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Role of the Lid Hydrophobicity Pattern in Pancreatic Lipase Activity*

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Pancreatic lipase is a soluble globular protein that must undergo structural modifications before it can hydrolyze oil droplets coated with bile salts. The binding of colipase and movement of the lipase lid open access to the active site. Mechanisms triggering lid mobility are unclear. The *KNILSQIVDIDGI* fragment of the lid of the human pancreatic lipase is predicted by molecular modeling to be a tilted peptide. Tilted peptides are hydrophobicity motifs involved in membrane fusion and more globally in perturbations of hydrophobic/hydrophilic interfaces. Analysis of this lid fragment predicts no clear consensus of secondary structure that suggests that its structure is not strongly sequence determined and could vary with environment. Point mutations were designed to modify the hydrophobicity profile of the [240–252] fragment and their consequences on the lipase-mediated catalysis were tested. Two mutants, in which the tilted peptide motif was lost, also have poor activity on bile salt-coated oil droplets and cannot be reactivated by colipase. Conversely, one mutant in which a different tilted peptide is created retains colipase dependence. These results suggest that the tilted hydrophobicity pattern of the [240–252] fragment is neither important for colipase binding to lipase, nor for interfacial binding but is important to trigger the maximal catalytic efficiency of lipase in the presence of bile salt.

The pancreatic lipase-colipase complex plays a key role in dietary fat absorption in the intestine by converting triglycerides into more polar products able to cross the brush-border membrane of enterocytes. The lipase is fully active on water-insoluble substrates that form lipid/water interfaces, a phenomenon called interfacial activation. *In vivo*, oil droplets consist of a bulk substrate phase surrounded by a monolayer of amphiphilic compounds, mainly biliary lipids (phosphatidylcholine, cholesterol and bile salts) that prevent lipase adsorption. To counteract the inhibitory effect of biliary lipids, the pancreas secretes a small protein, colipase, which anchors lipase to the biliary lipid-coated water/lipid interface. The water-soluble lipase must therefore partition between aqueous and lipid phases before lipolysis can occur.

Structural studies (1–3) have shown that lipases possess a two-domain organization, the N-terminal domain bearing the active site and the C-terminal domain bearing the colipase binding site. One specific feature of lipase is the shielding of its catalytic site by a surface loop (lid) controlling the access of the substrate. Therefore, movement of the lid domain is an absolute requirement for the lipase to adopt an active conformation.

The role of the lid in lipolysis has been investigated by site-directed mutagenesis, lid exchange, or lid deletion (4–6). It was first thought to account for the interfacial activation of pancreatic lipase but the presence of a full-length lid in most pancreatic lipase-related proteins that display no interfacial activation rules out this explanation. Actually, it is not the lid *per se* but rather its motion and stabilization in the open conformation that are implicated in the activation of the lipase (6, 7). Contradictory results were reported for the lid implication in the lipase binding to an interface. In one study (5), the deletion of the [240–260] region impeded lipase binding whereas, in another study (6), the binding was maintained but the mini-lid lipase had very weak activity. Investigation of crystal structures supported the idea that side chains of the [251–259] lid fragment might be involved in the hydrophobic groove interacting with the substrate (8).

Despite the fact that structures of both the closed and open conformations of lipase are now known, the mechanism of the lid opening is still unclear. Several activation processes have been proposed. The enzyme theory states that the lipase undergoes a conformational change while penetrating the oil-water interface. However, Hermoso *et al.* (9) have shown that the lid opening can occur in the absence of emulsified substrate through the formation of a ternary lipase-colipase-biliary lipids micelle complex. These authors support the idea that lipase adsorption to the emulsified oil droplets can be mediated by a preformed lipase-colipase mixed micelle complex. Conversely, Sugar *et al.* (10) support the idea that activation is a surface-mediated process in which lipase binding depends on a special organization of substrate molecules at the interface. The lipase would bind only to "nanodomains" created by colipase from which phospholipids are somewhat excluded. Both theories are complementary, not exclusive.

Also emerging from all these studies is the idea that modifications of the lid result in important decreases in lipase activity together with a significant loss of the anchoring effect of colipase. Both features have no straightforward explanation.

In the last decades, fragments of proteins called tilted peptides have been evidenced as responsible for the disruption of lipid interfaces (11–13). Tilted peptides are short fragments (10–20 residues) of proteins that display a special hydrophobicity profile when they are helical, hydrophobicity being distributed asymmetrically along and across the helix (11). This hydrophobicity gradient determines the inclination of the tilted peptide at a lipid/water interface (40–50°) and influences its effects on lipids. When the peptide is long and hydrophobic enough to dive among the acyl chains, it is likely to perturb their parallel stacking (12). Tilted peptides have been identified in many proteins and protein fragments interacting with lipids such as viral fusion proteins (14), signal peptides of membrane-translocated proteins (15), and proteins involved in lipid metabolism (16). In the latter, they may increase the accessibility of enzymes to hydrophobic substrates (12, 17, 18). Hence,

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in contrast with classical amphipathic helices currently found in proteins and supported to promote protein stability, tilted peptides are a signature for instability (13). An important feature of some tilted peptides already discovered is their occurrence in fragments in which the secondary structure is not uniformly predicted by computer algorithms. Such variations support the idea that the structure is not determined solely from the sequence but rather could vary with the environment.

In this paper, we made a first approach to test the hypothesis whether a special hydrophobicity pattern might be involved in the function of the lipase lid. In this perspective, we analyzed the lipase sequence to detect tilted peptides. We focused our attention on one tilted peptide located in the lid. We looked for its structure instability characteristics. Mutants were designed to change the hydrophobicity profile of this peptide and different steps of the lipase-mediated catalysis were compared in the native and mutant enzymes.

MATERIALS AND METHODS

Predictions of Secondary Structures—Predictions were obtained from the Network Protein Sequence Analysis (19). The HNNC, PHD, Predator, SIMPA96, and SOPM algorithms were used.

Sequence Screening for Tilted Peptides—Tilted peptides were detected by a systematic screening of the human pancreatic lipase sequence (Protein Data Bank Code 1LPA). The sequence was cut into fragments of 10–15 residues. All fragments were calculated to be α-helix and structures were optimized by a Simplex energy minimization procedure. The behavior of each helix at the IMPALA[6] hydrophobic/hydrophilic interface (20) was then calculated by inserting the peptide at every Å of the model membrane from −40 Å (water medium) to 0 Å (membrane center), rotating the helix around its axis and rotating the helix axis with respect to the interface plane. At each step, the best position was selected as the lowest IMPALA restraint value. For this configuration, the restraint value (kcal/mol), the tilt angle (°), and the z value (Å) of the helix center were calculated.

IMPALA Procedure—IMPALA describes a hydrophobic interface as made of three layers, the water phase (water content = 1), the full hydrophobic phase (water content = 0), and a transition phase between where the water content varies with a z ordinate. The interactions between the peptide and the interface are approached by two energy restraint terms that vary with z. As previously described (20, 21), one restraint term describes the hydrophobicity effect that pushes hydrophilic atoms in water and draws hydrophobic atoms in the lipid phase, whereas the other one, the lipid perturbation, pushes any molecule out of the bilayer. In our assay, the peptide structure remains helical throughout the test. Therefore, the peptide energy remains constant, whereas the insertion restraints vary.

The restraint hydrophobicity term is as follows,

$$E_{\text{int}} = -\sum_{i=1}^{N} S_{(i)} E_{tr(i)} C_{(z_i)} \qquad \text{(Eq. 1)}$$

where N is the total number of atoms, $S_{(i)}$ the solvent accessible surface of atom i, $E_{tr(i)}$, its transfer energy per unit of accessible surface area, and $C_{(zi)}$, the value of $C_{(z)}$ at the position of the atom i.

The lipid perturbation term is as follows,

$$E_{\text{lip}} = a_{\text{lip}} \sum_{i=1}^{N} S_{(i)}(1 - C_{(z_i)}) \qquad \text{(Eq. 2)}$$

where α_{lip} is an empirical factor of 0.018 and $C_{(z)}$ the function describing the interface properties. $C_{(z)}$ is constant in the xy plane and varies along the z axis.

$$C_{(z)} = 1 - \frac{1}{1 + e^{\alpha(z - z_0)}} \qquad \text{(Eq. 3)}$$

Because IMPALA is currently used to mimic membrane properties, $z = 0$ is set at the center of a membrane. Ranges are $\pm\infty < z < \pm 20$ Å for the water phase, ± 20 Å $< z < \pm 15.5$ Å for the transition phase, and ± 15.5 Å $< z < 0$ Å for the water-free phase. a is a constant equal to 1.99 and z_0 corresponds to the middle of transition phase (17.75 Å).

Homology Modeling—The mutant lipase three-dimensional structures were calculated by substituting residues in the three-dimensional structures of 1LPA and 1N8S (PDB codes). Structure energies were minimized using the conjugate gradient of HyperChem and the AMBER force field.

Structure Properties—Residues in interaction and accessible surface area residue energy (van der Waals and Mean Force Potential) were calculated using the Pex program (Biosiris, Parc Crealys, 5032 Gembloux, Belgium) (22).

Construction, Expression, and Production of Modified Lipases—Mutations were introduced in the pVL1393HuPL plasmid by the PCR overlap extension technique (23) with two internal oligonucleotides carrying the specific mutations and two external oligonucleotides located in the pVL1393 vector. The presence of the desired mutation was ascertained by sequencing using the dideoxy chain termination method (24). The subsequent plasmids were then purified using the QIAfilter Plasmid Midi Kit (Qiagen) and used for cotransfection of *Sf*21 cells with the BD BaculoGold™ linearized DNA (BD Pharmingen). Expression and purification of the recombinant proteins were performed as previously described (25). Purification of lipases was followed by activity measurements, SDS-gel electrophoresis, and Western blotting. The protein concentrations were determined by UV spectrophotometry at 280 nm using the extinction coefficient of porcine lipase ($E^{1\%} = 1.33$) (26).

Gel Electrophoresis and Western Blotting—Electrophoresis on 12% polyacrylamide gels was carried out in the presence of SDS as described by Laemmli (27). Western blots were performed according to Burnette (28). The membranes were incubated with polyclonal anti-lipase antibodies from rabbit, and immunodetection was performed using alkaline phosphatase-labeled goat anti-rabbit IgG.

Activity Measurements—Lipase activities were potentiometrically determined at pH 7.5 and 25 °C using 0.11 M emulsified triacylbutyrylglycerol (tributyrin, Sigma) as substrate in 1 mM Tris-HCl buffer containing 0.1 M NaCl and 5 mM $CaCl_2$ in the presence of different concentrations of NaTDC (0 to 4 mM). When required, a 5-fold molar excess of colipase was added. One unit of lipase activity corresponds to the release of 1 μmol of fatty acid per minute. For the determination of the kinetic parameters, the experiments were performed as described above using various tributyrin concentrations (1–220 mM) in the presence of a 5-fold molar excess of colipase and in the presence (4 mM) or absence of NaTDC. The lipolytic activity was also measured on an olive oil emulsion made up of 95% olive oil, 4% L-α-phosphatidylcholine from egg yolk, and 1% free cholesterol (both from Sigma). The lipids (500 mg) were solubilized in 5 ml of chloroform/methanol (2:1, v/v). An aliquot (500 μl) of the mixture was dried under nitrogen, resuspended in 3 ml of

[6] The abbreviations used are: IMPALA, integral membrane protein and lipid association; TP, tilted peptide; NaTDC, sodium taurodeoxycholate; HuLip, human pancreatic lipase; E600, diethyl *p*-nitrophenyl phosphate; PLRP2, pancreatic lipase-related protein 2; ASA, accessible surface area.

a 1 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$, and sonicated for 5 min at 95% power level and a frequency of 20.178 Hz (Sonoreactor Undatim, Japan) in a ice/ethanol bath. The assays were performed as described above using 1 ml of olive oil emulsion as substrate in the presence of 6 mM NaTDC and a 5-fold molar excess of colipase.

Interfacial Binding of Native and Mutant Lipases on Tributyrin Emulsion—The interfacial binding of HuPL was assayed as previously described by Borgström (29). Briefly, a tributyrin emulsion (0.11 M) was formed in a pH-stat vial in 1 mM Tris-HCl buffer, pH 7.5, 0.1 M NaCl, and 5 mM $CaCl_2$ with or without 0.5 or 4 mM NaTDC. When required a 5-fold molar excess of colipase was added. The emulsion was continually stirred for stabilization and pH monitoring. After 5 min, lipase (50 units) was added and after a 2-min incubation, the mixture was centrifuged at low speed (1,200 rpm) for 10 min to separate the oil phase from the water phase. The residual lipase activity in the water phase was determined on a 200-μl aliquot of the supernatant, using tributyrin as substrate, in the presence of 0.5 mM NaTDC and excess colipase.

TABLE ONE

Total, hydrophilic (Phi) and hydrophobic (Pho) accessible surface areas (Å^2) of open and closed lipases as monomer or as a complex with colipase

Values were extracted from Pex and calculated using the Shrake and Ripley algorithm (30). The last column is the Pho/Phi surface area ratio.

		Phi ASA	Pho ASA	Total	Pho/Phi ratio
Closed lipase	1N8S	9,059	9,278	17,732	1.02
Open lipase	1LPA	8,083	10,303	18,386	1.27
Closed complex	1N8S	11,123	11,982	23,105	1.08
Open complex	1LPA	8,853	12,327	21,180	1.39

RESULTS

Structural Study

Accessible Surface Areas of the Pancreatic Lipase Structures: Closed and Open Conformations—Accessible surface area (ASA) of globular proteins, in general, is equally hydrophobic and hydrophilic (30). Distribution of hydrophobicity corresponds to the alternate dispersion of small patches at the protein surface. Analysis of the pancreatic lipase surface shows that the closed lipase structure has a Pho/Phi ASA ratio of 1.02, characteristic of soluble proteins (TABLE ONE).

In the open conformation, this ratio was raised to 1.27, indicating an increase in hydrophobic surface. This increment is restricted to one part of the structure close to the active site (Fig. 1, TABLE TWO).

Similar observations can be made for the ASA of the lipase-colipase complex. The total surface is larger because of the presence of colipase but the hydrophobicity ratio is still 1.08 for the closed conformation and increases to 1.39 for the open conformation (TABLE ONE). Hence, the hydrophobic character of the lipase alone and the lipase-colipase complex is not changed by the colipase binding *per se* but is rather increased by the lid opening.

Considered as an individual domain, the lid ASAs are 1625 Å^2 in the closed structure and 1731 Å^2 in the open structure, but the hydrophobic

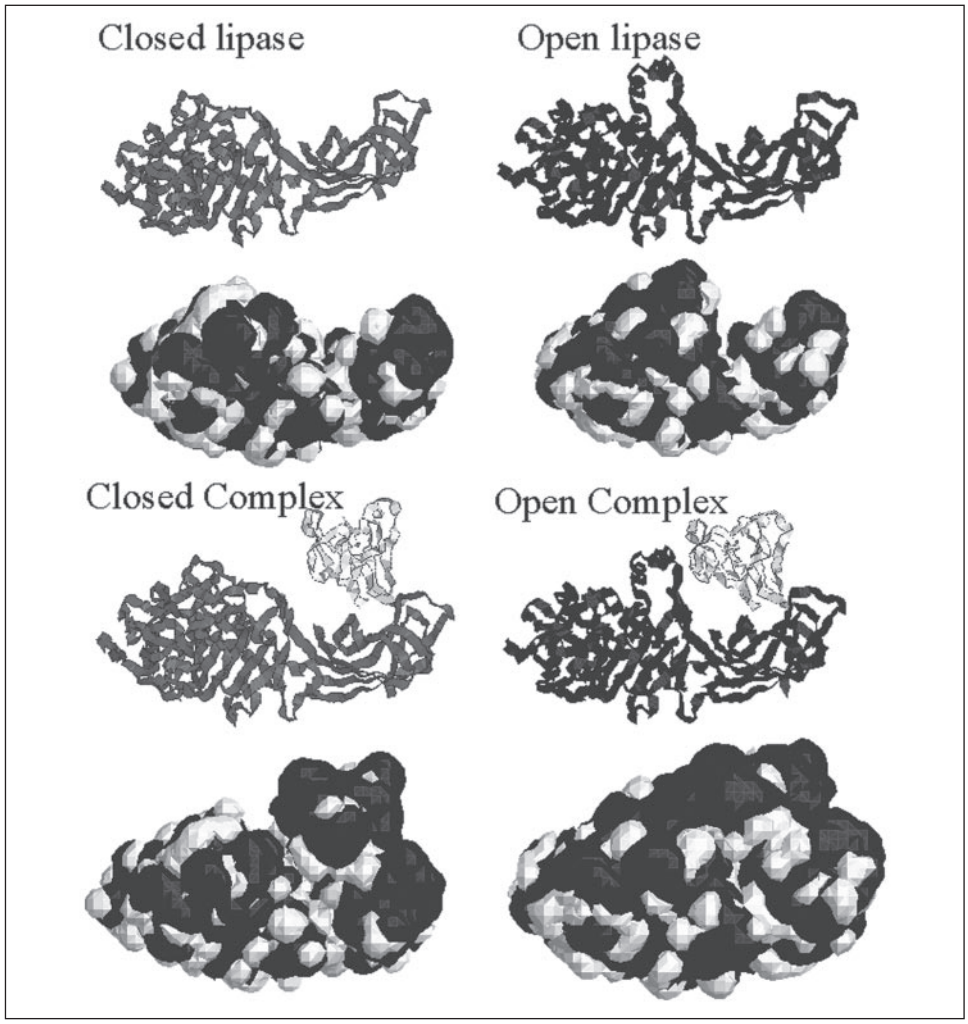

FIGURE 1. **Structure and molecular hydrophobicity potential surface of the closed and open pancreatic lipases.** *Top*, closed (PDB code 1N8S) and open (PDB code 1LPA) lipases. *Bottom*, closed and open lipase-colipase complexes. Hydrophilic (0.1 kcal/mol) and hydrophobic (−0.1 kcal/mol) isopotential surfaces were calculated according to Brasseur *et al.* (11) and are represented in *white* and *black*, respectively.

TABLE TWO

Accessible surface area variations (Å²) of individual residues in the open and closed lipase structures

Values were extracted from Pex files (30).

Amino acid	
Lys^{1}	−71
Asn^{409}	+61
Cys^{449}	−104
Lid	
Gln^{244}	−122
Ile^{245}	−90
Ile^{251}	+100
Trp^{252}	+102
Arg^{256}	−122
Asp^{257}	−139
Phe^{258}	+65
Ala^{259}	+55
Hydrophobic groove	
Pro^{211}	+70
Leu^{213}	+100
Phe^{215}	+71
β5 loop	
Ile^{8}	+75
Lys^{80}	−146
Glu^{83}	−91

parts of these areas are 800 and 1226 Å², respectively. Hence, the accessible surface area of the open lid is more hydrophobic as evidenced by the molecular hydrophobicity potential profile shown in Fig. 2 where the surface of the lid is turned outside up. The largest changes in ASA are observed for residues in the lid domain, in the hydrophobic groove close to the active site, and in the β5 loop (TABLE TWO).

Unmasked residues are mostly hydrophobic, whereas buried ones are more hydrophilic. This accounts for the hydrophobicity increase of the accessible surface and is in good agreement with stabilization of the open lid by intra- and inter- (with colipase) molecular polar interactions.

The large lid movement at the lipase surface is accompanied by a change in its secondary structure (Fig. 2). Mostly β-extended in the closed conformation, the structure is more than 50% helical in the open one.

Sequence Analysis—The tilted peptide candidates in human lipase were detected by screening the hydrophobicity patterns of the sequence (PDB code 1LPA). All sequence stretches of 10 to 15 residues (1970 fragments) were calculated as α-helical structures and tested for the best position of insertion at a hydrophobic interface. Most of the 1970 fragments adsorb parallel (angles 0 to 20°) (Fig. 3). Only 5.7% of the fragments insert tilted with an angle of 40–50°.

Among all the tilted peptides detected, we selected one located in the lid, TP1, which spans residues ^{240}KNILSQIVDIDGI^{252}. Indeed this peptide fulfils the two criterions of our search.

First, the sequence should not determine a definite structure. As shown in TABLE THREE, the predicted secondary structure of the TP1 fragment largely varies with the predictions algorithms. Moreover, in agreement with the idea of a structural susceptibility to the environment, TP1 has different conformations in the open and closed structures. It is mainly extended in the closed lipase, whereas in the open conformation, a short helix LSQIVD bordering a turn (IDGI) is formed. Second, the energy minimization by partition of hydrophobicity should not give a symmetric amphipathic α helix. After testing the α helix

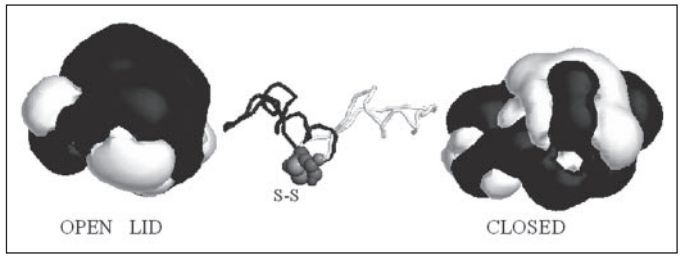

FIGURE 2. **Lid movement around the S-S bridge and molecular hydrophobicity potential profiles of closed and open lid conformations.** In the *middle figure*, the open (*black ribbon*) and closed (*white ribbon*) lid structures are adjusted by fitting their Cys-Cys residues. The lipase external side is up, the lipase core is down. In the two *side figures*, the molecular hydrophobicity potential profiles of the lids are calculated according to Brasseur *et al.* (11) (*black*, hydrophobic surface (−0.1 kcal/mol) and *white*, hydrophilic surface (+0.1 kcal/mol)).

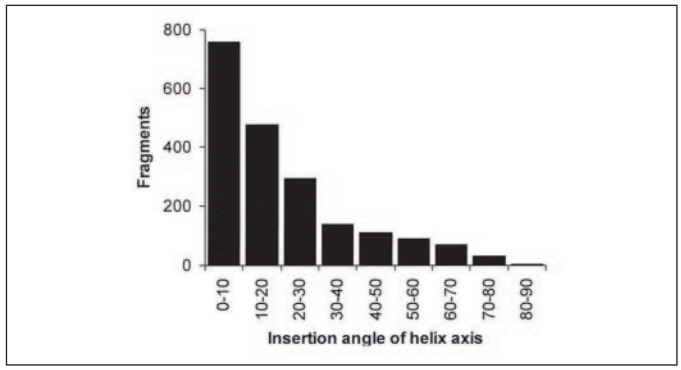

FIGURE 3. **Distribution of amphipathic helices based on their angle of insertion at a model water/lipid interface.** The human pancreatic lipase sequence was cut into fragments of 10–14 residues. All fragments were calculated as α helices and tested for interfacial adsorption in IMPALA as described under "Materials and Methods." For each fragment, the best position was selected as giving the minimal insertion restraint value. It corresponds to an angle between the helix axis and the interface plane. All fragments were classified according to this angle.

conformation of this peptide at the interface, the best TP1 position corresponds to a peptide with an insertion angle of 42° with respect to the plane of this interface and a mass center at the level of the interface (Fig. 4 and TABLE FOUR).

Mutant Design—TP1 mutants were designed by residue permutation to keep the mean hydrophobicity of the fragment constant but to vary its distribution in the sequence to destroy all possibilities of tilted insertion in a hydrophobicity interface.

The mutant TP1_A was obtained by permuting Leu^{243} for Asp^{248}, the mutant TP1_B by exchanging Ile^{242} for Asp^{248}, and the mutant C was obtained by permutation of Gln^{244} and Trp^{253}, although Trp^{253} is not in the tilted peptide sequence *per se*. In each case, IMPALA analysis was performed on a sequence longer than TP1, residues [238–256], to ascertain that residue permutations will not create new tilted peptides.

Permutation of the Leu^{243} for Asp^{248} residue in mutant A transforms the native TP1 into an amphipathic helix lying flat at the lipid interface (Fig. 4 and TABLE FOUR). Furthermore, the insertion angles of all fragments spanning the [238–256] sequence now range from 0 to 27°. This indicates that the possibility for any fragment of this sequence to insert oblique at the lipid interface has disappeared. The possibility for generating a hydrophobic gradient is lost in mutant A.

In TP1_B, permuting Ile^{242} for Asp^{248} transforms the native TP1 into a peptide with a tilt angle higher than 50° inserting deeper in the hydrophobic phase (TABLE FOUR). IMPALA analysis of the [238–256] sequence shows that the fragments apart from TP1_B are either inserted deeper in the hydrophobic phase than the native peptide or lie flat at the interface (tilt angles less than 24°). Hence, the behavior of

TABLE THREE

Prediction of secondary structures of the [238–256] sequence using a series of computer algorithms

The predictions are extracted from the 1LPA sequence analysis: c is the random coil; h is the helix; and e is the β extended structures.

	Residue No.																	
	Cys^{238}	Lys^{239}	Lys^{240}	Asn^{241}	Ile^{242}	Leu^{243}	Ser^{244}	Gln^{245}	Ile^{246}	Val^{247}	Asp^{248}	Ile^{249}	Asp^{250}	Gly^{251}	Ile^{252}	Trp^{253}	Glu^{254}	Gly^{255}
HNNC	c	c	h	c	e	e	e	e	e	e	e	c	c	c	h	h	h	h
PHD	c	c	c	e	e	e	e	e	e	e	e	c	c	c	c	c	c	c
Predator	c	c	h	h	c	c	c	c	c	c	h	h	h	h	c	c	c	c
SIMPA96	h	h	h	h	h	h	h	h	c	c	c	c	c	c	c	c	c	c
SOPM	c	c	h	h	h	h	h	h	e	e	e	h	h	c	e	h	t	c

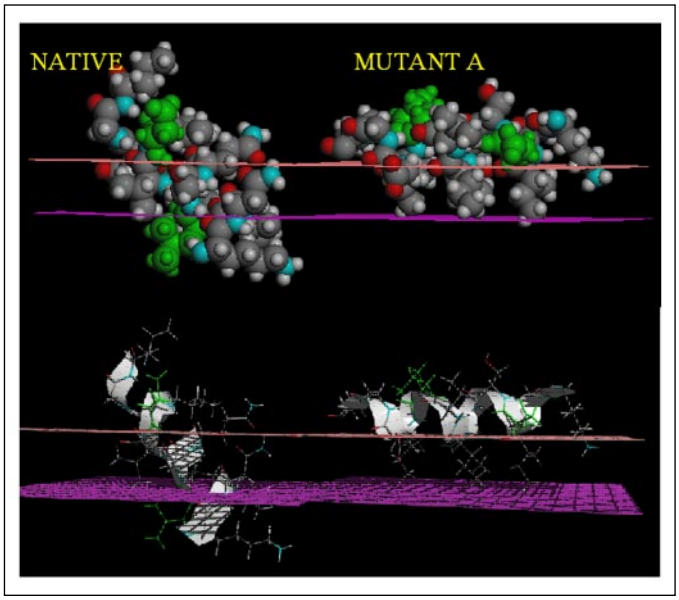

FIGURE 4. **Insertion of the wild type TP1 and of the mutant TP1_A peptides at the IMPALA interface.** The fragments of sequence were calculated as α-helices and tested for insertion in IMPALA by the systematic assay described under "Materials and Methods." The positions of the lowest IMPALA restraint energy are shown on the figure for the wild type sequence and the mutant A sequence. The *pink* plane is the membrane-water interface, the *violet* plane corresponds to the lipid polar head/acyl chain interface. In *green* are the residues that were permuted (Leu and Asp). On *top*, CPK views; at the *bottom*, ribbon and stick views. The angle between the helix axis and the water/lipid interface of the wild type peptide is 42°, that of the mutant is 4°.

mutant B is different from that of the native sequence and two configurations, parallel and perpendicular, are energetically possible.

In mutant TP1_C, the [240–252] peptide corresponding to TP1 is a classical amphipathic helix with an angle of insertion of 8°. However, several fragments spanning the [238–256] sequence, including TP1_C, populate the tilted region supporting the idea that the permutation Gln^{244} for Trp^{253} has shifted rather than destroyed the hydrophobicity gradient capacity. Hence, we concluded that the permutation has created a new tilted peptide (^{240}KNILSWIVDIDGIQ^{253}) (TABLE FOUR). It must be pointed out that the [240–253] fragment in the native, mutant A and mutant B sequences is not tilted (TABLE FOUR).

In previous studies on a series of proteins (13), parallel and perpendicular mutants of tilted peptides were shown to impair the functional capacities of their parent protein. Hence, if the hydrophobicity gradient plays a role in the lipase lid function, the TP_1A and TP_1B mutants should display modified activity, whereas the TP1_C mutant might display properties similar to those of the native enzyme.

By constructing computer models of the open and the closed mutant structures, we verified that no steric clash was created and that open and closed conformations of mutants were energetically possible. Consequently, the above permutations were introduced in the human lipase (HuLip) and properties of the variant lipases were investigated.

Biological Activities

Effect of NaTDC and Colipase on Native and Mutant Lipases Activity—In the absence of both NaTDC and colipase, measurements of initial velocities were inaccurate for all enzymes because of nonlinear kinetics (no "zero" point in Fig. 5). This property, already reported by Brockerhoff (31) and Vandermeers *et al.* (32), is described because of an interfacial denaturation of proteins. By contrast, in the absence of NaTDC but in the presence of colipase, the native lipase is stabilized and displays full activity. This is true also for the three mutants that retain more than 90% of the native lipase activity in these conditions (Fig. 5). This result indicates that the mutations are well supported by the protein and that the active site is operational. It also supports that the three mutants, as the native lipase, require the presence of colipase to be stabilized and active in the absence of bile salt.

As reported for native HuLip, the activity of HuLipTP1_A, -B, and -C are inhibited by increasing concentrations of NaTDC in the absence of colipase. Interestingly, the inhibitory effect of NaTDC on HuLipTP1_A activity is observed even at a NaTDC concentration below the critical micellar concentration (<1 mM), suggesting an effect different from the interfacial detergent activity. However, in contrast with what occurs for the native enzyme, mutants A and B are only weakly reactivated by addition of colipase. HuLipTP1_A is the most affected because its activity is not restored at 4 mM NaTDC, whereas HuLipTP1_B recovers 33% of the native lipase activity. HuLipTP1_C, on the other hand, displays a behavior more similar to that of native lipase because 50–60% of its activity is restored by colipase at 4 mM NaTDC. Hence, disrupting the hydrophobicity pattern of the [240–252] fragment of the lid does not affect the lipase activity *per se* but prevents the colipase regenerating effect.

Effect of Mutations on the Lipase/Colipase Apparent Affinity—Because in its open conformation the lid interacts with colipase, introducing mutations in this region might induce a modification in the lipase/colipase affinity. The rate of tributyrin hydrolysis was determined at 4 mM NaTDC, a concentration for which the presence of colipase is an absolute requirement. The tributyrin concentration was close to the substrate saturating concentration; thus experimental values of V_m should approach the limiting rate. The experimental points measuring lipase activity at different colipase concentrations were fitted to hyperbolic curves corresponding to a 1:1 stoichiometry between lipase and colipase. The $K_{d(app)}$ (concentration of colipase required for half-maximal activity) and V_m (maximal activity rate at 100% lipase complexed with colipase) were extrapolated from curves.

As shown in TABLE FIVE, all three mutants display an apparent colipase affinity similar to that of native lipase. This indicates that the residue permutations do not affect colipase binding. On the other hand,

TABLE FOUR

Analysis of the IMPALA results for the sequence fragments of 13 amino acids around the TP1 sequence (amino acids 240–252)

For the sake of comparison, fragment 240–253 was added for all sequences because this fragment is tilted in mutant C. Columns list the first and last residue numbers, the peptide length, the IMPALA restraint value (kcal/mol), the *Z* ordinate of the helix mass center (*Å*), and the tilt angle of the helix axis with respect to the interface planes (degrees). *Z* values are $\pm\infty < z < \pm 20$ Å for the water phase, ± 20 Å $< z < \pm 15.5$ Å for the transition phase, and ± 15.5 Å $< z < 0$ Å for the water-free phase; *Z* values over 20 Å correspond to pure water phase, and *z* values under 15.5 correspond to pure hydrophobic phases.

	From aa	To aa	Peptide length	IMPALA restraint	IMPALA z ordinate	Helix/interface angle	Sequence
Wild type	238	250	13	−21.4	17.3	23	CKKNILSQIVDID
	239	251	13	−21.2	18.3	25	KKNILSQIVDIDG
TP_1	**240**	**252**	**13**	**−15.5**	**17.0**	**42**	**KNILSQIVDIDGI**
	241	253	13	−15.0	13.0	27	NILSQIVDIDGIW
	242	254	13	−16.9	19.0	35	ILSQIVDIDGIWE
	243	255	13	−15.0	17.0	16	LSQIVDIDGIWEG
	240	253	14	−15.9	13.8	65	KNILSQIVDIDGIW
Mutant A	238	250	13	−14.8	19.8	0	CKKNIDSQIVLID
	239	251	13	−17.2	19.5	3	KKNIDSQIVLIDG
TP1_A	**240**	**252**	**13**	**−13.9**	**19.5**	**4**	**KNIDSQIVLIDGI**
	241	253	13	−13.8	18.5	3	NIDSQIVLIDGIW
	242	254	13	−16.7	16.0	19	IDSQIVLIDGIWE
	243	255	13	−13.5	17.3	0	DSQIVLIDGIWEG
	240	253	14	−14.8	19.0	6	KNIDSQIVLIDGIW
Mutant B	238	250	13	−13.1	18.0	20	CKKNDLSQIVIID
	239	251	13	−14.8	15.0	54	KKNDLSQIVIIDG
TP1_B	**240**	**252**	**13**	**−15.8**	**14.0**	**55**	**KNDLSQIVIIDGI**
	241	253	13	−16.2	12.0	58	NDLSQIVIIDGIW
	242	254	13	−10.5	14.3	30	DLSQIVIIDGIWE
	243	255	13	−16.0	12.5	27	LSQIVIIDGIWEG
	240	253	14	−17.2	12.8	53	KNDLSQIVIIDGIW
Mutant C	239	251	13	−17.0	18.3	1	KKNILSWIVDIDG
	240	252	13	−13.3	17.8	8	KNILSWIVDIDGI
	241	253	13	−15.3	18.0	6	NILSWIVDIDGIQ
	242	254	13	−17.9	11.5	58	ILSWIVDIDGIQG
	243	255	13	−17.4	17.3	19	LSWIVDIDGIQGT
TP1_C	240	253	14	−14.0	17.8	43	**KNILSWIVDIDGIQ**

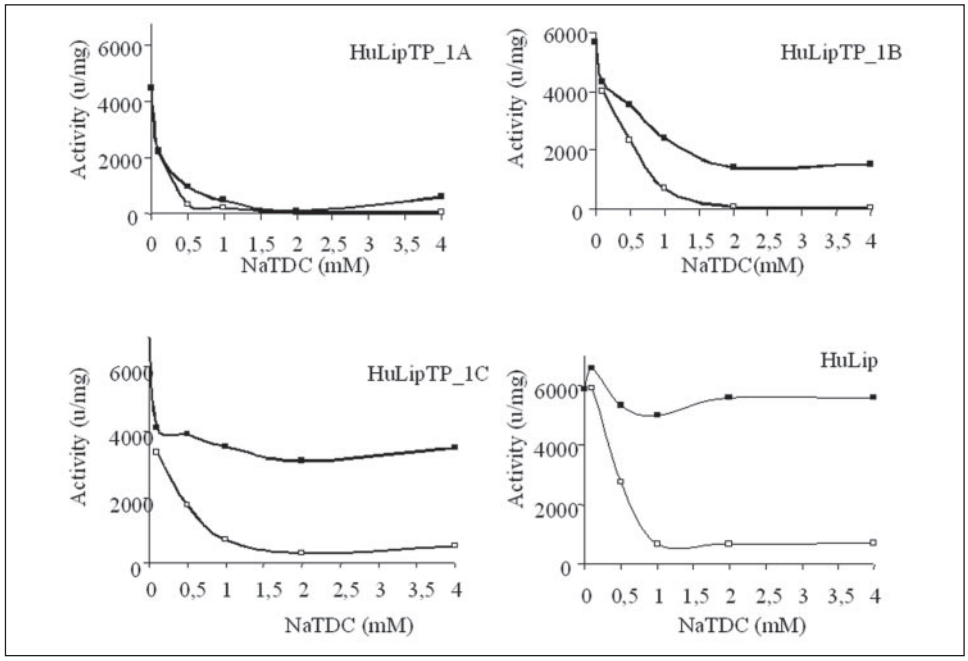

FIGURE 5. **Effects of NaTDC concentration and colipase on the lipase activity of HuLip and mutants on emulsified tributyrin.** Lipase activity was potentiometrically determined at 25 °C using 0.11 M emulsified tributyrin, pH 7.5, in the absence (○) or presence (■) of a 5-fold excess of colipase. The values of *V* are expressed as units (μmol of released fatty acids/min)/mg of either native or mutant lipases.

a noticeable decrease of the hydrolysis rate is observed for mutants A and B in agreement with results from Fig. 5.

Adsorption of Native and Mutant Lipases on Tributyrin Emulsion—The variations of the interfacial binding of native HuLip, HuLipTP1_A, and HuLipTP1_C were investigated using a tributyrin emulsion in the presence of two different NaTDC concentrations (0.5 and 4 mM) and in the presence or absence of colipase. After separation of the oil droplets from the water phase, the residual lipase activity remaining in the water phase was measured on tributyrin in the presence of 0.5 mM NaTDC and a 5-fold molar excess of colipase.

At the NaTDC concentration below the critical micellar concentration (0.5 mM), the three lipases display a similar interfacial binding profile irrespective of the presence of colipase, more than 50% of lipase being adsorbed to the lipid/water interface (Fig. 6). At 4 mM NaTDC, as observed for native lipase, about 95% of HuLipTP1_A are recovered in the water phase in the absence of colipase and the colipase anchoring effect is clearly visible.

Surprisingly, only 25 ± 9% of HuLipTP1_C is recovered in the water phase at 4 mM NaTDC without colipase. Addition of colipase only improves the adsorption. This result supports the conclusion that, in contrast with native lipase, variant C is able to bind to a bile salt-coated interface without the help of colipase.

Effect of Mutations on the Kinetic Parameters—We have measured the lipase activity of all enzymes as a function of substrate concentration in the presence of an excess of colipase with and without NaTDC. In each case, the experimental points were fitted to a hyperbolic plot. It is known that lipase-mediated catalysis cannot be treated using the classical Michaelis-Menten theory. However, because the experiments have been performed in the same conditions for all lipases, we should to be able to compare native and mutant lipases results. The values extrapolated from the hyberbola, which are k_{cat} and K_m in the Michaelis-Menten model, do not have the usual meaning but rather a more complex one, taking into account all steps of this heterogeneous catalysis (activation, adsorption, binding of substrate, and desorption of products). They were named apparent K_m and k_{cat}.

As shown in TABLE SIX, in the absence of NaTDC, the $K_{m(app)}$ values determined for HuLipTP1_A and -B are slightly lower than the value determined for the native lipase. On the other hand, all apparent k_{cat} values are quite similar. This lower $K_{m(app)}$ value results in a somewhat higher catalytic efficiency for mutants A and B.

In the presence of NaTDC, the $K_{m(app)}$ values for the native lipase and HuLipTP1_A and -B are roughly the same but the apparent k_{cat} values of mutants are 5–10-fold lower. This results in a sharp decrease of the catalytic efficiency of both HuLipTP1_A and HuLipTP1_B as compared with the native HuLip efficiency. These data support the idea that the low activity of mutants A and B is not correlated to a decreased apparent affinity of the lipase-colipase complexes for the bile salt-coated interface. By contrast, in the case of HuLipTP1_C, the $K_{m(app)}$ value is slightly lower than that of the native lipase whether or not bile salts are present, whereas the apparent k_{cat} are similar. This results in a 2-fold higher catalytic efficiency of mutant C in these conditions.

Thorough analysis of all these results reveals that, despite a conserved apparent affinity for colipase and emulsified substrates as well as an operational active site, HuLipTP1_A and -B display a decreased cata-

TABLE FIVE

Apparent affinity of native and mutant lipases for colipase

The rate of hydrolysis (V_m) was measured using a saturating concentration of emulsified tributyrin in the presence of 4 mM NaTDC and various concentrations of colipase. The values of K_{dapp} and the limiting value of V_m were determined by plotting V_m *versus* colipase concentration.

Proteins	K_{dapp}	V_m
	nM	*units/mg*
HuLip	2.8 ± 0.45	5800 ± 200
HuLipTP1_A	3.5 ± 0.05	600 ± 70
HuLipTP1_B	5.3 ± 0.2	1625 ± 53
HuLipTP1_C	3.5 ± 0.09	3527 ± 180

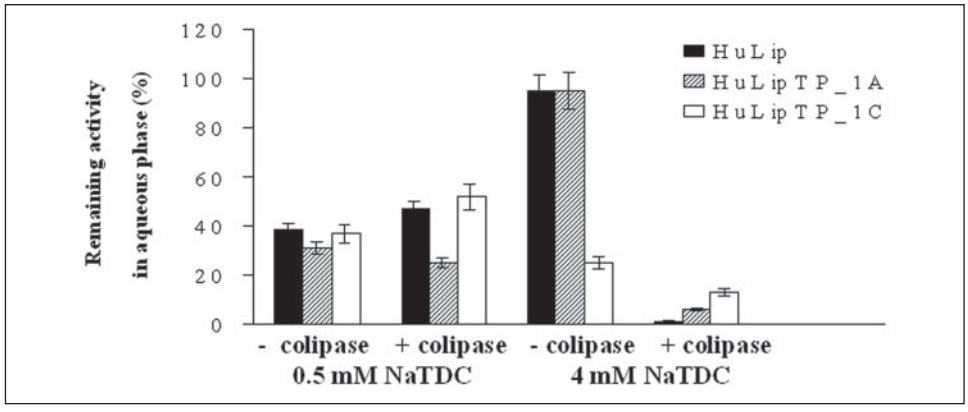

FIGURE 6. **Effect of bile salts and colipase on the interfacial binding of HuLip, HuLipTP1_A, and HuLipTP1_C.** The experiments were performed as described under "Materials and Methods" using tributyrin as substrate, two different NaTDC concentrations (0.5 and 4 mM), and in the presence or absence of a 5-fold molar excess of colipase. After separation of the oil phase from the water phase by centrifugation, the residual lipase activity in the water phase was determined by measuring lipase activity in the presence of 0.5 mM NaTDC and an excess of colipase.

TABLE SIX

Kinetic parameters of native and mutant lipases on tributyrin

The rate of hydrolysis was measured using various concentrations of tributyrin above its saturation point (0.5 mM) in the presence of a 5-fold molar excess of colipase and in the presence or absence of NaTDC.

	No NaTDC + colipase			4 mM NaTDC + colipase		
	k_{cat}	K_{mapp}	k_{cat}/K_M	k_{cat}	K_{mapp}	k_{cat}/K_M
	$10^3\ min^{-1}$	*mM*		$10^3\ min^{-1}$	*mM*	
HuLip	306 ± 10	7 ± 1.5	40 800	229 ± 12	2.8 ± 1	81 800
HuLipTP1_A	228 ± 8.8	1.5 ± 0.3	152 000	24 ± 1.8	3 ± 1.5	8 000
HuLipTP1_B	276 ± 9	1.5 ± 0.2	184 000	46 ± 2.5	7 ± 2.5	6 600
HuLipTP1_C	295 ± 23	4 ± 1.5	73 750	232 ± 6	1.2 ± 0.2	193 000

TABLE SEVEN

Lipolytic activity of native and mutant lipases on emulsified tributyrin and olive oil

The activities were determined potentiometrically at 25 °C in the presence of 6 mM NaTDC and a 5-fold molar excess of colipase.

Proteins	Activity on tributyrin	Activity	Activity on olive oil	Activity
	units/mg	*%*	*units/mg*	*%*
HuLip	5800 ± 215	100	1000 ± 150	100
HuLipTP1_A	850 ± 72	14	86 ± 13	14
HuLipTP1_B	1500 ± 125	26	277 ± 30	27
HuLipTP1_C	3000 ± 130	51	764 ± 85	50

lytic efficiency in the presence of a micellar concentration of NaTDC and that colipase is no longer able to restore their activity. On the other hand, although able to bind to a bile salt-coated interface in the absence of colipase, HuLipTP1_C displays only 14% of the optimal lipase activity in these conditions.

These results indicate that binding of lipase to a bile salt-coated interface does not necessarily result in the hydrolysis of substrate. They also confirm that the colipase is required for full activity. The decreased activities of mutants A, B, and C might be because of a modified ability of the lid to promote, in conjunction with colipase, some required change in substrate/bile salt interactions.

Substrate Specificity—The lid has been shown to contribute to one acyl chain binding site (3). Therefore, modifications in the lid could alter the substrate preference. We investigated the activity of mutants on a physiological emulsion made up of a bulk phase of long chain triglycerides (olive oil) surrounded by a monolayer of phospholipids, bile salts, and cholesterol.

As shown in TABLE SEVEN, mutations have no significant effect on the chain length specificity because all mutants retain the same relative activity on short and long chain substrates compared with the native enzyme.

Tilted Peptides in Pancreatic Lipase-related Protein 2 (PLRP2)—New pancreatic lipases such as PLRP2 have been identified in the last few years. Despite their similar amino acid sequences and three-dimensional structures, pancreatic lipase and PLRP2 differ in substrate specificity, bile acid inhibition, and requirement for colipase. Moreover, some of them do not elicit any interfacial activation (low activity on monomeric substrates and enhanced activity on water-insoluble substrates presenting an oil/water interface) despite the presence of a full-length lid (43, 44). This is interpreted as meaning that they efficiently hydrolyze monomers of partly water-soluble substrates. It rules out the idea of a strict correlation between the interfacial activation process and the presence of a lid. However, it must be kept in mind that some authors emphasize the ambiguity of the phenomenon of interfacial activation (33, 34). We have looked for tilted peptides in the lid sequences of several PLRP2 displaying or not an interfacial activation process.

As shown in TABLE EIGHT, a tilted peptide was detected in all lids. It is worth noting that tilted peptides of PRLP2 are more hydrophobic than the tilted peptide of lipase. The relevance of this higher hydrophobicity remains to be explored. One can raise the assumption that the higher hydrophobicity could have a role in the different behavior of PLRP2, in particular their ability to act on different types of lipid structures as micelles, vesicles, or emulsion.

[7] I. Crenon, unpublished data.

TABLE EIGHT

Predicted tilted peptides in the lids of a series of pancreatic lipases

The same procedure as for the pancreatic lipase, *i.e.* systematic screening in IMPALA of fragments of 10–15 residues, was used. Only peptides inserting at the phospholipid polar head interface (z between 14 and 18 Å) with tilt angles between 30 and 60 were selected. When several fragments overlapping similar residues were in the selection, the longest fragment was retained.

Lipase name	Sequence	Z value (Å)	Tilt angle	Interfacial activation
Human lipase	KNILSQIVDIDGI	17.5	42°	Yes
Porcine lipase	KNILSQIVDIDGI	17.5	42°	Yes
Human PLRP2	IDGIWEGIGG	16	47.4°	Yes (41)
Rat PLRP2	ILSTIVDINGI	15.8	49°	Yes (42)
Horse PLRP2	INGIWQGAQD	16.5	47.5°	No[a]
Coypu PLRP2	VNGFLEGITS	17	42	No (43, 44)

[a] Footnote 7.

DISCUSSION

Action of lipase on dietary triglycerides requires its intimate interactions with lipids. How are interactions triggered? This question remains a challenge for understanding lipase activity.

Tilted peptides, initially characterized from sequence analysis and then demonstrated to have functional relevance are energy motifs (13, 35, 36). They are short (10–18 amino acids) stretches of sequences likely to lie tilted at a hydrophobic/hydrophilic interfaces when helical. In many instances, they display no definite secondary structures. Hence, they require low energy to change conformation to adapt to the environment.

What is the significance of a tilted fragment in a protein structure? Folding of proteins implies a minimization of energy. This can come either from the creation of favorable intramolecular interactions (hydrophobic-hydrophobic or charged+/charged− . . .) or from the spatial separation of atoms of opposite polarity. This occurs in amphipathic secondary structures. Amphipathy has a crucial role because the core of proteins is hydrophobic but the type of amphipathic architectures made during folding is not random. Chou *et al.* (37) have demonstrated that most helices in protein three-dimensional structures are amphipathic and lie flat on a hydrophobic/hydrophilic interface. In tilted peptides, the partition of hydrophobicity makes the helix axis oblique with respect to the hydrophobicity interface. The interesting hypothesis in the story of tilted peptides is that tilt might have functional consequences. Tilt was first postulated and further demonstrated to be related to the fusogenic ability of fusion proteins of viruses (13, 38), to the secretion yield of secreted proteins (13, 15), and to the catalytic activity of some enzymes involved in lipid metabolism (17, 35). The reason for this effect was suggested to be in the ability of such fragments to adsorb on and penetrate into the hydrophobic interfaces, and to destabilize the packing of acyl chains. In the lipase catalysis, such activities are somehow required.

It is now clear that the lipase activation results from the displacement of the lid that opens the active site. This displacement goes with a modification of the lid secondary structure.

Comparing the hydrophobic and hydrophilic surface areas of both the closed and open lipase monomers and lipase-colipase complexes reveals that, although the overall surfaces are somewhat equivalent, the hydrophobic patch around the active site is larger in the open than in the closed structures. This argues that hydrophobic collapse might be a driving force of lipase adsorption onto substrates. Computational studies on microbial lipases have suggested that the lid motion is enhanced in a hydrophobic environment (39) and depends on changes in the dielectric constant of the medium, emphasizing also the role of electrostatic interactions (7).

Because the lid sequence does not encode for a definite secondary structure and undergoes large conformational change upon activation, we made the hypothesis that a fine adjustment of the hydrophobic/hydrophilic balance of the lid structure to the environment plays an important role in lipase activity and we tested the hypothesis that tilted peptides are involved.

Based on this remark, we have looked for tilted peptides in lipase. We have screened the human pancreatic lipase sequence. Most sequence fragments can structure themselves as amphipathic helices lying parallel to the interface and less than 6% of them are standing tilted at a lipid interface. One of these tilted peptides, TP1, residues ^{240}KNILSQIV-DIDG^{252}, is in the lid domain. Its helical form lies oblique at a hydrophobicity interface with an angle of 42° between the hydrophobicity interface and the helix axis.

Secondary structure predictions for this fragment are diverse and, in the lipase three-dimensional structures, the fragment undergoes a large structural rearrangement. This led us to suggest that TP1 might have a role via its hydrophobicity pattern and its large secondary structure choice. Mutations in the [^{240}KNILSQIVDIDG^{252}] lid fragment were designed to destroy the hydrophobicity tilt and turn the fragment into a parallel and perpendicular amphipathic structure when helical while retaining the same mean hydrophobicity. This goal was obtained by residue permutations (TP1_A and -B). Alternatively, we also erased native TP1 while creating a new tilted peptide with a different sequence (TP1_C).

These calculations were all theoretical but we knew from previous works that introduction of calculated mutations in the proteins could modify its function (14, 37). In the case of protein secretion, calculations were shown to explain the lower activity of natural mutants (40). Site-directed mutagenesis was then performed on the human lipase and properties of mutants (HuLipTP_1A, -B, and -C) were investigated.

E600 inhibition experiments demonstrated that, in the presence of colipase and micellar concentrations of NaTDC, the native lipase (2) and the three mutant lipases similarly displayed an open active conformation, with the catalytic serine accessible to E600 (data not shown). Hence mutations neither impaired the occurrence of the open conformations nor the lid opening in the presence of both colipase and bile salts. Moreover, in the absence of bile salts but in the presence of colipase, mutants were all as active as the native lipase. This indicates that the active site of all mutants is not altered and that the absence of a hydrophobicity gradient in the [240–252] peptide has no impact on the catalytic activity *per se*.

HuLipTP1_A and -B have lost all or part of their activity on bile salt-coated interfaces in the presence of an excess of colipase. Thus, HuLipTP1_A and –B, which have lost their peculiar hydrophobicity pattern in TP1, have also lost their ability to be reactivated by colipase. This suggests that both points are related. Interestingly, HuLipTP1_C, in which a different tilted peptide has been created retains 50–60% of the native lipase activity and the property to be reactivated by colipase.

We cannot completely rule out the hypothesis that residue permutations in HuLipTP1_A and -B affect the orientation of the lipase-colipase complex at the lipid interface. However, we suspect that an orientation change should induce a modification in the apparent lipase/colipase affinity and/or in the $K_{m(app)}$ values. No significant changes were observed in our study.

Our result supports the conclusion that neither the apparent affinity for colipase and oil droplets nor the "catalytic step" in the absence of bile salt are affected by the mutations. Therefore, two hypotheses were made. Either the mutations introduced in the [240–252] peptide decrease the lipase adsorption to the bile salt-coated interface, or impair a lid mobility required for activity.

Interactions of lipases with a lipid interface was investigated by kinetic studies and adsorption experiments. Altering the hydrophobicity gradient of TP1 affected neither the interfacial binding of lipase *per se*, nor the colipase anchoring effect, but rather affected the lipase catalytic turnover in the presence of bile salts. This was suggested by the apparent K_m and k_{cat} values of all mutants. The results also support the conclusion that interfacial binding and the catalysis are partly independent steps. Indeed, the binding of a catalytically functional lipase to a bile salt-coated interface in the presence of colipase does not systematically result in the hydrolysis of the substrate.

From the above, we suggest that the low capability of the [240–252] stretch of the lid to self-stabilize should be necessary for an efficient catalysis in the presence of bile salts, either to help extract a substrate molecule from its vehicle site and to bring this substrate to the active site or to discard the product from the hydrophobic patch of the lipase surface after hydrolysis is over.

The lower catalytic efficiency of HuLipTP1_A and -B in the presence of bile salts may be a consequence of a decreased mobility of the lid because of the suppression of the hydrophobicity pattern of the [240–252] fragment. When this tilted peptide is mutated into a classical amphipathic helix, the fragment might be less susceptible to change the structure and position because the energy barrier to go from one conformation to the other is higher because of the stabilization of a conformation.

Since in parallel mutants, colipase is no longer able to restore lipase activity when the lipid interface is coated with bile salts, we also support the conclusion that a coordinated mechanism requiring the lid movement and the colipase presence on the bile salt-substrate complexes is required for full activity. This hypothesis grants the lid with a functional role more important than simply opening the active site. In the future, mutations of the more hydrophobic tilted peptides of PRLP2 might be helpful to better understand which roles may be attributed to the pancreatic lipase lid and colipase.

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