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Val-407 and Ile-408 in the β5′-Loop of Pancreatic Lipase Mediate Lipase-Colipase Interactions in the Presence of Bile Salt Micelles*

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Abstract

In a previous study, we demonstrated that the β5′-loop in the C-terminal domain of human pancreatic triglyceride lipase (hPTL) makes a major contribution in the function of hPTL (Chahinian *et al.* (2002) *Biochemistry* 41, 13725–13735). In the present study, we characterized the contribution of three residues in the β5′-loop, Val-407, Ile-408, and Leu-412, to the function of hPTL. By substituting charged residues, aspartate or lysine, in these positions, we altered the hydrophilic to lipophilic ratio of the β5′-loop. Each of the mutants was expressed, purified, and characterized for activity and binding with both monolayers and emulsions and for binding to colipase. Experiments with monolayers and with emulsions suggested that the interaction of hPTL with a phospholipid monolayer differs from the interaction of the hPTL-colipase complex with a dicaprin monolayer or a triglyceride emulsion (*i.e.* neutral lipids). Val-407, Ile-408, and Leu-412 make major contributions to interactions with monolayers, whereas only Val-407 and Ile-408 appear essential for activity on triglyceride emulsions in the presence of bile salt micelles. In solutions of taurodeoxycholate at micellar concentrations, a major effect of the β5′-loop mutations is to change the interaction between hPTL and colipase. These observations support a major contribution of residues in the β5′-loop in the function of hPTL and suggest that a third partner, bile salt micelles or the lipid interface or both, influence the binding of colipase and hPTL through interactions with the β5′-loop.

The three-dimensional structure of human pancreatic triglyceride lipase (hPTL)[2] revealed two functional domains, a globular N-terminal domain and a β-sandwich C-terminal domain (residues 336–449) (1). Since the determination of the structure, functional roles have been described for each domain. The N-terminal domain consisting of an α β-hydrolase fold contains the catalytic site. A surface loop, the lid, delineated by a disulfide bond between Cys-237 and Cys-261, covers the active site and controls the activity of hPTL (2, 3). In the inactive conformation, the lid sterically hinders access of substrate to the active site. In the active conformation, the lid adopts a new position that opens and configures the active site.

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[2]The abbreviations used are: hPTL, human pancreatic triglyceride lipase; hPTLS153G, hPTL mutant in which the active site serine 153 has been substituted with a glycine residue; TDC, taurodeoxycholate; LPL, lipoprotein lipase.

The C-terminal domain, which has structural similarity to the C2 domain of other lipid-binding proteins, contributes the majority of the binding surface for colipase, a necessary cofactor for hPTL function in the duodenum (4). Until recently, colipase binding was the best documented function for the C-terminal domain.

Over the past several years, several lines of evidence suggested an additional role for the C-terminal domain. Examination of the colipase-PTL crystal structure reveals an exposed hydrophobic loop, the β5′-loop, including residues 405–414, of the C-terminal domain that orients in same plane as the hydrophobic plateau of colipase and the lid (5). This orientation positions the loop to interact with the oil-water surface of the lipid substrate (Fig. 1). Next, a number of studies indicate that a homologous loop in lipoprotein lipase, a protein with structural similarity to hPTL, contributes to the lipid-binding properties of lipoprotein lipase. For instance, mutagenesis of three tryptophans in the lipoprotein lipase loop abolished binding of the lipase to substrate (6). Finally, our earlier study demonstrated that a mutant of hPTL in which the hydrophobicity and size of the β5′-loop was increased by introducing the homologous lipoprotein lipase β5′-loop into hPTL had altered interactions with phospholipid monolayers and with trioctanoin emulsions (7). These findings support a major role for the β5′-loop in the function of hPTL, possibly in the interaction with substrate.

Another finding in our previous study was that the hPTL-β5′LPL mutant had decreased affinity for colipase in the presence of bile salts (7). Because colipase does not interact with the β5′-loop in the crystal structure of the hPTL-colipase complex, we considered several alternative explanations for the effect of the mutation on the affinity for colipase. One possibility arises from the observation that colipase, PTL, and bile salt micelles can form a complex with the micelle contacting the β5′-loop and residues in colipase (8, 9). β5′-Loop mutations could impair the interaction between PTL and bile salt micelles thereby decreasing the affinity of the hPTL- β5′LPL mutant for colipase. Another potential explanation is that the larger size of the LPL β5′-loop might sterically hinder binding of the PTL mutant to colipase. Alternatively, the LPL loop could perturb the protein structure near the binding site of colipase resulting in decreased affinity for colipase even though the overall structure of the hPTL-β5′LPL mutant is not grossly altered.

In this study, we have addressed the possibility that the β5′-loop interacts with a third partner to increase the affinity for colipase by introducing mutations in the β5′-loop that increase the hydrophilic/lipophilic balance of the loop without greatly altering the size. To accomplish these studies, we created mutants of hPTL with substitutions of a positively or negatively charged residue for a hydrophobic residue. Each mutant was expressed in yeast, purified, and characterized for interfacial binding and lipase activity in both bulk phase and monolayer assays. The effects of bile salts and colipase on activity and binding were studied and compared with the properties of hPTL.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis

Manipulations of DNA were done by using standard methods (10). Mutations were introduced into the wild-type hPTL cDNA in the pHILS1 vector by PCR using the Stratagene QuikChange site-directed mutagenesis kit per the manufacturer's protocol (11). Oligonucleotide primers were designed to introduce the desired mutations and were generally 27 bp long. Transformations into Epicurian Coli XL-1 Supercompetent cells were performed as described in the QuikChange protocol. Minipreps were performed using either the Qiagen Plasmid Mini kit or the Qiagen QIAprep Miniprep kit. Presence of the desired mutation was confirmed by dideoxynucleotide sequence analysis using the ABI Prism Big

Dye Terminator Cycle Sequencing Ready Reaction kit. The DNA was transformed into yeast by electroporation, and positive clones were selected as previously done (11, 12).

Protein Methods

The mutant lipases were expressed and purified as previously described (11). Protein concentrations were determined by amino acid analysis and by spectrophotometry at 280 nm using an extinction coefficient of $E_{1\%}^{1\ \mathrm{cm}}$=1.2. The purified proteins were analyzed by SDS-PAGE and staining with GELCODE Blue stain (Pierce) to confirm the homogeneity of the isolated protein.

Lipase Activity Measurements

The lipase activity of wild-type and mutant hPTL was determined in bulk by measuring the release of fatty acids from mechanically stirred emulsions of tributyrin, trioctanoin, or triolein as previously described (7, 13). Unless otherwise stated, pure colipase from porcine pancreas or pure recombinant, human colipase was added at a molar excess of 5. The lipolytic activities are expressed in International Units per milligram of enzyme. One unit corresponds to 1 μmol of fatty acid released per minute.

Binding of hPTL and the Mutants to Tributyrin and Trioctanoin Emulsions

The interfacial binding of hPTL and the mutants was assayed by mixing the enzyme with a trioctanoin or tributyrin emulsion as previously described (14, 15). A 5-fold molar excess of colipase and varying amounts of taurodeoxycholate were included in the incubations as indicated.

Monolayer Experiments

Lipase activities were measured by the monolayer technique using monomolecular films of 1,2-dicaprin (Fluka, Paris, France) spread over an aqueous phase made of 10 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA, and placed in a zero order trough (16). The hydrolysis rates were expressed in moles of substrate hydrolyzed per cm^2 of the reaction compartment, per minute, and for a theoretical enzyme concentration of 1 M in the reaction compartment.

The kinetics of the adsorption of hPTL and $\beta 5'$-loop mutants were studied by recording the changes with time of the surface pressure of a monomolecular film of egg phosphatidylcholine (PC, Sigma, Saint Quentin-Fallavier, France) spread at the air-water interface in a cylindrical Teflon trough (volume: 5 ml; surface area: 7 cm^2). The surface pressure was measured using the Wilhelmy method with a thin platinum plate (perimeter 3.94 cm) attached to an electromicrobalance (Beckman LM 600). The trough was filled with 10 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA, and the monolayer was spread using various volumes (1–10 μl) of a 1 $mg.ml^{-1}$ phospholipid solution in chloroform. Once the initial surface pressure had stabilized, the lipase was injected into the aqueous subphase, which was stirred for 30 s at 250 rpm with a magnetic rod to homogenize the aqueous phase. The increase in the surface pressure was then recorded for 60 min. Experiments with PC monolayers were performed using 5 μg of lipase (20 nM overall concentration in the trough).

Modeling of β5′-Loop Mutations and Calculating the Water-accessible Surfaces

Three-dimensional models of the hPTL-$\beta 5'$-loop mutants were built from the known x-ray three-dimensional structure of open hPTL (accession number 1LPA in the Protein Data Bank). The water-accessible surfaces were calculated using the DSSP option in the Turbo-Frodo software program (17, 18).

RESULTS

Design and Expression of Single Substitution Mutants in the β5′-Loop

Examination of the three-dimensional structure of hPTL revealed that three hydrophobic residues, Val-407, Ile-408, and Leu-412, reside on the surface of the β5′-loop (Fig. 1). To determine the effect of increasing the hydrophilic to lipophilic ratio of the β5′-loop without greatly changing the size of the loop, we substituted each site with an aspartic acid to create I407D, V408D, and L412D or with lysine to create I407K, V408K, and L412K. Introduction of a single charged residue changed the accessible surface area minimally, 2.5% or less for the aspartate mutants and 7% or less for the lysine mutants (Table 1). In contrast, the hydrophilic to lipophilic ratio was increased from 50% to 135%, with substitutions at position 408 causing the greatest effect.

Each mutant was expressed in *Pichia pastoris* and purified as described under "Experimental Procedures." The yeast secreted each mutant protein into the medium indicating that the proteins were not misfolded. The yield for each mutant ranged from 5 to 10 mg/liter of culture. Analysis of the purified proteins by SDS-PAGE revealed a single band for each mutant (data not shown). By this analysis, there was no evidence for degradation of any of the mutants. We further confirmed the structural integrity of each mutant by determining the temperature stability of the mutants. After incubation at 50 °C for 20 min, similar residual activity, ~50% of the original activity in 0.5 mM TDC, remained for wild-type hPTL and each of the hPTL mutants. Additionally, the activity and binding of the mutants did not change over time indicating that degradation or alterations such as isoaspartate formation in the substituted residues did not occur (19).

Effect of Colipase and Bile Salts on the Activity of the β5′-Loop Mutants against Three Different Substrates

To begin our analysis of the mutants, we measured the activity of hPTL and each mutant against emulsions of tributyrin, trioctanoin, and triolein in the standard pH-stat assay containing increasing concentrations of TDC. In the absence of colipase, hPTL and all of the mutants had activity at 0.5 mM TCD, and these activities ranged from 500 to 4,000 units/mg depending on the substrate (Fig. 2). There were no differences in activity among the mutants and wild-type hPTL indicating that the mutations did not grossly affect the folding of the catalytic N-terminal domain. Above the critical micelle concentration of TDC (~1.1 mM) all of the lipases were inhibited. When colipase was included in the assay, the activity below the critical micelle concentration of TDC increased for all mutants with each substrate and equaled the activity for hPTL. At higher TDC concentrations, colipase restored the activity for the L412D and L412K mutants to 80% or more of the hPTL activity with all of the substrates. Colipase restored activity to the Ile-407 and Val-408 mutants only with tributyrin, but the levels were significantly lower than that for wild-type hPTL. At 4 mM taurodeoxycholate and tributyrin, V407D and I408D had 16%, V407K had 13%, and I408K had 55% of wild-type hPTL activity. None of these mutants were active against emulsions of the water-insoluble triglycerides, trioctanoin, or triolein, under these conditions. Thus, mutations in the β5′-loop alter hPTL function but only in the presence of micellar solutions of bile salts.

During the standard 5-min assays with triolein, we noted the presence of a lag time with the L412D mutant and determined if the other mutants also exhibited lag times (Table 2). Each of the mutants except for L412K showed a lag time when assayed against triolein in 4 mM TDC. The times ranged from 2.0 min for L412D to 32.5 min for I408D. After the lag time, the mutants showed a burst of activity. The activities of all mutants except those at position 408 were near normal. This result shows that denaturation of the mutants in 4 mM TDC

cannot explain the decreased activity of the loop mutants in the standard assay. The long lag times suggest that the mutants have altered partitioning onto the substrate interface.

Partitioning of the β5′-Loop Mutants in Tributyrin and Tri-octanoin

We next tested the ability of the β5′-loop mutants to partition with lipid substrate at both 0.5 and 4.0 mM TDC in the presence of colipase (Table 3). At 0.5 mM TDC, the mutants partitioned with tributyrin and trioctanoin to the same extent as did wild-type hPTL. In the presence of 4.0 mM TDC, clear differences among the mutants were seen. V407D, I408D, and V407K had decreased partitioning with both tributyrin and trioctanoin, a finding consistent with the decreased or absent activity of these mutants against these lipids. I408K partitioned well in tributyrin and has ~60% of wild-type activity (Fig. 2). I408K also partitioned normally in trioctanoin even though the mutant had no activity against trioctanoin in 4.0 mM TDC and colipase (Fig. 2). The L412D and L412K mutants had normal partitioning in both lipids as expected for their near normal activity against tributyrin and trioctanoin. These results support a role for the β5′-loop in the function of hPTL in the presence of micellar solutions of bile salts.

Interaction of the β5′-Loop Mutants with Colipase

One explanation for the decreased activity and partitioning of the mutants in 4.0 mM TDC is that the β5′-loop mutations impair the interactions of the mutant lipases with colipase. To test the effects of the mutations on the colipase-lipase interaction, we first determined the ability of colipase to activate the β5′-loop mutants over a range of colipase concentrations in 0.5 or 4.0 mM TDC and excess substrate with a constant amount of each lipase (Fig. 3). In 0.5 mM TDC, the mutants had normal activity suggesting normal interactions with colipase and with the lipid interface (Fig. 3, *upper panels*). Furthermore, these findings indicate that mutations in the β5′-loop did not affect the overall folding of the C-terminal domain. A different situation was observed when the TDC concentration was increased to 4.0 mM where several of the mutants had decreased activity. Because hPTL activity in 4.0 mM depends on colipase, which is not the situation in 0.5 mM TDC where hPTL has activity in the absence of colipase, we were able to calculate the apparent dissociation constant (K_d) and apparent binding constant (B_{max}) for the colipase-hPTL complex in 4.0 mM TDC by nonlinear regression with a rectangular hyperbola function (Table 4). The apparent B_{max} was similar to hPTL for all of the mutants. The two Leu-412 mutants had values for apparent K_d similar to hPTL. In contrast, the I408K mutant had ~a 5-fold increase in K_d and the V407K, V407D, and I408D mutants had 15-fold increases in K_d values. These results indicate that mutations in positions 407 and 408 influence the interaction of colipase and hPTL in the presence of bile salt micelles but not in the presence of bile salt monomers.

We next tested the ability of each mutant to compete for colipase with hPTL or with S153G, an inactive mutant of hPTL with normal colipase binding. In each of these assays, we included equimolar amounts of the β5′-loop mutants with a lipase:colipase ratio of 1.0: 0.5. Because the V407D, I408D, and V407K mutants have no activity against trioctanoin in 4 mM TDC, we tested their ability to compete with hPTL in an assay against trioctanoin in 4 mM TDC. None of these mutants inhibited hPTL under these conditions indicating that they have a much lower affinity for colipase than does hPTL (Table 5). For the mutants with activity at 4.0 mM TDC (I408K, L412D, and L412K), we estimated their affinities for colipase by their ability to compete with S153G lipase for colipase. If these mutants have normal or near normal affinities for colipase, S153G should decrease their activity by 50%. In this assay, S153G decreased L412D activity to 47.0 ± 8.2%, I408K activity to 46.2 ± 17.1%, and L412K activity to 31.3 ± 21.4%. Although there was considerable variability in the assay for the I408K and L412K mutants, it is clear these mutants can compete with S153G for colipase and have relatively preserved affinity for colipase. The two methods

concur that the V407D, I408D, and V407K mutants have decreased affinity for colipase in 4.0 mM TDC, whereas the L412D, I408K, and L412K mutants have normal or near normal affinities for colipase.

Effect of the β5′-Loop Mutations on Lipase Activity against Monolayers

To more directly determine the interaction of the mutants with a lipid interface, we turned to studies with monolayers. Monolayers allow the variation and control of the interfacial quality of the interface and allow assays in the absence of colipase and bile salts (16). We first measured the activity of the mutants against monolayers of dicaprin. Each mutant was assayed at various surface pressures ranging from 15 to 35 millinewtons/m (Fig. 4). In general, the mutants behaved similarly to hPTL. Although the V407D, V407K, and L412D mutants had increased activity compared with hPTL, the differences were not statistically significant. These results indicate that the introduction of single mutations into the β5′-loop did not greatly alter the interaction of the mutant lipases with a neutral lipid monolayer and suggest that the β5′-loop plays a greater role in the activity against neutral lipids in the presence of colipase and bile salts.

We next measured the interaction of the β5′-loop mutants with a zwitterionic monolayer of phosphatidylcholine. For our analysis of interactions with phosphatidylcholine, we determined the increase in surface pressure as a function of the initial surface pressure of the monolayer under which the lipase was injected (Fig. 5). Phosphatidylcholine is not a substrate for hPTL or the mutants, and the recorded variations in surface pressure only reflect the ability of the test lipase to bind and penetrate the phospholipid monolayer. Each of the β5′-loop mutants behaved similarly. They all showed decreased penetration compared with hPTL. None of the mutants penetrated the monolayer above a surface pressure of 4 millinewtons/m, whereas the critical surface pressure of penetration for hPTL was above 12 millinewtons/m.

DISCUSSION

In our previous study, we concluded that the β5′-loop plays a critical role in the function of hPTL (7). In the present study, we provide evidence that the β5′-loop, particularly the region containing Val-407 and Ile-408, contributes to the function of hPTL by influencing the binding of colipase in micellar concentrations of bile salts. Additionally, experiments with monolayers and with emulsions suggest that the interaction of hPTL with a phospholipid monolayer differs from the interaction of the hPTL-colipase complex with a dicaprin monolayer or a triglyceride emulsion (*i.e.* neutral lipids).

Importance of Residues Val-407 and Ile-408

The substitution mutants of Val-407 and Ile-408 had greater effects than the replacement of Leu-412. Both the aspartate and lysine substitutions at position 407 greatly impaired the activity of the mutant PTL in the presence of bile salt micelles. In contrast, the aspartate substitution at position 408 made a greater change in activity than did the lysine substitution. Furthermore, the I408K mutant had relatively preserved partitioning with tributyrin and trioctanoin as well as only minimal effects on the interaction with colipase. The differences between I408D and I408K lipases can be explained by repulsion of the negatively charged aspartate side chain from the negatively charged surface of an emulsion particle coated with TDC or of TDC micelles. Conversely, the positively charged side chain of lysine might interact favorably with these same surfaces. Alternatively, the hydrophobic portion of the lysine side chain might stretch or snorkle into the hydrophobic portion of the emulsion or bile salt micelle and thereby allow the I408K mutant to have near normal function (20).

Regardless, the data are consistent with the conclusion that the region of the β5′-loop containing Val-407 and Ile-408 contributes to the function of hPTL.

Binding to Phospholipid Monolayers

A major difference between the results of experiments with a phospholipid monolayer and with triglyceride emulsions is the importance of Leu-412 in the interactions with phospholipid monolayers and the relative lack of effect with emulsions and dicaprin monolayers. Like the other mutants, the Leu-412 mutants showed decreased penetration of a phosphatidylcholine monolayer compared with hPTL. In contrast, Leu-412 does not appear to contribute significantly to the activity of hPTL in the presence of triglyceride emulsions with bile salts with or without colipase. Leu-412 mutations had minimal to no effect on the activity against tributyrin, trioctanoin, and triolein, on binding to tributyrin and trioctanoin, or on the interactions with colipase.

The varied behavior of the Leu-412 mutants depending on the nature of the lipid-water interface suggests that hPTL may orient differently on a phospholipid monolayer than it does on an emulsion particle. Molecular modeling of the hPTL indicates that Leu-412 should not be as close to the interface as are Val-407 and Ile-408 when the β5′-loop and the putative lipid binding regions of the N-terminal domain (lid and β9-loop) are in contact with the interface. If hPTL positions so that Leu-412 interacts with the interface, the catalytic site moves away from the interface creating an unfavorable orientation of hPTL for activity. Improper orientation of hPTL when bound to a phospholipid interface could explain, in part, the absence of activity of hPTL against phospholipids and the ability of phospholipids to inhibit hPTL activity in either monolayers or emulsions.

The β5′-Loop and Colipase

In our earlier study, where we replaced the β5′-loop of hPTL with the homologous loop from LPL, we also found that the mutant hPTL had reduced affinity for colipase (7). We offered several possible explanations for these findings. First, the larger lipoprotein lipase β5′-loop might sterically hinder the binding between the mutant hPTL and colipase. Alternatively, the presence of the larger β5′-loop could cause enough perturbation of the C-terminal domain structure to alter the critical colipase binding sites in this domain. The current study makes these interpretations less likely. The substitutions do not greatly alter the size of the β5′-loop, and the single-site substitutions are less likely to perturb the colipase binding sites of the C-terminal domain. Examination of the crystal structure of the hPTL-co-lipase complex indicates that the substitution of either aspartate or lysine at Val-407 or Ile-408 should not allow interactions of the new side chains with colipase. More importantly, each of these mutants showed normal activity and normal partitioning with colipase in 0.5 mM TDC. These results suggest that the β5′-loop mutants hPTL can form normal interaction with colipase under these conditions. Thus, the single-site substitutions we created and characterized do not hinder the interaction between colipase and hPTL because of their size or by perturbing the structure of the C-terminal domain.

Another explanation for the decreased affinity of the LPL β5′-loop mutant for colipase arose because we only measured the interaction between colipase and the mutant by competition with S153G hPTL. Under these conditions, the S153G-colipase complex may have a stronger affinity for the interface than does the lipoprotein lipase β5′-loop hPTL-colipase complex. Even if the mutant hPTL has normal affinity for colipase in the bulk phase, the accumulation of the S153G-colipase complex at the interface would deplete the concentration of colipase in solution and induce the dissociation of the LPL β5′-loop hPTL-colipase complex thereby decreasing the amount of the complex at the interface. If this were true, the mutants should have a normal apparent K_d for colipase and a decreased apparent

B_{max}. V407D, V407K, and I408D had increased apparent K_d values and normal apparent B_{max} values indicating that the mutants have decreased affinity for colipase and that, once formed, the mutant hPTL-colipase complex functions efficiently at the interface.

The last hypothesis to explain our results is that colipase and hPTL form a ternary complex with another partner. Because the mutations affect activity only in micellar solutions of bile salts, the partner could be bile salt micelles. Many older studies have demonstrated binding between bile salts and colipase or lipase (21–25). More recently, examination of the hPTL-colipase complex by neutron diffraction identified a detergent micelle contacting both colipase and the C-terminal domain of hPTL, including the $\beta 5'$-loop (26).

For several reasons, binding to bile salt micelles seems unlikely. First, the dissociation of bile salts from the micelles occurs at a millisecond rate, and it is difficult to accept that pure bile salt micelles contribute to a stable ternary complex with colipase and hPTL. Second, multiple studies have shown that TDC reduces the binding between colipase and hPTL and decreases the dissociation constant from $3–5 \times 10^{-7}$ to $0.3–1 \times 10^{-5}$ (24, 27, 28). The concentrations of hPTL and colipase in our assays are well below this range, 1.3×10^{-9} and 6.5×10^{-9}, respectively. Importantly, the dissociation constants in the earlier studies were not measured in the presence of lipid substrate, and the presence of a lipid-water interface may influence the formation or stability of a hPTL-colipase-bile salt micelle ternary complex.

Another potential partner in the formation of a ternary complex with colipase and hPTL are mixed micelles. In contrast to pure bile salt micelles, fatty acid-bile salt micelles increase the binding between colipase and hPTL 1000-fold (29). In fact, several studies have shown the formation of a hPTL-colipase complex with mixed micelles or with the lipoprotein particles present in bile (22, 30–32). In our experimental system, fatty acid-bile salt micelles may form rapidly during a short and lag period or the bile salt micelle-hPTL-colipase complex may form at the lipid-water interface. *In vivo*, the hPTL-colipase complex likely forms on the mixed micelles present in bile (22). Pancreatic and biliary secretions mix before they enter the duodenum. Because pancreatic secretions contain colipase and hPTL at concentrations of 10^{-6} M and the dissociation constant for the hPTL-colipase complex in the presence of mixed micelles is 10-fold lower, binding to the mixed micelles in bile is likely to be rapid.

Although there is evidence that colipase and hPTL associate with mixed micelles, there is no evidence that colipase and hPTL remain associated with the mixed micelles at the lipid-water interface. It is possible that the interface also plays the role of the third partner that stabilizes the colipase-hPTL complex and allows optimal activity. Whatever the nature of the complex at the lipid-water interface, the present results confirm that the colipase-hPTL association is stabilized in a ternary complex influenced by residues Val-407 and Ile-408 (Fig. 1).

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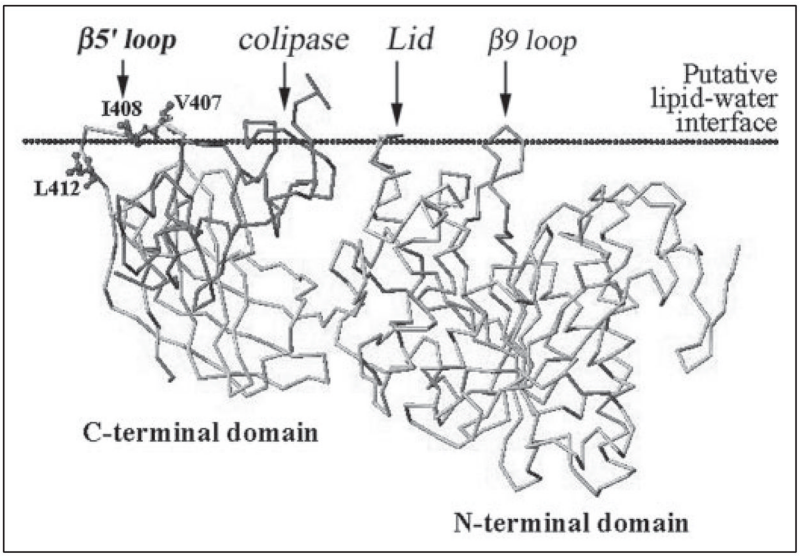

FIGURE 1. Schematic representation of the orientation of the PTL-colipase complex at a putative lipid interface

A side view of the PTL-colipase complex is presented as an α-carbon model. The lid domain, the β9-loop, and the β5′-loop of PTL and the hydrophobic fingers of colipase define a plane that can align with the lipid-water interface. In this view, the β5′-loop residues Val-407 and Ile-408 interact with the lipid interface, whereas the side chain of Leu-412 points away from the surface.

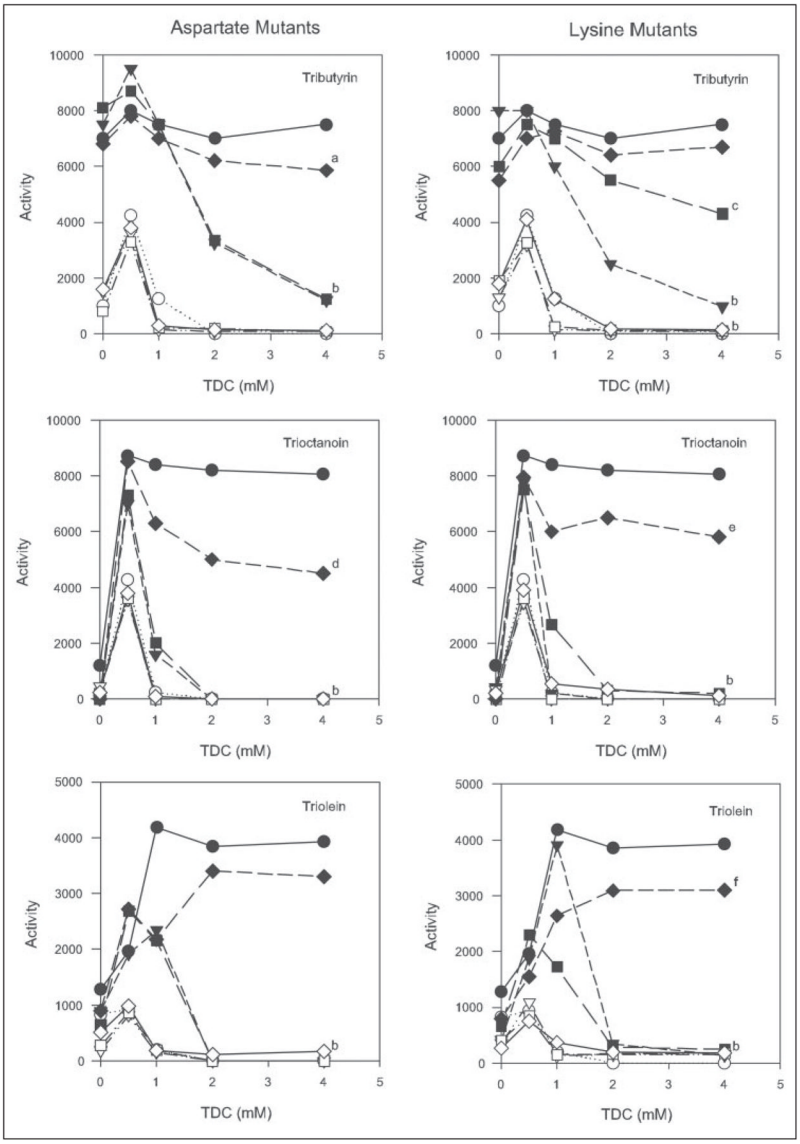

FIGURE 2. The bile salt and colipase dependence of the β5′-loop mutants activity against various triglyceride substrates

The assays were done with 1 μg of each lipase in the pH-stat at various concentrations of TDC and in the absence or presence of a 5 M excess of colipase. Activity is micromoles of fatty acid release per min per mg of lipase and represents the initial velocity of lipolysis measured during the pH-stat assay. *Circles*, hPTL; *triangles*, Val-407; *squares*, Ile-408; *diamonds*, Leu-412; *open symbols*, no colipase; *closed symbols*, colipase. The values are the average of three to four determinations. *Error bars* were not included for clarity. The significance of differences between each mutant and hPTL in the presence of 4.0 mM TDC was determined by *t* test: *a*, $p = 0.02$; *b*, $p \leq 0.001$; *c*, $p = 0.002$; *d*, $p = 0.003$; *e*, $p = 0.02$; and *f*, $p = 0.03$.

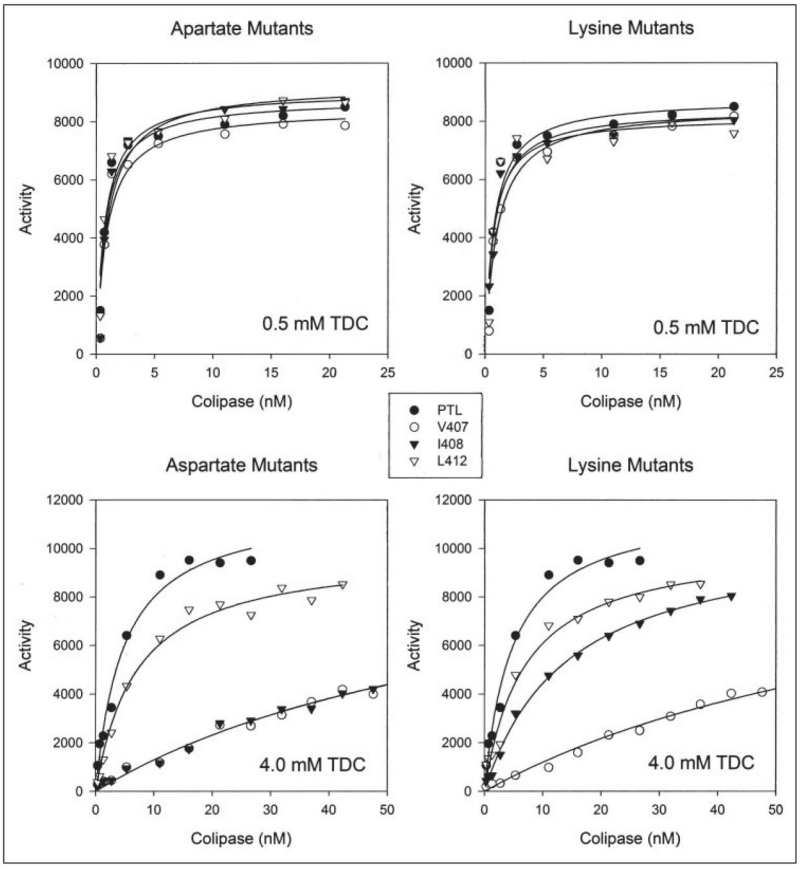

FIGURE 3. The colipase dependence of the β5′-loop mutants

The assays were done by the pH-stat method. Each assay included 1.3 nM lipase, 310 mM tributyrin, and the indicated concentrations of colipase and TDC. Activity is micromoles of fatty acid released per min per mg lipase. The *symbols* are defined in the *inset*.

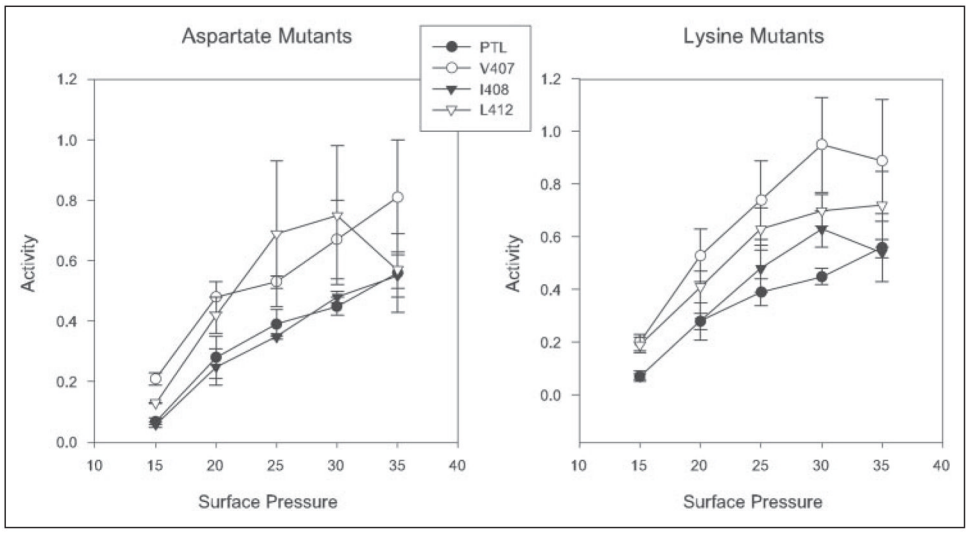

FIGURE 4. Activity of the β5′-loop mutants against a dicaprin monolayer

The activities were determined on a KSV-2200 barostat using a zero order Teflon trough as described under "Experimental Procedures." Each lipase was injected to a final concentration of 0.04 – 0.065 nM. The change in surface pressure was determined at various starting pressures for each lipase. Each point is the mean ± S.D. of three or more determinations. The *symbols* are defined in the *inset*.

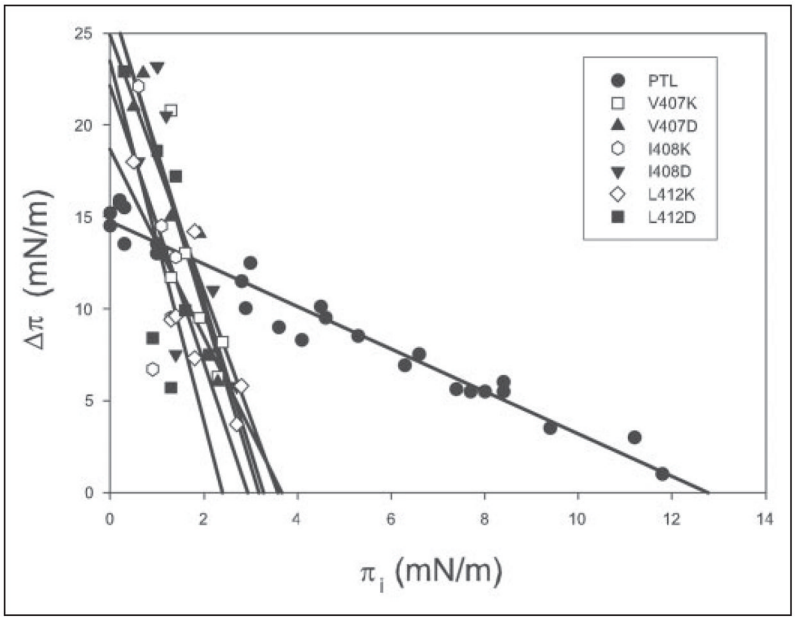

FIGURE 5. Penetration/adsorption of the β5′-loop mutants onto an egg phosphatidylcholine monolayer

17 μg of lipase was injected under the monolayer, and the increase in surface pressure was measured. The maximum increase in surface pressure ($\Delta\pi$) was plotted as a function of the initial surface pressure (π_i). Both $\Delta\pi$ and π_i are expressed in millinewtons per meter. The linear curves were obtained by linear regression using the least squares method. The *symbols* are defined in the *inset*.

TABLE 1

Water-accessible surfaces (Å^2) of the β5′-loop in hPTL and the β5′-loop mutants

The values are the water-accessible surfaces of charged residues (Arg, Asp, Glu, His, and Lys), semipolar residues (Gln, Gly, Asn, Cys, Ser, and Thr), and hydrophobic residues (Ala, Phe, Ile, Leu, Met, Pro, Val, Trp, and Tyr).

	hPTL	V407D	I408D	L412D	V407K	I408K	L412K
				Å^2			
Charged residues	105	277	289	286	283	290	286
Semipolar residues	284	206	261	185	246	311	237
Hydrophobic residues	488	409	315	384	409	320	384
Total surfaces	877	892	865	855	938	921	907
HLB[a]	0.80	1.18	1.75	1.23	1.29	1.88	1.36

[a]HLB is the hydrophilic/lipophilic balance and is defined by the (charged plus semipolar/hydrophobic) ratio.

TABLE 2

Lag times and activity against triolein of β5′-loop mutants in 4 mM TDC

All assays were done by the pH-stat method with triolein in the presence of 4 mM TDC and a 5-fold molar excess of colipase. The lag time was determined from the plot of fatty acids released *versus* time by extrapolating the slope of the burst phase to the time axis and reported as minutes. Activity (units/mg) was determined from the slope of the burst phase. Each result is presented ± S.D.

Lipase	Lag time	Activity
	min	*units/mg*
Wild type	0.0 ± 0.0	3900 ± 280
V407D	23.6 ± 4.0	3100 ± 400
I408D	32.5 ± 3.2	1500 ± 230
L412D	2.0 ± 0.5	3400 ± 370
V407K	12.9 ± 1.3	3200 ± 290
I408K	6.0 ± 1.0	2500 ± 380
L412K	0.0 ± 0.0	3100 ± 260

TABLE 3
Partitioning of lipase mutants with tributyrin and trioctanoin

All assays were done as described under “Experimental Procedures.” Colipase was present in a 5-fold molar excess for all assays.

Lipase	Tributyrin[a]		Trioctanoin[a]	
	0.5 mM	4.0 mM	0.5 mM	4.0 mM
	%			
Wild type	83 ± 7.4	92 ± 4.0	88	91
V407D	84 ± 6.3	47 ± 5.3	75	5
I408D	82 ± 5.9	37 ± 2.5	75	5
L412D	89 ± 4.0	86 ± 6.2	82	88
V407K	87 ± 5.0	52 ± 5.7	88	5
I408K	78 ± 9.2	80 ± 7.1	78	87
L412K	92 ± 8.7	87 ± 4.8	84	86

[a]The percentage of the original lipase amount that partitions with each lipid is given. Values for tributyrin are the average ± S.D. of three determinations.

TABLE 4

Apparent K_d and B_{max} of β5′-loop mutants for colipase with 4 mM TDC

All assays were done by the pH-stat method with tributyrin in the presence of 4 mM TDC. Values were determined by nonlinear regression with a rectangular hyperbola function. The regression analysis for V407D, I408D, and V407K was done with values out to 210 nM colipase (data not shown). B_{max} is given as micromoles of fatty acid released/min ± 1 S.D., and the K_d is reported as nM ± 1 S.D.

Lipase	Apparent K_d	Apparent B_{max}
Wild type	4.7 ± 0.8	11,800 ± 640
V407D	72.0 ± 6.0	10,740 ± 450
I408D	74.0 ± 4.6	10,855 ± 245
L412D	6.9 ± 0.9	9,890 ± 360
V407K	84.9 ± 5.6	11,380 ± 400
I408K	14.0 ± 1.1	10,790 ± 310
L412K	6.8 ± 1.2	10,270 ± 565

TABLE 5

Affinity of β5′-loop mutants for colipase by competition assays

All of the pH-stat experiments were performed with trioctanoin as substrate, 4 mM TDC. In procedure A, 10 μg of hPTL, 10 μg of mutant lipase, and 1 μg of colipase were premixed before their injection into the pH-stat vessel. In procedure B, the same amounts of mutant lipase and colipase were premixed and injected into the pH-stat vessel. The release of fatty acids was monitored for 2 min, 10 μg of S153G was added to the vessel, and the reaction continued for another 2–3 min. Each assay was performed in duplicate or triplicate.

Lipase	Residual lipase activity
	%
A) Incubation of the mutant lipase with hPTL and colipase	
V407D	107.6 ± 7.8
I408D	116.0 ± 16.7
V407K	117.8 ± 27.6
B) Incubation of the mutant lipase with colipase followed by the addition of S153G hPTL	
L412D	47.0 ± 8.2
I408K	46.2 ± 17.1
L412K	38.9 ± 18.5