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 240-KNILSQIVDIDGI-252 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 which 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) which prevents lipase adsorption. To counteract the inhibitory effect of biliary lipids, the pancreas secretes a small protein, colipase, which anchors lipase to the biliary lipids 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 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 either 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 the 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 into 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 the 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 the 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, 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 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 in order 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 NPS@ server; http://npsa-pbil.ibcp.fr (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 (PDB code:1LPA). The sequence was cut into fragments of 10 to 15 residues. All fragments were calculated to be α-helix and structures were optimized by a Simplex energy minimization procedure. The behaviour of each helix at the IMPALA hydrophobic/hydrophilic interface (20) was then calculated by inserting the peptide at every angstrom 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 in between where the water content varies with a z ordinate. The interactions between the peptide and the
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interface are approached by two energy restraint terms which vary with \( z \). As previously described (20,21), one restraint term describes the hydrophobicity effect which pushes hydrophilic atoms in water and draws hydrophobic atoms in the lipid phase while 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 while the insertion restraints vary.

Restraint hydrophobicity term:

\[
E_{\text{int}} = - \sum_{i=1}^{N} S(i) E_{\text{tr}}(i) C(z_i)
\]

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

Lipid perturbation term:

\[
E_{\text{lip}} = a_{\text{lip}} \sum_{i=1}^{N} S(i) (1 - C(z_i))
\]

where \( a_{\text{lip}} \) 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

\[
C(z) = 1 - \frac{1}{1 + e^{\alpha(z-z_0)}}
\]

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

**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 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, Germany) and used for cotransfection of Sf21 cells with the BD BaculoGold™ linearized DNA (BD Biosciences 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 (\( E_{\text{1%}} = 1.33 \)) (26).

**Homology modeling**

The mutant lipase 3D structures were calculated by substituting residues in the 3D structures of 1LPA and 1N8S (PDB codes). Structure energies were minimised 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).

**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 concentration (1 mM to 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% Lα-phosphatidylcholine from egg yolk and 1% free cholesterol (both from Sigma, La Verpilliére, France). The lipids (500mg) 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 a 1 mM Tris/HCl buffer pH 7.5 containing 150 mM NaCl and 5 mM CaCl2 and sonicated for 5 min at 95% power level and a frequency of 20.178 Hz (Sonoreactor Undatim, Japan) in ice/ethanol bath. The assays were performed as described above using 1mL 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 a 1 mM Tris/HCl buffer pH 7.5, 0.1 M NaCl and 5 mM CaCl2 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 (1200 rpm) for 10 min in order 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 an excess of colipase.

**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 1).

**Table 1**

Unmasked residues are mostly hydrophobic while buried ones are more hydrophilic. This accounts for the hydrophobicity increase of the accessible surface and is in good agreement with the 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 pattern of the sequence (PDB: 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).

**Figure 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 fulfills the two criterions of our search:
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- the sequence should not determine a definite structure. As shown in table 3, the predicted secondary structure of the TP1 fragment largely varies with the predictions algorithms.

Table 3
Moreover, in agreement with the idea of a structural susceptibility to the environment, TP1 has different conformations in the open and in the closed structures. It is mainly extended in the closed lipase while in the open conformation, a short helix LSQIVD bordering a turn (IDGI) is formed.

- the energy minimization by partition of hydrophobicity should not give a symmetric amphipathic α helix. After testing the α helix 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 4).

Figure 4

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 in order to destroy all possibilities of tilted insertion in a hydrophobicity interface.

Table 4
The mutant TP1_A was obtained by permuting L243 for D248, the mutant TP1_B by exchanging I242 for D248 and the mutant C was obtained by permutation of Q244 and W253, although W253 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 L243 for D248 residue in mutant A transforms the native TP1 into an amphipathic helix lying flat at the lipid interface (Fig. 4 and Table 4). 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 L243 for D248 transforms the native TP1 into a peptide with a tilt angle higher than 50° inserting deeper in the hydrophobic phase (Table 4). IMPALA analysis of the [238-256] sequence shows that the fragments apart 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 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 Q244 for W253 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 4). It must be pointed out that the [240-253] fragment in the native, mutant A and mutant B sequences is not tilted (Table 4).

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 TP1A and TP1B mutants should display modified activity while 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 due to non linear kinetics (no “zero” point in figure 5). This property, already reported by Brockerhoff (31) and Vandermeers et al (32), is described as due to 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 which 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.

Figure 5
As reported for native HuLip, the activity of HuLip TP1_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 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. HuLip TP1_A is the most affected since its activity is not restored at 4 mM NaTDC while HuLip TP1_B recovers 33% of the native lipase activity. HuLip TP1_C, on the other hand, displays a behavior more similar to that of native lipase since 50 to 60% of its activity is restored by colipase at 4mM 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
Since 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.

Table 5
As shown in Table 5, 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, a noticeable decrease of the hydrolysis rate is observed for mutants A and B in agreement with results from figure 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 fivefold molar excess of colipase.

Figure 6
At 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 the 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 classical Michaelis-Menten theory. However, since the experiments have been performed in the same conditions for all lipases, we ought to be able to compare native and mutant lipases results. The values extrapolated from the hyperbola 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, desorption of products). They were named apparent K_m and k_cat.

Table 6
As shown in table 6, in the absence of NaTDC, the $K_{\text{m,app}}$ values determined for HuLipTP1_A and B are slightly lower than the value determined for the native lipase. On the other hand, all apparent $k_{\text{cat}}$ values are quite similar. This lower $K_{\text{m,app}}$ value results in a somewhat higher catalytic efficiency for mutants A and B. In the presence of NaTDC, the $K_{\text{m,app}}$ values for the native lipase and HuLipTP1_A and B are roughly the same but the apparent $k_{\text{cat}}$ values of mutants are 5 to 10 fold lower. This results in a sharp decrease of the catalytic efficiency of both HuLipTP1_A and HuLipTP1_B as compared to 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 $K_{\text{m,app}}$ value is slightly lower than that of the native lipase whether or not bile salts are present while the apparent $k_{\text{cat}}$ 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 catalytic efficiency in the presence of micellar concentration of NaTDC and that colipase is not anymore 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 due to 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 mutants activity 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 7, mutations have no significant effect on the chain length specificity since all mutants retain the same relative activity on short and long chain substrates compared to the native enzyme.

**Tilted peptides in pancreatic lipase related proteins 2**

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, in bile acid inhibition and in 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. 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 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.

**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 aa) stretches of sequences likely to lie tilted at a hydrophobic/hydrophilic interfaces when helical. In many instances, they display no definite
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What is the significance of a tilted fragment in a protein structure? Folding of proteins implies a minimisation 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 since 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 3D 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 in 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 which 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 the open lipase monomers and lipase/coliepase 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 the driving forces 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).

Since 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 the 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-KNILSQIVDIDG-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 3D 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 mutageneses were 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 did neither impair 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...
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active as the native lipase. This indicates that the active site of all mutants is not altered and that the absence of an 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 to 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 HuLip TP1_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 \( K_m \) and \( K_{app} \) values. No significant changes were observed in our study.

Our result support 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 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 turn over 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 the 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.

Hypothesis and conclusion: 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 extracting 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 due to 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 structure and position because the energy barrier to go from one conformation to the other is higher due to the stabilization of a conformation.

Since in parallel mutants, colipase is no longer able to restore the 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.
REFERENCES

A tilted peptide in the lipase lid

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FOOTNOTES

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1 The abbreviations used are: TP, tilted peptide; IMPALA, integral membrane protein and lipid association; MHP, molecular hydrophobicity potential, NaTDC, sodium taurodeoxycholate; HuLip, human pancreatic lipase; E600, diethyl p-nitrophenyl phosphate; PLRP2, pancreatic lipase related protein 2.

FIGURE LEGENDS

Fig. 1. Structure and MHP surface of the closed and the open pancreatic lipases. Top: closed (PDB: 1N8S) and open (PDB: 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.

Fig. 2. Lid movement around the S-S bridge and MHP 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 MHP profiles of the lids are calculated according to Brasseur et al (11) (black is the hydrophobic surface (-0.1 kcal/mol) and white the hydrophilic surface +0.1 kcal/mol).

Fig. 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-to 14 residues. All fragments were calculated as $\alpha$ helices and tested for interfacial adsorption in IMPALA as described in 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.

Fig. 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 $\alpha$-helices and tested for insertion in IMPALA by the systematic assay described in Materials and Methods. The positions of 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 (L and D). 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°.

Fig. 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 (o) 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.

**Fig. 6.** Effect of bile salts and colipase on the interfacial binding of HuLip, HuLipTP1_A and HuLipTP1_C. The experiments were performed as described in Materials and Methods using tributyrin as substrate, two different NaTDC concentrations (0.5 mM and 4 mM) and in the presence or in the 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.

**TABLES**

**Table 1:** Total, hydrophilic (Phi) and hydrophobic (Pho) accessible surface areas (Å²) 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). Last column is the Pho/Phi surface area ratio.

<table>
<thead>
<tr>
<th></th>
<th>Phi ASA</th>
<th>Pho ASA</th>
<th>Total</th>
<th>Pho/Phi ratio</th>
</tr>
</thead>
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<tr>
<td>closed lipase</td>
<td>1N8S</td>
<td>9059</td>
<td>9278</td>
<td>17732</td>
</tr>
<tr>
<td>open lipase</td>
<td>1LPA</td>
<td>8083</td>
<td>10303</td>
<td>18386</td>
</tr>
<tr>
<td>Closed complex</td>
<td>1N8S</td>
<td>11123</td>
<td>11982</td>
<td>23105</td>
</tr>
<tr>
<td>Open complex</td>
<td>1LPA</td>
<td>8853</td>
<td>12327</td>
<td>21180</td>
</tr>
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</table>

**Table 2:** Accessible surface area variations (Å²) of individual residues in the open and closed lipase structures. Values were extracted from Pex files (30).

<table>
<thead>
<tr>
<th></th>
<th>Lid</th>
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<th>Hydrophobic groove</th>
<th>h5 loop</th>
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<tr>
<td></td>
<td>Q 244</td>
<td>-122</td>
<td>P 211</td>
<td>T 78</td>
</tr>
<tr>
<td></td>
<td>I 245</td>
<td>-90</td>
<td>L 213</td>
<td>K 80</td>
</tr>
<tr>
<td></td>
<td>I 251</td>
<td>+100</td>
<td>F 215</td>
<td>E 83</td>
</tr>
<tr>
<td></td>
<td>W 252</td>
<td>+102</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 256</td>
<td>+122</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D 257</td>
<td>-139</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 258</td>
<td>+66</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>A 259</td>
<td>+55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3:** 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 random coil; h is helix; and e is beta extended structures.
Table 4: Analysis of the IMPALA results for the sequence fragments of 13 amino acids around the TP1 sequence (aa 240-252). For sake of comparison, fragment 240-253 was added for all sequences since this fragment is tilted in mutant C. Columns list the first and last residue number, the peptide length, the IMPALA restraint value (kcal/mol), the Z ordinate of the helix mass center (Å), 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, $\pm 20 \text{ Å} < z < \pm 15.5$ Å for the transition phase and $\pm 15.5 \text{ Å} < z < 0$ Å for the water-free phase. Z values over 20 Å correspond to pure water phase z values under 15.5 to pure hydrophobic phases.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>From aa</th>
<th>To aa</th>
<th>peptide length</th>
<th>IMPALA restraint</th>
<th>IMPALA z ordinate</th>
<th>helix / interface angle</th>
</tr>
</thead>
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<tr>
<td>WILD TYPE</td>
<td>238 250</td>
<td>13 -21.4</td>
<td>13 -21.4</td>
<td>17.3</td>
<td>23 CKKNILSQIVDID</td>
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</tr>
<tr>
<td></td>
<td>239 251</td>
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<td></td>
</tr>
<tr>
<td>TP_1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>241 253</td>
<td>13 -15.0</td>
<td>13.0</td>
<td>27 NLSQIVDIDIW</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>242 254</td>
<td>13 -16.9</td>
<td>19.0</td>
<td>35 LSQIVDIDIWEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>243 255</td>
<td>13 -15.0</td>
<td>17.0</td>
<td>16 LSQIVDIDIWEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>240 253</td>
<td>14 -15.9</td>
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<td></td>
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<tr>
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<td>0 CKKNIDSQIVLID</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>239 251</td>
<td>13 -17.2</td>
<td>19.5</td>
<td>3 KKNIDSQIVLIDG</td>
<td></td>
<td></td>
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<tr>
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<td>19.5</td>
<td>4 KNIDSQIVLIDG</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>241 253</td>
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<td>18.5</td>
<td>3 NDSQIVLIDIGW</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>242 254</td>
<td>13 -16.7</td>
<td>16.0</td>
<td>19 IDSQIVLIDIGE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>243 255</td>
<td>13 -13.5</td>
<td>17.3</td>
<td>0 DSQIVLIDIGE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>240 253</td>
<td>14 -14.8</td>
<td>19.0</td>
<td>6 KNIDSQIVLIDIGW</td>
<td></td>
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<tr>
<td>MUTANT B</td>
<td>238 250</td>
<td>13 -13.1</td>
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<tr>
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<td>239 251</td>
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<tr>
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<td>13 -15.8</td>
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<td></td>
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<td>13 -16.2</td>
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<td>MUTANT C</td>
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<td></td>
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<td>58 ILSWVIDDIGO</td>
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<tr>
<td></td>
<td>243 255</td>
<td>13 -17.4</td>
<td>17.3</td>
<td>19 LSWVIDDIQGT</td>
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<tr>
<td>TP1_C</td>
<td>240 253</td>
<td>14 -14.0</td>
<td>17.8</td>
<td>43 KNILSWVIDDIQ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5: 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 $K_d$ app and the limiting value of $V_m$ were determined by plotting $V_m$ versus colipase concentration.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$K_d$ app (nM)</th>
<th>$V_m$ (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuLip</td>
<td>2.8 ± 0.45</td>
<td>5800 ± 200</td>
</tr>
<tr>
<td>HuLipTP1_A</td>
<td>3.5 ± 0.05</td>
<td>600 ± 70</td>
</tr>
<tr>
<td>HuLipTP1_B</td>
<td>5.3 ± 0.2</td>
<td>1625 ± 53</td>
</tr>
<tr>
<td>HuLipTP1_C</td>
<td>3.5 ± 0.09</td>
<td>3527 ± 180</td>
</tr>
</tbody>
</table>

Table 6: 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.

<table>
<thead>
<tr>
<th>No NaTDC + Colipase</th>
<th>4 mM NaTDC + Colipase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ ($10^3\text{min}^{-1}$)</td>
</tr>
<tr>
<td>HuLip</td>
<td>306 ±10</td>
</tr>
<tr>
<td>HuLipTP1_A</td>
<td>228 ±8.8</td>
</tr>
<tr>
<td>HuLipTP1_B</td>
<td>276 ±9</td>
</tr>
<tr>
<td>HuLipTP1_C</td>
<td>295 ±23</td>
</tr>
</tbody>
</table>

Table 7: 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.

<table>
<thead>
<tr>
<th>Lipase name</th>
<th>Sequence</th>
<th>$Z$ value (Å)</th>
<th>Tilt angle</th>
<th>Interfacial activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human lipase</td>
<td>KNILSQIVDIDI</td>
<td>17.5</td>
<td>42°</td>
<td>yes</td>
</tr>
<tr>
<td>Porcine lipase</td>
<td>KNILSQIVDIDI</td>
<td>17.5</td>
<td>42°</td>
<td>yes</td>
</tr>
<tr>
<td>Human PLRP2</td>
<td>IDGIWEGIGG</td>
<td>16</td>
<td>47.4°</td>
<td>yes (41)</td>
</tr>
<tr>
<td>Rat PLRP2</td>
<td>ILSTIVDINGI</td>
<td>15.8</td>
<td>49°</td>
<td>yes (42)</td>
</tr>
<tr>
<td>Horse PLRP2</td>
<td>INGIWQGAQD</td>
<td>16.5</td>
<td>47.5°</td>
<td>No*</td>
</tr>
<tr>
<td>Coypu PLRP2</td>
<td>VNGFLEGITS</td>
<td>17</td>
<td>42</td>
<td>No (43,44)</td>
</tr>
</tbody>
</table>

*Crenon (unpublished data)
Figure 1

Closed lipase  Open lipase

Closed Complex  Open Complex
A tilted peptide in the lipase lid

Figure 2
Figure 3

![Graph showing the insertion angle of helix axis vs. fragments]

- **Axes:**
  - **Y-axis:** Insertion angle of helix axis
  - **X-axis:** Number of fragments

- **Legend:***
  - Bars represent different insertion angle ranges.
Figure 4

A tilted peptide in the lipase lid
Figure 5
Figure 6

Remaining activity in aqueous phase (%)

- colipase + colipase

0.5 mM NaTDC

- colipase + colipase

4 mM NaTDC

- 

+ 

HuLip

HuLip TP_1A

HuLip TP_1C