homology in primary structure between GPL and the human pancreatic lipase (HPL), with one striking exception, viz., the presence in GPL of a short lid domain, five residues long (Figures 1A and 2). In HPL a stretch of 23 residues is found at the equivalent position. This so-called lid domain has been shown by X-ray analysis to cover the active site as a short one-turn α-helix (Winkler et al., 1990). A similar lid domain, delineated by two conserved cysteines, is seen in the primary structure of other mammalian pancreatic triglyceride lipases (Figure 1B). By inspection of the 3-D structure of two crystallized lipases, Winkler et al. (1990) and Brady et al. (1990) proposed independently that interfacial activation probably involved a reorientation of the lid domain, not only in HPL and in the *Rhizomucor miehei* lipase but also in the homologous hepatic and lipoprotein lipases. The noticeable deletion of precisely this domain in GPL prompted us to investigate the kinetic properties of this unusual lipolytic enzyme.

The recombinant like the native (data not shown) GPL displayed no interfacial activation using tributyroylglycerol as substrate, in contrast to dog pancreatic lipase (Figure 3a). The catalytic activity of GPL on a partially soluble triglyceride, such as tributyroylglycerol, is thus clearly more typical of an esterase than of a pancreatic lipase (Sarda & Desnuelle, 1958).

Another interesting kinetic feature of GPL (native and recombinant) is its insensitivity to the presence of bile salts (up to a final concentration of 10 mM) when using emulsified tributyroylglycerol as substrate (Figure 3B). This behavior contrasts sharply with the well-known inhibition by bile salts of all other lipases tested so far (Verger, 1984). As expected,

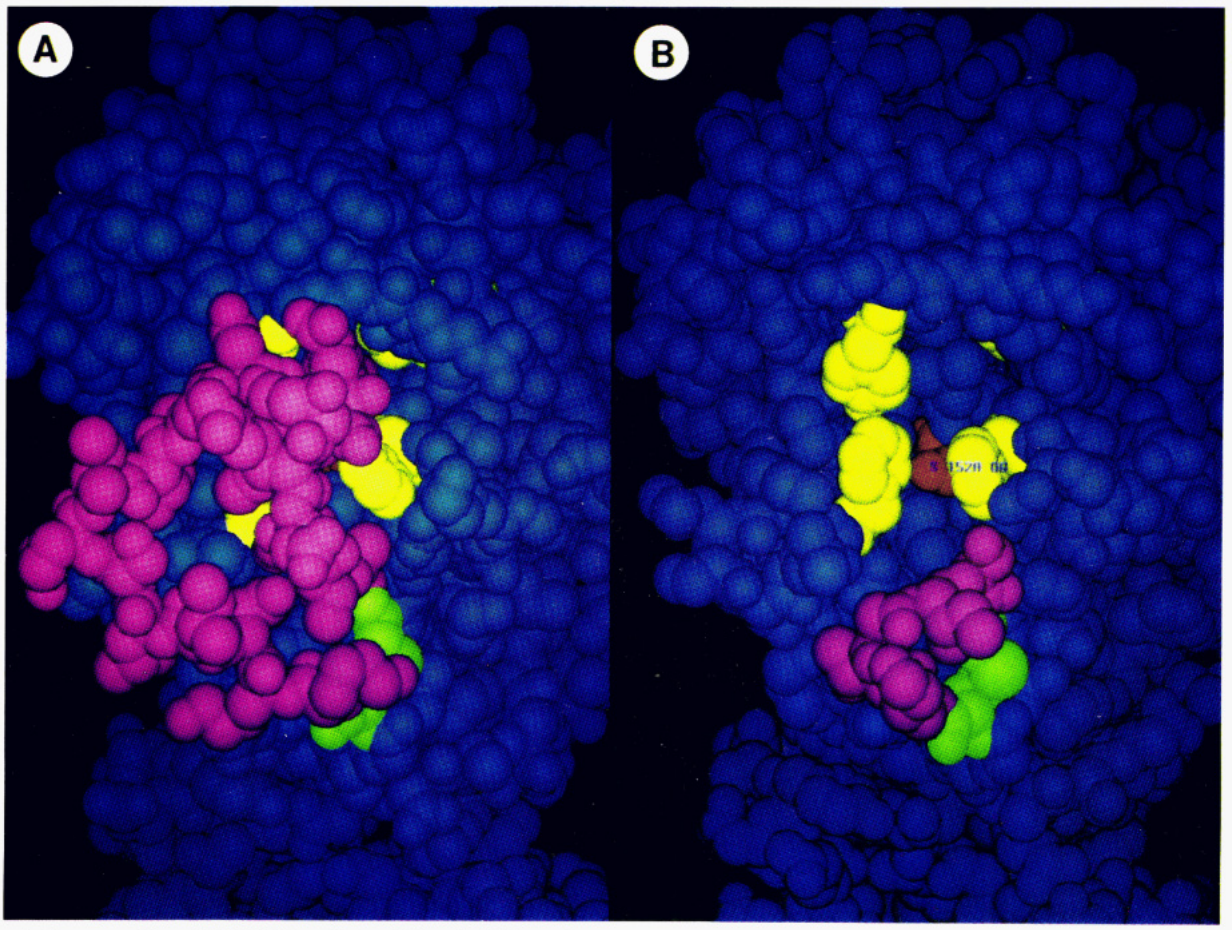

FIGURE 4: Compact view of HPL (A) and GPL (B). The lid (HPL) or the mini-lid (GPL) between the two cysteine residues (green) is purple, the four aromatic residues in the hydrophobic channel (Phe 77, Tyr 114, Trp 106, Phe 215) are yellow, and the active serine (Ser 152) is red. The mini-lid in GPL facilitates access to the active serine via a hydrophobic channel.

from the absence of the bile salt effect on tributyroylglycerol hydrolysis, the addition of colipase (from porcine or guinea pig origin) had no effect on the catalytic activity of GPL (Figure 3B).

Finally, as illustrated in Figure 3C,D, native and recombinant GPL hydrolyze monomolecular films of various phospholipids at rates comparable to those observed using 1,2-diglyceride as a substrate. Yet, it is worth noting that the optimal hydrolysis of the diglyceride substrate is found at a higher surface pressure compared to that seen with phospholipid substrates. It is a common property of pancreatic lipases to be more susceptible to the surface density of electrically charged chemical groups such as phospholipid polar heads than to the surface density of acyl chains of neutral lipids (Verger, 1984). These surface properties can explain the fact that the enzymatic activity of GPL on a phospholipid monolayer decreases more rapidly than the enzymatic activity on a diglyceride monolayer when the surface pressure is increased.

In view of these kinetic observations, the question arises as to whether there might be a direct structure–function relationship between the existence of a mini-lid structure in GPL and the unusual kinetic characteristics of this enzyme: its lack of interfacial activation, insensitivity to bile salts, and high catalytic activity on phospholipids.

In recent reports (Brzozowski et al., 1991; Derewenda et al., 1990; Martinez et al., 1992), the presence of a lid domain has been postulated to be associated with the phenomena of interfacial activation. Below the substrate saturation point, lipase reacts poorly with monomeric substrate since the lid domain covers the active site. In a lipase containing a "mini-lid", the catalytic site is, however, freely accessible to monomeric short-chain triglycerides as illustrated in Figure 4 by the molecular model of GPL. The lack of interfacial activation in GPL is probably a consequence of a maximal enzymatic activity being displayed on monomeric substrates. In fact, interfacial activation of classical lipases may be viewed as a depressed action on monomeric esters rather than an increased interfacial activity on aggregated substrates. In that sense, GPL is a true lipase distinguished by the absence of interfacial activation. The traditional classification of lipases and esterases is thus weakened.

The lack of bile salt inhibition and colipase effect on GPL activity on tributyrin is probably indirectly related to the presence of a mini-lid. In fact, the maximal enzymatic activity of GPL on monomeric sustrates does not allow one to see any effect of bile salts. Even if the enzyme is desorbed from the interface, it will remain active on monomers.

It is worth noting that the activity of a novel HPL-related protein (HPLRP2) cloned recently (Giller et al., 1992) likewise is relatively insensitive to bile salts, yet this enzyme possesses a lid domain. The activity of HPLRP2 was, however, assayed using an unsoluble long-chain triglyceride, suggesting that this enzyme is still capable of binding to the interface in the presence of bile salts. Since this enzyme only shows an amino acid sequence identity to HPL of 65%, another structural difference might explain its insensitivity to bile salts.

Yet, the question remains as to whether there is a simple structure–function relationship between the presence of a mini-lid domain and the unusually high phospholipase activity of

GPL. From the GPL modeling based on the HPL 3-D structure, the core of the N-terminal domain appeared to be conserved overall, with the exception of the lid domain. Furthermore, within a 10-Å-radius sphere of the active serine (Ser 152 Oγ), there are no insertions/deletions and only four minor residue changes pointing away from the active site [Ile 78 (HPL) → Thr (GPL)] or buried [(Ala 157 (HPL) → Thr (GPL); Leu 264 (HPL) → His (GPL); Tyr 267 (HPL) → Ile (GPL)]. One is therefore tempted to speculate that the lipase lid is indeed involved in substrate selectivity of these enzymes. It is known that pancreatic lipase can hydrolyze negatively charged phospholipids with a very low specific activity (Verger et al., 1977). Accordingly, the active center in pancreatic lipase can catalyze the hydrolysis of ester bonds in both triglycerides and phospholipids. Here again, the lack of high phospholipase activity in pancreatic lipase may be viewed as a depressed action on phospholipids due to the presence of the lid domain, rather than a total absence of catalysis.

It is worth noting that hepatic lipase, which belongs to the pancreatic lipase gene family (Komaromy & Schotz, 1987) and has a lid domain, also has a high phospholipase activity (Kinnunen, 1984). The amino acid sequence of the lid domain is, however, distinctly different from the HPL lid sequence. Thus the conclusions drawn from pancreatic lipase characterization might not be relevant for hepatic lipase. Furthermore, the role of the lid domain in substrate specificity is strengthen by a recent study concerning lipoprotein lipase, which is also related to pancreatic and hepatic lipases. Mutations in the lid domain of lipoprotein lipase affect differently the hydrolysis of short- and long-chain triglycerides (Dugi et al., 1992).

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