The open lid mediates pancreatic lipase function

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Abstract **Pancreatic triglyceride lipase (PTL) and the homologous pancreatic lipase related protein 2 (PLRP2) provide a unique opportunity to understand the molecular mechanism of lipolysis. They differ in substrate specificity, sensitivity to bile salts, and colipase dependence despite their close amino acid and tertiary structure identity. One important structure, present in both lipases, is the lid which occupies different positions in the inactive and active forms of PTL. We investigated the role of the lid in lipase function by site-specific mutagenesis. By exchanging the lids between PTL and PLRP2, we created two chimeric lipases. Additionally, we made multiple substitution mutations in the PTL lid. PLRP2 with the PTL lid had kinetic properties similar to PLRP2. PTL with the PLRP2 lid was greatly impaired and had no activity at micellar bile salt concentrations even in the presence of colipase. Both chimeras showed interfacial activation suggesting that the closed lid position was maintained. A series of substitution mutations were made in positions Arg257 and Asp258. These mutations demonstrated the importance of these two residues to maintaining the normal activity, triglyceride acyl chain specificity, and colipase interaction of PTL. The preserved interfacial activation in the chimeras, the similar crystal structure of the two lids in the closed position, and the importance of Arg257 and Asp258 in mediating the open conformation of the lid argue that the position of the open lid influences the differences in activity against triglycerides, in sensitivity to bile salts, and in colipase dependence between PTL and PLRP2.**—Yang, Y., and M. E. Lowe. **The open lid mediates pancreatic lipase function.** ***J. Lipid Res.*** **2000.** 41: **48–57.**

Supplementary key words pancreatic lipase-related protein • colipase • site-directed mutagenesis • bile salts

The pancreas expresses three closely related members of the lipase gene family (1). One, pancreatic triglyceride lipase (PTL), was recognized many years ago (2). The other two were described recently and were named pancreatic lipase related proteins 1 and 2 (PLRP1 and PLRP2) because of their high amino acid sequence homology to PTL (3). Of the three homologues, only PTL and PLRP2 have known lipase activity (4–6). Even though PTL and PLRP2 have lipase activity and homologous protein structures, they differ in enzymatic properties, such as substrate specificity, sensitivity to inhibition by bile salts, and colipase dependence. PTL hydrolyzes only triglycerides whereas PLRP2 hydrolyzes triglycerides, phospholipids, and galactolipids (4–7). Bile salts inhibit PTL, but do not inhibit PLRP2. PTL depends on colipase for activity in the presence of bile salts and PLRP2 does not. These functional differences between PTL and PLRP2 must be based on structural differences between the two pancreatic lipases.

The close identity of the two lipases and the wealth of data about protein structure for both lipases provide a unique opportunity to identify the structures that account for their functional differences. One region of the proteins, revealed by the tertiary structures of PTL and of PLRP2, that could influence the function of PTL and PLRP2 is a 23 amino acid surface loop, the lid overlying the active site (8, 9). In this position, the lid would prevent substrate from entering the active site. These lipases would not be active, a conclusion that led several authors to predict a conformational change in the lid (9–11). Subsequently, the tertiary structures of several lipases, including colipase and PTL crystallized in the presence of mixed micelles, demonstrated the predicted movement of the lid away from the active site (12). The new position of the lid opens the active site to substrate, creates a binding site for an acyl chain, exposes a potential interfacial binding site, and forms new interactions between the lid and colipase. These findings imply that lipase function depends on reorganization of the lid.

Several other studies support the hypothesis that the lid influences the function of lipases. Exchanges of the lid between two other members of the lipase gene family, lipoprotein lipase and hepatic lipase, produced chimeras with altered substrate specificity (13–15). A guinea pig lipase, GPLRP2, lacks a lid and possesses high phospholipase activity (4). The addition of the PTL lid to GPLRP2 decreases, but does not abolish, the phospholipase activity, suggesting that the lid contributes to substrate specificity (16). In another study, deletion of the lid in human PTL

Abbreviations: PCR, polymerase chain reaction; PLRP2, pancreatic lipase-related protein 2; PTL, pancreatic triglyceride lipase; PLRP2/PTL lid, PLRP2 with the lid sequence from PTL; PTL/P2 lid, PTL with the lid sequence from PLRP2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TDC, taurodeoxycholate.

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decreased activity against triglycerides and impaired binding to mixed micelles of bile salt and triglyceride (17). Site-directed mutagenesis of colipase indicates that colipase interacts with the lid and that lipase activity in the presence of bile salts depends on this interaction (18). Together these studies implicate the lid domain in several functions of lipase, substrate specificity, interfacial binding, and colipase interaction.

To determine whether the lid influences substrate preference, colipase dependence, and response to bile salts, we exchanged the highly homologous lids of PTL and PLRP2 and introduced mutations at divergent sites in the lid. The mutant lipases were expressed in a yeast system, purified to homogeneity, and characterized for stability and enzymatic properties. Our findings demonstrate that PTL function depends on the lid and identify two residues that are critical for the function of the lid.

METHODS

Construction of the lid chimeras

All manipulations of DNA were done by standard methods unless otherwise noted (19). The nucleotide sequence encoding the rat PLRP2 lid was introduced into the cDNA encoding rat PTL by the polymerase chain reaction (PCR) overlap extension method (20). The internal primers for the initial reactions were two synthetic 69 bp oligonucleotides. The two complementary oligonucleotides overlapped by 30 bp and together comprised the entire coding sequence for the PLRP2 lid and the PTL sequence flanking the lid. The flanking primers were complementary to the 5′- and 3′-ends of the PTL sequence. PTL cDNA isolated from pGEM4z by EcoRI and BamHI restriction digestion and agarose gel purification (QIAquick, Qiagen) was the template. The PCR conditions were 20 cycles at 94°C for 1 min, 45°C for 1 min, and at 72°C for 1 min in a Thermolyne TEMPTRONIC cycler. The PCR products were isolated by agarose gel electrophoresis and were used as the template in a second PCR reaction with the flanking primers using the conditions described above. The final PCR product was purified by agarose gel electrophoresis and extraction. The product was digested with BalI and KpnI and the resulting restriction fragment was ligated into BalI and KpnI digested PTL pGEM and transformed into TB1 cells. Several colonies were selected at random. Plasmid DNA was prepared and sequenced through the region encoding the lid by the Sequenase method. The chimeric DNA was excised from pGEM by digestion with EcoRI and BamHI and ligated into pVL1392. The presence of the expected sequence was confirmed by nucleotide sequencing of the entire cDNA insert. Recombinant baculoviruses were made as previously described (21).

PRLP2/PTL lid was made with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's directions. PLRP2 cDNA was cloned into the yeast expression vector pHIL-S1 as described for PTL (22). In this vector the native signal peptide was replaced by the yeast PHO1 signal peptide. The chimeric cDNA was made in two steps. In the first step, complementary synthetic oligonucleotides corresponding to bp 801–837 of the PTL cDNA were the primers in the first QuickChange reaction. The mutant was isolated as described in the QuickChange instructions and the sequence was confirmed by dideoxynucleotide sequencing. This plasmid containing the partial PTL lid in the PLRP2 cDNA was the template for a second reaction. The primers in the second reaction were complementary to bp 839–869 of the PTL cDNA. After isolating individual plasmids, the presence of the mutations was confirmed by dideoxynucleotide sequencing.

Construction of PTL mutants

All single or double amino acid substitutions were made with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's directions. PTL cDNA in pHIL-S1 was the template. Synthetic oligonucleotides containing the desired mutation were the primers. Each mutation was confirmed by dideoxynucleotide sequencing.

Recombinant proteins

PTL, PLRP2, and PTL/P2 lid were expressed in sf9 cells and purified as previously described (21). PTL, PLRP2, PLRP2/PTL lid, and the PTL lid mutants were expressed in yeast cells and purified as described (22). Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein concentrations were measured by the Bradford assay (23) using purified, native human pancreatic lipase as the standard. Recombinant human procolipase was also produced in yeast cells and converted to colipase by immobilized trypsin as previously described (24).

Lipase and phospholipase assays

Triglyceride lipase assays were done in a Radiometer VIT 90 pH-stat as described (25). Triglyceride substrates were tripropionin, tributyrin, trioctanoin (Fluka, Ronkonkoma, NY) and triolein (Sigma, St. Louis, MO). These triglycerides differ in acyl chain length and water-solubility and were utilized to determine whether lid mutations changed the acyl chain preference of the mutant PTL. Phospholipase activity was assayed with a radioactive substrate, [^{3}H]phosphotidylcholine (Amersham Pharmacia Biotech, Piscataway, NJ) as described (26). Interfacial activation was determined with *p*-nitrophenylbutyrate following a published method (27). The results are expressed as mmoles fatty acid released/min per mg of purified protein as determined by a Bradford assay (23).

Binding to tributyrin

Binding assays were done in 0.1 m Tris-Cl, pH 8.0, 0.1 m NaCl, 2 mm $CaCl_2$ and the stated concentration of taurodeoxycholate (TDC). Tributyrin was added to the binding buffer in a ratio of 0.5 ml tributyrin to 14.5 ml buffer and the mixture was emulsified by homogenization. Assays were done in 1.7-ml microfuge tubes. One ml of buffer and a 10-fold excess of colipase were added to a tube. Immediately after adding lipase (2–3 μg), the tube was shaken at room temperature for 1 min. After the incubation, the phases were separated by centrifugation in a microfuge at high speed for 20 min. Five hundred μl of the upper, aqueous phase was assayed by the titrametric method. PTL and PLRP2 were assayed with 4.0 mm TDC and PTL/P2 lid was assayed with 0.5 mm TDC. The total activity for each lipase was determined in an identical tube containing buffer without tributyrin and handled in parallel with the other samples.

Temperature stability

Each lipase (5 μg) was incubated in 10 μl 10 mm Tris-Cl, pH 8.0, 0.15 m NaCl for 20 min at the indicated temperatures. An aliquot, 2.5 μg, was assayed by the pH-STAT method with 5-fold molar excess of colipase and 4.0 mm TDC for PTL and PLRP2 and 0.5 mm TDC for the lid chimeras. The results were expressed as the percentage of the activity in the original sample.

RESULTS

Expression of lid chimeras

We began our investigation into the contribution of the lid to lipase function by exchanging the lids between PTL and PLRP2. The exchanges created two chimeras, PTL with the PLRP2 lid (PTL/P2 lid) and PLRP2 with the PTL lid (PLRP2/PTL lid). To produce adequate amounts of protein for kinetic analysis, we initially expressed PTL, PLRP2, and PTL/P2 lid in baculovirus infected sf9 cells, but later switched to a yeast expression system to produce PLRP2/PTL lid, PLRP2, and PTL because the yeast were easier to manipulate and larger amounts of protein were expressed (22). We expressed each protein in large scale and purified 2–10 mg of each as described in Methods. We analyzed the purified proteins by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. The purified lid chimeras ran as a single, major band on SDS-polyacrylamide gel electrophoresis and were suitable for further analysis (**Fig. 1**).

Lipolytic activity

Because PTL and PLRP2 differ in response to bile salts and to colipase, we measured the activity of the purified wild-type lipases and of the lid chimeras against tributyrin emulsified in varying concentrations of TDC. The assays were done with and without colipase. As previously reported, micellar concentrations of TDC inhibited PTL activity whereas PLRP2 activity showed little variation with increasing TDC concentrations (**Fig. 2**, PTL and PLRP2). Colipase restored activity to PTL and increased PLRP2 activity about 4-fold (Fig. 2, PTL and PLRP2). Of the two lid chimeras, only the activity of the PTL/P2 lid was affected by exchanging the lids. The PTL/P2 lid chimera had only about 10% of wild-type activity at low TDC concentrations. Higher TDC concentrations completely inhibited PTL/P2 lid like the parent lipase, PTL. But, in contrast to its effect on PTL, colipase did not restore activity to PTL/P2 lid (Fig. 2, PTL/P2 lid). The P2/PTL lid had the same activity and response to colipase as did the parent lipase, PLRP2 (Fig. 2, P2/PTL lid). These findings demonstrate that PLRP2 tolerates changes in the lid sequence better than does PTL, suggesting that the conformation of the lid may be critical to PTL function.

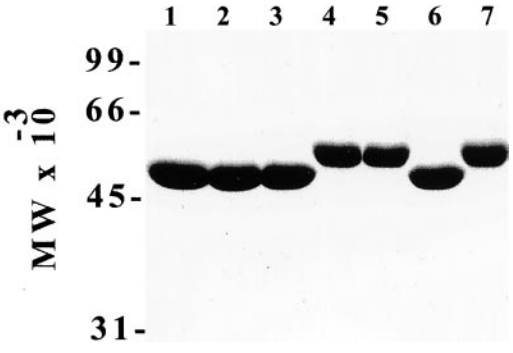

Fig. 1. SDS PAGE of purified PTL, PLRP2, and the lid chimeras. Ten μg of each protein was dissolved in SDS sample buffer and separated by SDS PAGE as described in Methods. The gel was stained with Coomassie Blue. The positions of the molecular weight markers are given on the left of the figure: 1, PTL from human pancreas; 2, PTL from sf9 cells; 3, PTL from yeast; 4, PLRP2 from sf9 cells; 5, PLRP2 from yeast; 6, PLT/P2 lid chimera; 7, P2/PTL lid chimera.

Although the decreased activity of the PTL/P2 lid chimera may be caused by conformational changes in the lid alone, more global conformational changes in the protein could be induced by the mutation and alter the chimeras activity to produce the results in Fig. 2. Whenever recombinant proteins, particularly mutant proteins, are analyzed, the possibility that they have not folded properly must be tested. The robust secretion of the chimera by the yeast cells argues against any large changes in folding. We further assessed the conformational stability of the mutants and the wild-type proteins by determining the temperature denaturation curves for each protein (**Fig. 3**). The denaturation curves for PTL and PTL /P2 lid were identical with a $T_{1/2}$ of 50°C. Likewise, the denaturation curve for the P2/PTL lid mutant paralleled the PLRP2 denaturation curve. These two lipases were more sensitive to increasing temperature than was PTL and had a $T_{1/2}$ of 43°C. We concluded that the amino acid changes introduced in the lid had no significant effect on conformational stability that could explain the results with the PTL/P2 lid chimera.

Another explanation for the inability of colipase to restore PTL/P2 lid activity at higher TDC concentrations is that the chimera may be more sensitive to denaturation by TDC. We tested the stability of the PTL/P2 lid in TDC by incubating the chimera in 10 mm Tris-Cl, pH 8.0, 0.15 m NaCl, and 4 mm TDC buffer at room temperature. At various times, we removed an aliquot and determined the activity in 0.5 mm TDC. Full activity remained after a 45-min incubation showing that PTL/P2 lid is stable in TDC and eliminating denaturation by TDC as an explanation for the colipase-independent, inhibition of PTL/P2 lid at higher TDC concentrations.

The absent activity of the PTL/P2 lid in the presence of TDC could also occur if the PTL/P2 lid chimera cannot bind colipase on interfaces containing bile salts. The interaction between colipase and PTL includes contacts with colipase and the open lid of PTL. If the lid on PTL/P2 lid does not move into the proper conformation, its interaction with colipase may be inefficient and the lipase may not bind to the interface. To test whether the PTL/P2 lid chimera interacted normally with colipase, we measured the ability of colipase to mediate binding of the mutant to tributyrin emulsions of varying TDC concentrations. PTL, PLRP2, PTL/P2 lid, or P2/PTL lid were incubated for 1 min with the emulsions and a 5-fold molar excess of colipase. The organic and aqueous phases were separated by centrifugation and the activity remaining in the aqueous phase was determined by the pH STAT assay. In the presence of colipase, PTL separated with the organic phase under all conditions (**Fig. 4**). The majority of PLRP2 and P2/PTL also partitioned with the organic phase, but a larger fraction remained in the aqueous phase. Nearly all of the PTL/P2 lid chimera bound to the organic phase until the TDC concentration reached 1.0

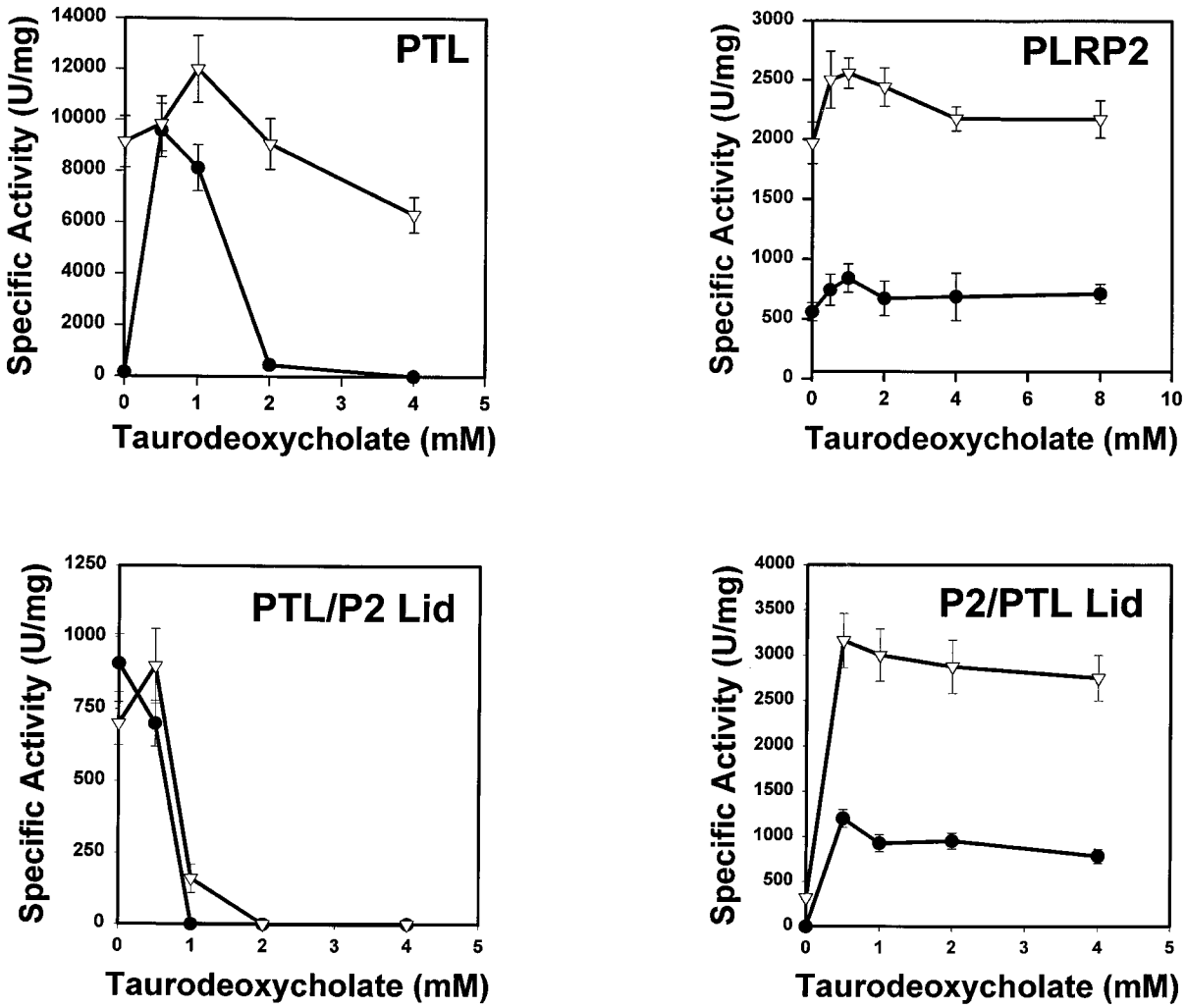

Fig. 2. Activity of the lipases against tributyrin in various taurodeoxycholate concentrations. Activity was determined in the pH-stat as described in Methods; 2.5 to 5 μg of lipase was assayed. Closed circles, lipase alone; open triangles, 5-fold molar excess of colipase added before the lipase. The lipase assayed is given in each panel.

mm where binding was completely inhibited. The binding data mirrored the effect of TDC observed with the activity assays. This result confirmed the hypothesis that colipase-mediated binding to substrate is disrupted in PTL/P2 lid.

Interfacial activation

The lid has been implicated in another property of PTL, interfacial activation, the preference of PTL for water-insoluble substrates with interfaces over water-soluble, monomeric substrates. Several studies support the hypothesis that a conformational change in the lid accounts for the kinetic phenomena of interfacial activation (10–12, 28). Although interfaces activate both PTL and PLRP2, the possibility remains that amino acid changes in the lid could alter conformation in a way that affects interfacial activation in the chimeras. We tested the lid chimeras for interfacial activation against PNPB (**Fig. 5**). The low activity of the PTL/P2 chimera against tripropionin precluded using that substrate for these studies. PLRP2 and the P2/PTL lid had similar activities against PNPB. Their activity was about 3-fold higher than that of PTL/P2 lid. All three lipases showed an increase in activity above the solubility limit of PNPB indicative of interfacial activation. The P2/PTL lid chimera also demonstrated interfacial activation with tripropionin (data not shown). The preserved interfacial activation of the chimeras argues that the chimeras have no significant alterations in conformation. More importantly, the presence of interfacial activation is consistent with the previous crystal structures showing that the stabilizing interactions between the lid and the core of the protein are similar for PTL and PLRP2 (8, 9).

Substrate specificity

Earlier studies suggested that residues in the lid contribute to substrate binding and substrate preference (13–16). Our lid chimeras provided the opportunity to determine whether the lid of either PTL or PLRP2 contributes to activity against several different substrates. The lid contributes to one acyl chain binding site and alterations in the lid might affect the substrate preference by changing that site. First, we assayed the activity of the native and chimeric lipases against various triglycerides containing dif-

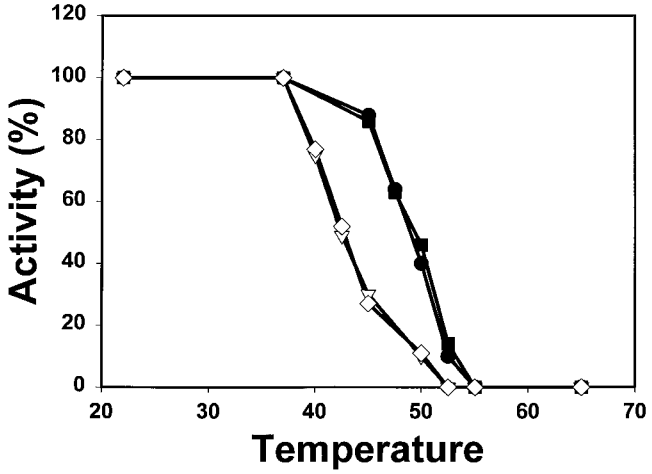

Fig. 3. Temperature denaturation curve for PTL, PLRP2, and the lid chimeras. Each lipase was incubated at various temperatures as described in Methods. An aliquot was measured for activity against tributyrin by the pH-stat method with a 5-fold molar excess of colipase. Data were expressed as a percentage of the starting activity. Closed circles, PTL; closed squares, PLT/P2 lid; open diamonds, PLRP2; open triangles, P2/PTL lid.

ferent acyl chain lengths (**Table 1**). Because the PTL/P2 lid chimera was completely inactive against all substrates above 2.0 mm TDC (Fig. 2 and data not shown), the assays were done in 0.5 mm TDC. Both PTL and PLRP2 hydrolyzed all of the triglyceride substrates at high rates. The source of the enzyme, be it tissue, insect cells, or yeast, did not affect the specific activities for either PTL or PLRP2. The PTL/P2 lid chimera had decreased activity against all substrates when compared to PTL. (Table 1). In contrast, the activity of the P2/PTL lid was preserved and, in fact, was higher than the activity of the parent lipase, PLRP2, against all substrates except for tributyrin which was slightly decreased (Table 1).

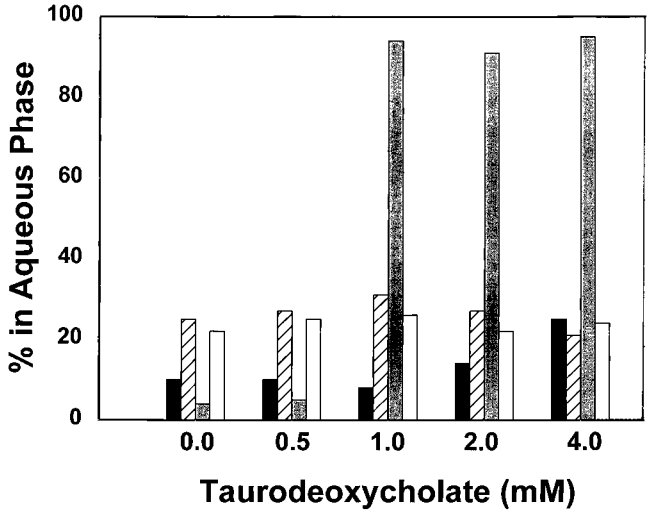

Fig. 4. Binding of PTL, PLRP2, and PTL/P2 lid chimera to tributyrin. Binding was done as described in Methods. The taurodeoxycholate concentration was varied in the binding buffer. Black bar, PTL; hatched bar, PLRP2; gray bar, PTL/P2 lid; white bar, P2/PTL lid.

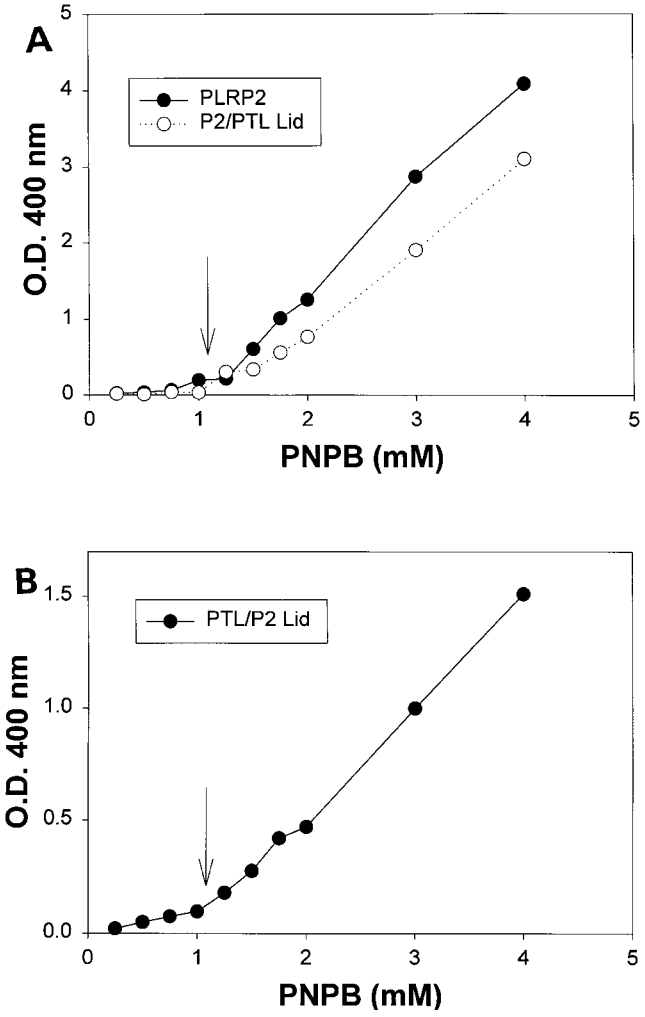

Fig. 5. Interfacial activation of the lid chimeras. Interfacial activation was determined with PNPB. Panel A: Interfacial activation of PLRP2 (closed circles) and P2/PTL lid (open circles). Panel B: Interfacial activation of PTL/P2 lid. The saturation point of PNPB is given by the arrow in each panel.

In addition to affecting the specific activity, the lid exchanges altered the substrate preference of the chimeras. The activity of PTL/P2 lid against tripropionin was most affected, with over a 300-fold decrease in activity (**Table 2**). Next was triolein with a 20-fold decrease followed by tributyrin with about a 10-fold decrease in activity. Activity against trioctanoin was the best preserved, about 8-fold lower for the chimera. For the P2/PTL lid chimera, the activity against trioctanoin was increased disproportionately to the other substrates (Table 2). These results show that residues in the lid play a significant role in determining the substrate preference for both PTL and PLRP2. The contribution of the lid residues to substrate activity and preference was much greater for PTL than for PLRP2, again suggesting the greater role of the lid in the lipolytic mechanism of PTL compared to PLRP2. Even so, the lack of a simple correlation between the lid and activity profiles indicates that multiple regions of both lipases contribute to substrate binding and hydrolysis.

TABLE 1. Activity of PTL, PLRP2, and lid chimeras against various triglyceride substrates

Lipase	Specific Activity			
	Tripropionin	Tributyrin	Trioctanoin	Triolein
	units/mg protein			
PTL (tissue)	7200 ± 650	10,000 ± 850	7500 ± 640	3230 ± 410
PTL (sf9 cells)	6800 ± 700	9750 ± 900	7120 ± 580	3150 ± 375
PTL (yeast)	7000 ± 675	10,520 ± 1150	7750 ± 895	3080 ± 360
PLRP2 (sf9)	600 ± 50	2600 ± 280	700 ± 50	640 ± 70
PLRP2 (yeast)	620 ± 65	2740 ± 330	760 ± 60	710 ± 55
PTL/P2 lid	22 ± 5	900 ± 80	1000 ± 60	160 ± 30
P2/PTL lid	1000 ± 130	2340 ± 295	2280 ± 240	1110 ± 90

Activities were determined by the pH-stat method in the presence of 0.5 mm taurodeoxycholate with a 5-fold molar excess of colipase. The average and standard deviations of 3–4 determinations are given.

Phospholipase activity

In addition to the differences with triglycerides, PLRP2 and PTL differ in reactivity against other lipids. PLRP2 will hydrolyze phospholipids and galactolipids and PTL will not (5–7, 12, 21, 29). We determined whether the lid contributed to phospholipase activity by measuring the ability of the chimeras to hydrolyze phosphatidylcholine. As expected, PLRP2 hydrolyzed phosphatidylcholine with a specific activity of 3.0 ± 0.5 nmol/min per mg and PTL did not. Only the P2/PTL lid chimera had phospholipase activity and its specific activity, 3.5 ± 0.3 nmol/min per mg, was similar to that of PLRP2. These results demonstrate that the lid does not contribute significantly to phospholipid hydrolysis.

Identification of the critical lid residues

Amino acids that differ between the PTL and PLRP2 lids must be responsible for the detrimental effects of lid exchange on the activity of the PTL/P2. Alignment of the lid amino acid sequences for the PTL and PLRP2 lipases from various species shows conservation of most lid residues (**Fig. 6**). Several exceptions occur at positions 245, 257, and 258, where the PTL lipases have a conserved residue that differs from the residue found in all PLRP2 lipases, and at position 250, where the PTL lipases have an aspartic acid and all but one PLRP2 lipase have an asparagine. Human PLRP2 has aspartate at position 250. Of these, Arg257 and Asp258 are the most attractive candidates for mediating the conformation of the open lid, the conformation most likely affected in the PTL/P2 lid. When the PTL lid opens, Arg257 and Asp258 form salt bridges with two residues in the core of the protein, Tyr268 and Lys269 (**Fig. 7**). These interactions stabilize the open conformation of the lid. In PLRP2, phenylalanine and glutamic acid occupy positions 268 and 269. The differences between PTL and PLRP2 at these four positions may permit the respective lids to occupy different conformations, which, in turn, would influence the function of the two lipases. These changes could account for the functional differences between PTL and the PTL/P2 lid chimera.

TABLE 2. Relative activity of the lid chimeras to the parent lipase

Lipase ratio	Ratio of Specific Activities			
	Tripropionin	Tributyrin	Trioctanoin	Triolein
PTL/P2lid to PTL	0.003	0.09	0.13	0.05
P2/PTL lid to PLRP2	1.7	0.87	3.26	1.59

The specific activities from Table 1 are expressed as a ratio of the chimera to the parent wild-type lipase.

Expression of single and multiple substitution mutants

To determine whether lid residues Gln245, Arg257, Asp258, and core residues Tyr268 and Lys269 affect the function of PTL, we introduced mutations at these positions. Both multiple and single mutants were introduced. Alanine and the residues occupying these positions in PLRP2 lipases were substituted into PTL. Each of the mutant proteins was expressed in yeast and 2–4 mg of each was purified as described in the Methods. The purified proteins migrated as a single band on SDS polyacrylamide gels (data not shown). All mutants were active against tributyrin at 0.5 mm TDC (see below). The activity allowed us to determine the temperature denaturation curves for the mutants. All of the mutants had half-maximal activity after heat denaturation at the $T_{1/2}$ for PTL indicating that the mutation did not profoundly affect protein folding (**Table 3**).

Activity of the lid domain mutants

We measured the activity of each mutant against tributyrin, trioctanoin, and triolein at two concentrations of TDC, 0.5 mm and 4.0 mm. Only one of the tested mutants, Q245T, had normal specific activity against tributyrin, trioctanoin, and triolein (6500, 3600, and 1800 nmol/min per mg, respectively) and colipase dependence. Gln245 is located in a short irregular helix that forms when the lid opens. In the crystal structure of the PTL–colipase complex, Gln245 does not contribute to the substrate binding site or to the colipase binding site of the open lid. The normal activity of the Q245T mutant agrees with the structural data and demonstrates that the residue at position 245 does not account for the kinetic differences observed among PTL, the PTL/P2 lid chimera, and PLRP2.

The other mutations at positions 257 and 258 all decreased lipase activity although there was a broad range of effects. Replacing the naturally occurring residues with glycine residues decreased the activity of the R257G/D258G double mutant to 1–3% of wild-type activity against tributyrin and trioctanoin at both TDC concentrations (**Fig. 8**, lid 1). No activity of the R257G/D258G PTL mutant against triolein was detected under conditions where 0.1% of wild-type activity could be measured. The activity profile of this mutant was the same as that of the PTL/P2 lid chimera suggesting that positions 257 and 258 account for most if not all of the decreased activity of this chimera.

The influence of positions 257 and 258 may be mediated through the interactions that form between these residues in the open lid and amino acids in the core of PTL. The substitution of Arg257 and Asp258 with the corresponding residues from PLRP2 could alter the interac-

HPL	C	K	K	N	I	L	S	Q	I	V	D	I	D	G	I	W	E	G	T	R	D	F	A	A	C
RPL	C	Q	K	N	I	L	S	Q	I	V	D	I	D	G	I	W	E	G	T	R	D	F	A	A	C
PPL	C	Q	K	N	I	L	S	Q	I	V	D	I	D	G	I	W	E	G	T	R	D	F	V	A	C
HP2	C	K	K	N	V	L	S	T	I	T	D	I	D	G	I	W	E	G	I	G	G	F	V	S	C
RP2	C	Q	K	N	I	L	S	T	I	V	D	I	N	G	I	W	E	G	T	Q	N	F	V	A	C
MP2	C	Q	K	N	I	L	S	T	I	V	D	I	N	G	I	W	E	G	T	R	N	F	A	A	C
CP2	C	E	K	N	I	I	S	T	I	V	D	V	N	G	F	L	E	G	I	T	S	L	A	A	C

Fig. 6. Alignment of the lid domains from PTL and PLRP2 isolated from various species. The amino acid sequence for the lid is given in the one-letter amino acid code. The boxes highlight positions 245, 257, and 258 of PTL and the corresponding residues in PLRP2. HPL, human PTL; RPL, rat PTL; PPL, porcine PTL; HP2, human PLRP2; RP2, rat PLRP2; MP2, mouse PLRP2; CP2, coypu PLRP2.

tions with Tyr268 and Lys269 and prevent the lid from reaching the optimal conformation for activity. We removed this potential hindrance in the R257G/D258G PTL mutant by changing Tyr268 and Lys269 to the corresponding PLRP2 residues, Phe268 and Glu269. The resulting mutant PTL activity against tributyrin and trioctanoin increased to 10% of PTL activity with 0.5 mm TDC and to 5% of PTL activity with 4.0 mm TDC (Fig. 8, lid 2). Activity against triolein was now detectable at about 4% of wild-type levels. Although the quadruple mutant lipase had increased activity compared to the R257G/D258G PTL mutant, full activity was not restored, indicating that the mutation of Arg257 and Asp258 may influence other normal interactions of the lid.

Several single substitution mutations were made at positions 257 and 258 to determine the contribution of each residue to PTL function. In the presence of 0.5 mm TDC, all of the mutant lipases had activity against each of the three triglycerides (Fig. 8A). The activity against trioctanoin was the least affected in general and ranged from 30 to 58% of PTL activity. The exceptions were D258G and D258N which had slightly higher relative activities against tributyrin. For the other PTL mutants with single substitutions, tributyrin was the second best substrate with activities extending from 13 to 58% of PTL activity. Triolein activity decreased the most in all of the mutants with activities spanning 6 to 29% of PTL.

The activity profile changed in the presence of 4.0 mM TDC (Fig. 8B). Except for D258R PTL, the Asp258 mutants had relatively preserved activity, 40 to over 100% of PTL

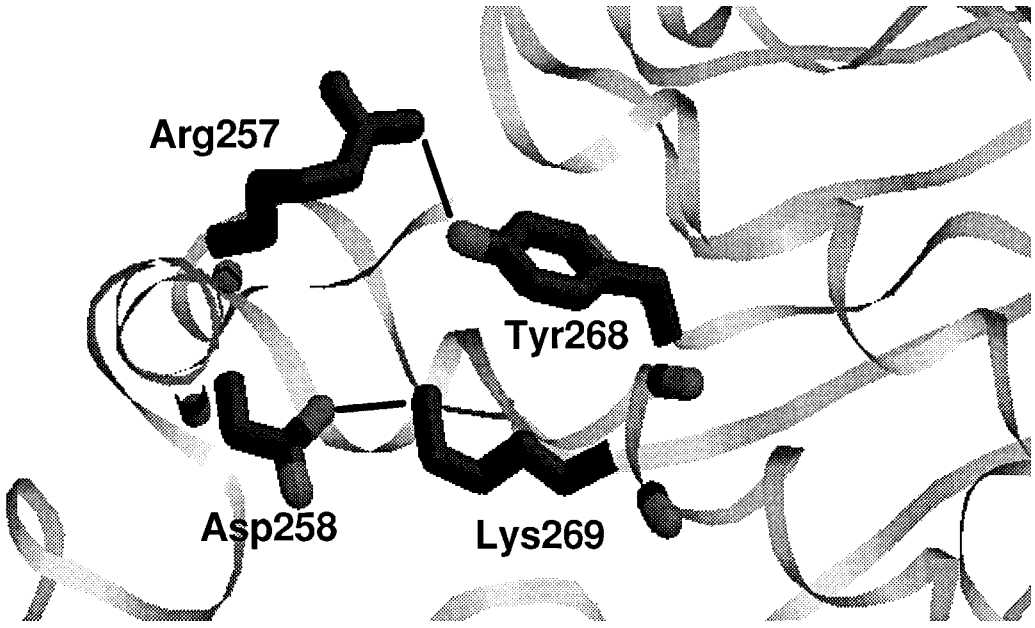

Fig. 7. The contacts of lid residues Arg257 and Asp258 with Tyr268 and Lys269. The orientation of the residues forming interaction between the lid and the core of PTL is given. The α-carbon backbone is represented by a ribbon and the side chains are represented by tubes. The interacting residues are connected by the solid lines. The figure was made with Alchemy 2000 (Tripos, Inc, St. Louis) using the coordinates for the PTL-colipase complex (PDB 1LPB).

TABLE 3. Temperature denaturation of PTL mutants

Lipase	Activity	Lipase	Activity
	%		%
PTL	50 ± 3.1	RD	47 ± 3.0
PLRP2	11 ± 3.8	RQ	49 ± 4.2
Lid 1	52 ± 3.5	DA	53 ± 3.3
Lid 2	50 ± 3.6	DG	54 ± 4.6
RA	47 ± 3.8	DN	48 ± 3.9
RG	52 ± 4.0	DR	52 ± 4.2

Each lipase was incubated at 50°C for 20 min and an aliquot was removed to determine activity by the pH-stat assay as described in the Methods. The activity is expressed as a % of the total activity remaining after incubation at 50°C. The average of three determinations and the standard deviation are given. There were no significant differences between any of the mutants and PTL by a paired *t*-test.

activity, against all three substrates. The D258R PTL had lower activities against all three substrates than the other Asp258 mutants but the D258R activities were still higher than the Arg257 mutants. These mutants had less than 2% of PTL activity regardless of the substrate. These data show that both Arg257 and Asp influence the activity and substrate preference of PTL and account for some of the differences between PTL and PLRP2.

Because the greater inhibition of the Arg257 mutants by TDC suggests that this residue is critical for the proper interaction of PTL with colipase, we measured the interaction between colipase and each of the Arg257 and Asp258 mutants with a binding assay. Each mutant was briefly incubated with tributyrin and a molar equivalent of colipase in 4.0 mm TDC at pH 8.0. The tributyrin phase was separated from the aqueous phase by centrifugation and the amount of lipase remaining in the supernatant determined in the pH STAT with 0.5 mm TDC and a molar excess of colipase. The Asp258 mutants, again D258R is an exception, behaved like wild-type PTL and 90 to 98% partitioned with the organic phase (**Fig. 9**). Seventy-five percent of D258R bound to the tributyrin phase. In contrast, the Arg257 mutants remained in the aqueous phase, unaffected by colipase. These results are consistent with the hypothesis that Arg257 is critical for the proper interaction of PTL with colipase.

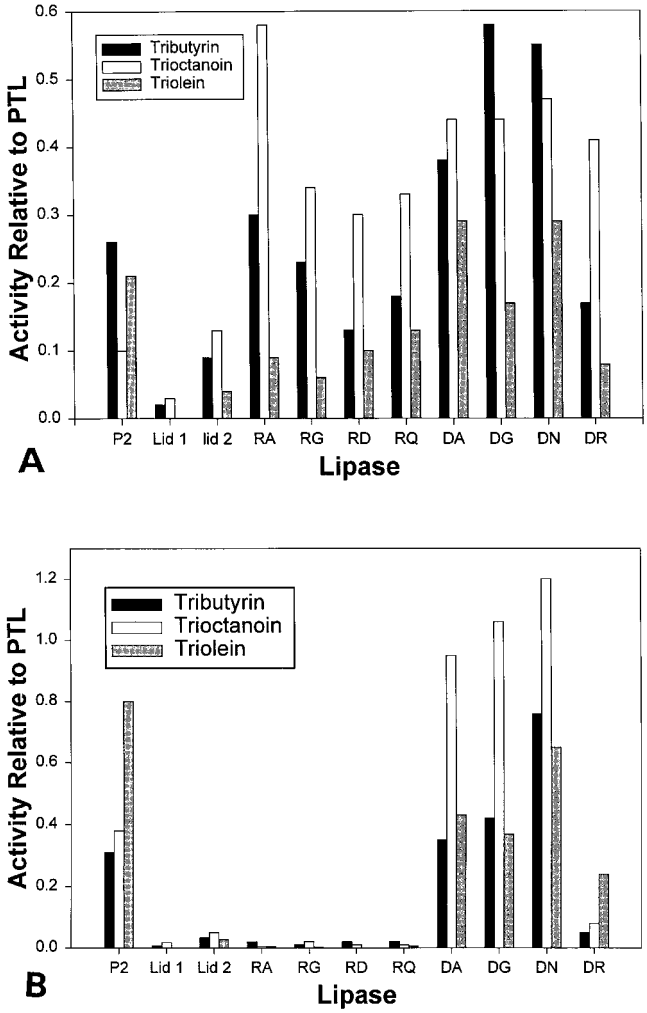

Fig. 8. The activity of the Arg257 and Asp258 mutants against various triglycerides. The activity was determined against tributyrin (black bars), trioctanoin (white bars), and triolein (gray bars) in the pH-stat assay as described in Methods. A 5-fold molar excess of colipase was included in each assay. Panel A: the taurodeoxycholate concentration was 0.5 mm. Panel B: the taurodeoxycholate concentration was 4.0 mm. P2, PLRP2; lid 1, Arg257Gly/Asp258Asp PTL; lid 2, Arg257Gly/Asp258Gly/Tyr268Phe/Lys269Glu PTL.; RA, Arg257Ala PTL; RG, Arg257Gly PTL; RD, Arg257Asp PTL; RQ, Arg257Gln PTL; DA, Asp258Ala PTL; DG, Asp258Gly PTL; DN, Asp258Asn PTL; DR, Asp258Arg PTL.

DISCUSSION

The crystal structures of PTL, PLRP2, and other lipases as well as mutagenesis studies have suggested that a surface loop plays an important function in the enzymatic mechanism of lipases. Residues in the lid of PTL can potentially influence substrate binding, substrate preference, and interactions with the substrate interface and with colipase. Because PTL and PLRP2 differ in these same properties, we predicted that differences in the lids of these lipases account for some or all of the functional differences between PTL and PLRP2.

We utilized the known sequence and structural homology of PTL and PLRP2 to examine the role of the lid in the differing functions of these two lipases (30). Only 4 out of 23 residues differ between PTL and PLRP2 compared to 15 of 23 residues for lipoprotein and hepatic lipase. The near identity of the lid amino acid sequence between PTL and PLRP2 suggested that the lids could be exchanged without disrupting protein structure as might have occurred with exchanges of lids containing more differences in primary sequence. The robust secretion of the lid chimeras, their normal temperature stability, and the preserved interfacial activation all argue that the lid exchanges did not greatly alter the overall structure of the chimeras.

Our analysis demonstrates that the lid contributes to activity, substrate preference, and colipase interactions. Interestingly, the properties of PLRP2 were little changed by exchanging the lid. Only minor changes in substrate pref-

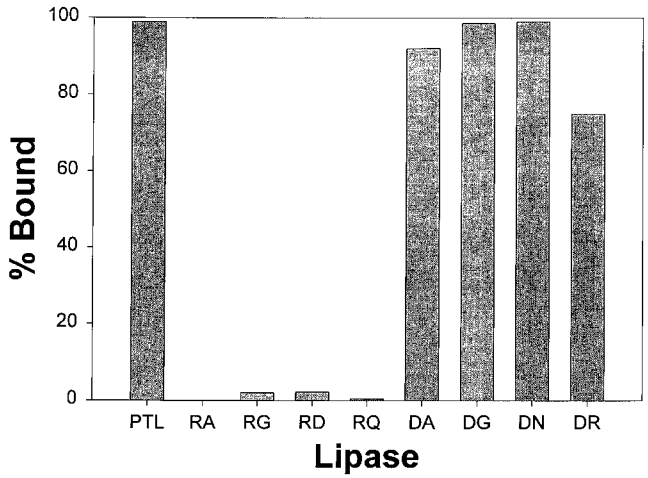

Fig. 9. Binding of the Arg257 and Asp258 mutants to tributyrin. The binding of each mutant PTL to tributyrin was determined as described in Methods. The abbreviations for each mutant are defined in the legend to Fig. 8.

erence were found in P2/PTL lid. In contrast, the properties of PTL were greatly altered by replacing the native lid with the PLRP2 lid. These findings suggest that the lid conformation may be more critical to the function of PTL than to PLRP2.

Most likely, it is in the open conformation where the lids must differ. The preserved interfacial activation of the lid chimeras indicates that the closed conformation of the chimeras is as stable as that of the parent lipases. This stability would be obtained if the closed conformations of the lids were similar or identical. The recently completed crystal structure of rat PLRP2 supports this conclusion. In the 3D structure, the closed PLRP2 lid occupies the same position and forms the same interactions as does the closed PTL lid (8, 9). Together, the preserved interfacial activation and the identical closed conformations of the two lids on the parent lipases suggest that the closed lid maintains the same conformation regardless of the lid origin. The effects on activity, substrate preference, and colipase activation of the PTL/P2 lid certainly reflect changes in the open lid conformation. The open lid contributes to one acyl chain binding site and also to the configuration of the catalytic site. Any alterations in lid conformation would affect these properties thereby changing activity or substrate preference or both. The apparent lack of an effect of colipase on the PTL/P2 lid is also consistent with an altered open lid conformation. Previous studies have shown that colipase interacts with residues in the lid of PTL and that these interactions are critical for colipase-dependent PTL activity (12, 18). The two lid residues, Asn240 and Val246, that interact with colipase are conserved in PTL and PLRP2. The simplest explanation for the inability of colipase to activate PTL/P2 lid is that the lid lies in a position that is unfavorable for the interaction of Asn240 and Val246 with colipase.

In this paper, we showed that two lid residues, Arg257 and Asp258, which stabilize the open conformation of PTL, are essential for efficient lipolysis. Substitution of any PLRP2 residue for the PTL residues at position 257 and 258 affects the activity of PTL significantly. Of these two, Arg257, is required for activity in the presence of TDC. Converting that residue to any of the corresponding PLRP2 amino acids decreased activity in the presence of TDC at least 100-fold. The best explanation for these results is that the proper open lid conformation of PTL requires the interactions of Arg257 and Asp258 with core residues. Any substitution at these positions would disrupt these interactions and prevent the lid from attaining the proper open conformation. Conversely, this interpretation predicts that similar interactions are not required to achieve the open conformation of PLRP2. The answer awaits the structure of PLRP2 in the open conformation.

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