The hydrophobic surface of colipase influences lipase activity at an oil–water interface

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Abstract **The interaction of pancreatic triglyceride lipase and colipase at an oil–water interface is required for efficient digestion of dietary fats and provides a model system for the interaction of proteins at biological membranes. Colipase has two important surfaces, a hydrophilic surface that interacts with lipase and a hydrophobic surface that presumably interacts with substrate. To begin our investigations into the role of the hydrophobic surface in the function of colipase, we replaced three neighboring tyrosine residues at positions 55, 58, and 59 in the hydrophobic surface with aspartic acid. Two of the three residues, Tyr55 and Tyr59, influenced the activity of colipase. Introducing aspartic acid at either position decreased the activity with long-chain triglycerides, but not with a short-chain triglyceride. Decreased ability of the mutants to anchor lipase to long-chain triglycerides did not explain the altered activity of the mutants. A mutant containing aspartic acid at positions 55 and 59 had no activity with any substrate and did not anchor lipase to either short- or long-chain triglycerides. These results identify the two tyrosine residues that interact with substrate and suggest that the hydrophobicity of the surface containing these tyrosines influences colipase function and the substrate selectivity of pancreatic triglyceride lipase.—**Cordle, R. A., and M. E. Lowe. **The hydrophobic surface of colipase influences lipase activity at an oil–water interface.** ***J. Lipid Res.*** **1998.** 39: **1759–1767.**

Supplementary key words enzyme • lipids • membranes • protein expression • protein purification • site-directed mutagenesis • yeast

Many biological events occur at lipid–water interfaces like those found in biological membranes, in lipoprotein particles, or in emulsions of dietary fats. An important class of enzymes, lipases, functions exclusively at the lipid–water interface. Phospholipases generate second messengers, participate in cytotoxicity, and digest phospholipids in the gastrointestinal tract. Triglyceride lipases are essential for transporting fats into cells for storage or for conversion into energy. Triglycerides in the diet, in lipoprotein particles, and in fat storage cells cannot be directly absorbed into cells. They must be hydrolyzed into free fatty acids and monoacylglycerols before they cross cellular membranes. The crucial role of lipases in many biological processes has stimulated interest in defining the molecular details behind the functional properties of lipases.

The kinetic mechanism of lipases differs from that of enzymes hydrolyzing water-soluble substrates in a homogeneous milieu by an additional, distinct step in the catalytic mechanism. The water-soluble lipase must move from the aqueous phase onto the lipid–water interface prior to diffusion of substrate into the substrate binding site of the lipase. Most lipases adsorb to the lipid–water interface readily, but there are several exceptions including lipoprotein lipase and pancreatic triglyceride lipase (PTL). Although efficient intraduodenal lipolysis by PTL requires bile salts, physiological concentrations of bile salts prevent binding of PTL to substrates (1). Another pancreatic protein, colipase, restores activity to bile salt inhibited lipase (2). The protein–protein interaction of colipase and PTL at the lipid–water interface is not only essential for efficient fat digestion, it also provides a model system for studying protein–protein interactions at biological membranes.

The molecular mechanism of colipase reactivation remains mostly speculative. Many studies indicated that colipase anchors PTL to the substrate interface, but these studies did not identify the protein–protein interactions between colipase and PTL at the interface nor did they define the molecular interactions of colipase with the lipid–water interface (3). A clue to the nature of these interactions was provided by the tertiary structure of the colipase-PTL complex (4, 5). In this structure, colipase is a flattened, rectangular molecule with a predominantly hydrophilic surface opposite a predominately hydrophobic surface. Residues in the hydrophilic surface interact with PTL. As a result, the hydrophobic surface points away from lipase in a position to interact with the lipid–water interface. Because of the orientation of the hydrophobic surface, the authors proposed that this surface binds to the lipid substrate, thereby anchoring PTL on the substrate.

Abbreviations: PTL, pancreatic triglyceride lipase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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To determine whether the hydrophobic surface contributes to the function of colipase, we used site-directed mutagenesis to alter the properties of this surface. Because various investigators have proposed that three neighboring tyrosines in this hydrophobic surface mediate colipase binding to the substrate, we started our investigation by replacing each of the neighboring tyrosines with a charged amino acid (3). We expressed the mutants in a yeast system, and fully characterized the recombinant, purified mutants. This approach gave control over the location of the modified residues and did not require the linkage of bulky groups to the tyrosine side chains. Our data demonstrate that both Tyr55 and Tyr59, but not Tyr58, and, by implication, the hydrophobic surface have an important role in the activity of colipase and lipase that extends beyond the simple ability to anchor lipase at an oil–water interface.

MATERIALS AND METHODS

Construction of tyrosine mutants

We introduced mutations into the full length cDNA of human procolipase by overlap polymerase chain reaction (PCR) (6, 7). The Protein and Nucleic Acids Chemistry Laboratory (Washington University School of Medicine, St. Louis, MO) synthesized primers of 24 nucleotides encoding the desired mutations. In order to clone the mutant cDNA into a yeast transformation vector pPIC9 (Invitrogen, San Diego, CA) we restricted the insert from pSVL and amplified the insert by PCR with 5′ and 3′ primers containing the appropriate restriction sites, XhoI and EcoRI. Additionally, the 5′-flanking primer overlapped the amino-terminus of mature procolipase and the carboxy-terminus of the yeast α factor signal sequence. After subcloning, the native procolipase signal peptide was replaced with the yeast α factor signal peptide and introduced an additional glutamate at the amino-terminus. Restriction digests and dideoxy nucleotide sequencing according to the instructions provided with Sequenase 2.0 (USB/Amersham, Cleveland, OH) confirmed the successful construction of the mutants.

Expression of tyrosine mutants in yeast

We transformed the pPIC9 vectors containing the mutant cDNAs into *Pichia pastoris* as previously described (8). Single colonies were selected and screened by small scale expression as described (8). Immunoblot of the medium after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) identified transformants. Large scale expression of the positive transformants as previously described produced milligrams of the mutant colipases after 5 days of methanol induction (8).

To confirm the recombination of the mutant cDNA into the yeast genome, we isolated yeast genomic DNA from procolipase-expressing clones as described in the Invitrogen manual for *Pichia pastoris*. PCR with primers unique to human procolipase and flanking the mutation site amplified a portion of the procolipase sequence from the yeast genome. The PCR fragment isolated from an agarose gel was sequenced with AmpliTaq DNA Polymerase, FS and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA). The sequence analyzed on an Applied Biosystems, Inc. automated sequencer confirmed the presence of the desired mutant cDNA.

Purification of the tyrosine mutants

We harvested the medium by centrifugation at 3000 *g* for 10 min, adjusted the pH to 8.0, and removed the resulting white precipitate by centrifugation. Immunoaffinity chromatography purified procolipase from the medium as previously described (8). After pooling the procolipase-containing fractions, we dialyzed them against distilled water. The sample was concentrated by lyophilization and resuspended in 0.5 ml of distilled water. Chromatography of the sample over a Superdex Peptide PE 7.5/300 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 30% acetonitrile and 0.1% trifluoroacetic acid and connected to an Akta explorer (Pharmacia Biotech, Uppsala, Sweden) removed small molecular weight contaminants. The absorption at 280 nm identified the fractions containing the eluted procolipase. We concentrated the pooled fractions by lyophilization and resuspended the final product in 50 mm Tris-Cl, pH 8.0.

Protein methods

Fifteen percent SDS polyacrylamide gel electrophoresis followed by staining with Coomassie Blue demonstrated the purity of the isolated proteins (9). Amino terminus sequencing performed at the Protein and Nucleic Acid Chemistry Laboratory (Washington University School of Medicine) using the Applied Biosystems, Inc. gas-phase sequencer confirmed the expected amino terminus sequence. Electro-spray mass spectrometry was performed at the same facility. Purified mutants determined the molecular mass of the proteins. An extinction coefficient of $E_1\%$ at 280 = 0.3 defined the concentration of colipase.

Activation of procolipase with trypsin

Incubation with trypsin converted procolipase to colipase. Trypsin 0.15 ml immobilized to Sepharose (Pierce, Rockford, Il) was washed 3 times with 0.5 ml of 0.05 m NH_4CO_3, pH 8.5. Each time centrifugation pelleted the Sepharose allowing removal of the supernatant. After the final centrifugation, we added 0.5 mg of procolipase in 0.4 ml of 0.05 m NH_4CO_3, pH 8.5, to the pellet, vortexed briefly, and incubated at room temperature for 6 h with occasional mixing. After the incubation, we removed the immobilized trypsin by centrifugation. Amino terminal sequencing, electro-spray mass spectrometry, and polyacrylamide gel electrophoresis on a 7.5% non-denaturing gel followed by Coomassie Blue staining confirmed the conversion to colipase.

Lipase assays

The yeast-expressed colipase was assayed with a pH STAT. For assays with tributyrin, we added 0.5 ml of the triglyceride to 14.5 ml of 1 mm Tris-HCl, pH 8.0, 2.0 mm $CaCl_2$, 150 mm NaCl, and 4 mm taurodeoxycholate at 37°C. For assays with triolein, 100 mg was added to 14.5 ml of assay buffer. Intralipid (0.5 ml) washed by repeated centrifugation and suspended in 150 mm NaCl, was added to 14.5 ml of reaction buffer (11). We purified human pancreatic triglyceride lipase from pancreas as described (12). Each figure legend lists the amounts of colipase and lipase added to the assay.

Binding assays

The ability of colipase to anchor lipase to tributyrin was done as described (8). Intralipid binding assays were done as follows. 0.5 ml of washed Intralipid was suspended in 14.5 ml of 50 mm Tris-HCl, pH 8.0, 2.0 mm $CaCl_2$, and 150 mm NaCl. Aliquots (0.5 ml) of the suspension were dispensed in 1.5-ml microfuge tubes. After adding the desired amount of colipase and lipase, we incubated the mixture at room temperature with shaking for 1–2 min. The mixture was transferred to an Amicon Micron-100 Ultrafiltration tube (Beverly, MA) and the tubes were centrifuged at 300 rpm for 15 min in an Eppendorf 5415 microcentrifuge. We assayed 100 μl of the clear filtrate in the pH STAT with tributyrin as the substrate and a molar excess of colipase added to the assay. The assay included controls with no lipase or colipase or with lipase alone.

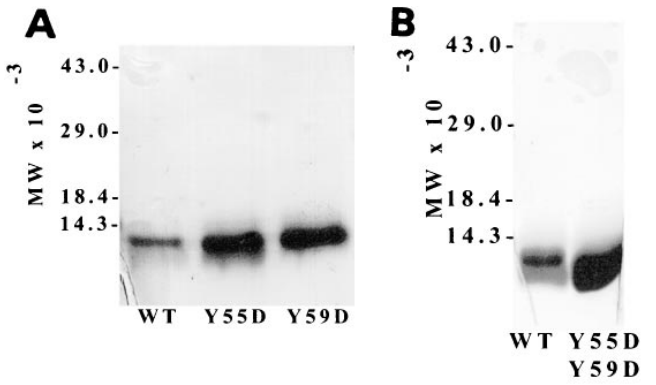

Fig. 1. SDS polyacrylamide gel electrophoresis of purified wild-type, Y55D, Y59D, and Y55D/Y59D colipase expressed in yeast. The recombinant proteins were expressed in yeast and purified as described in the Methods. The samples were run on a 15% acrylamide gel and the gel was stained with Coomassie blue. The sample is identified below the lanes and the positions of the molecular weight markers is on the side. Panel A: 5 μg of wild-type and 15 μg each of Y55D and Y59D were loaded onto the gel. Panel B: 10 μg of wild-type and 25 μg of Y55D/Y59D were loaded onto the gel.

RESULTS

Expression of the tyrosine to aspartate mutants in yeast cells

To maximally alter the properties in the hydrophobic surface on colipase, we chose to replace each of the neighboring tyrosines with a charged amino acid, aspartic acid. Each of the tyrosines was individually replaced to create single substitution mutants. One double substitution mutant Y55D/Y59D was also made. Because our earlier studies with wild-type colipase demonstrated the efficient expression of milligram quantities in *Pichia pastoris*, a yeast expression system, we expressed the Y55D, Y58D, Y59D and Y55D/Y59D mutants in yeast (8). Small scale expression of the mutants showed the yeast secreted each mutant into the medium, indicating that the mutation did not appreciably affect folding. With large scale expression, we found that Y58D consistently expressed at low levels. Less than 100 μg of Y58D could be isolated from 1 L of broth. In contrast, 1 L of expression medium yielded 3–6 mg of Y55D, Y59D, and Y55D/Y59D. Our further analyses concentrated on these three mutants.

After isolating the recombinant, single mutant proteins over an immunoaffinity column and the double mutant by size exclusion and reverse phase liquid chromatography, we demonstrated their purity and fidelity with several methods. The purified mutants migrated as a single band with the same mobility as wild-type colipase on SDS-polyacrylamide gel electrophoresis (**Fig. 1**). Amino acid sequence analysis of the purified proteins confirmed the successful conversion from procolipase to colipase. The sequence, GIINLENGE, matched the expected sequence for human colipase. The molecular masses of 9576 Da for Y59D and 9575 Da for Y55D measured by electro-spray mass spectroscopy agreed well with the predicted molecular mass of 9575 Da for colipase with one tyrosine substi-

TABLE 1. Activity of colipase mutants against tributyrin and triolein

Colipase	Specific Activity	
	Tributyrin	Triolein
	μmol FA/min/mg	
Wild-type	11,200	3200 ± 350
Y55D	13,100	0
Y58D	12,500	3000 ± 290
Y59D	11,800	0
Y55D/Y59D	0	0

The assay was done in the pH-stat with 2.4 μg of human PTL and a 2 molar excess of colipase. The tributyrin results are the average of two determinations. The triolein results are the average and standard deviation of 3 determinations.

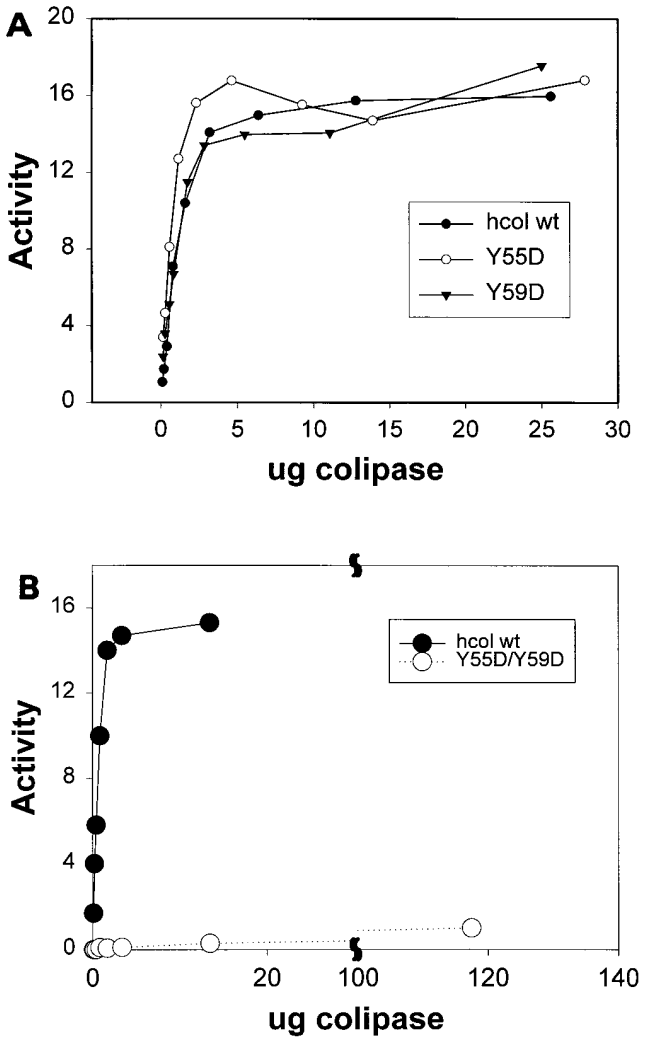

Fig. 2. Activity of the tyrosine mutants against tributyrin. The tributyrin assay was done in the pH STAT as described in Methods. Two μg of human pancreatic triglyceride lipase was added to each assay along with varying amounts of colipase. The measurements were done three times and a representative experiment is shown. Panel A: solid circles, wild-type colipase; open circles, Y55D; solid triangles, Y59D. Activity is μmoles fatty acid released per min. Panel B: solid circles, wild-type colipase; open circles, Y55D/Y59D.

tuted by aspartate. Electro-spray mass spectrophotometry showed a molecular weight of 9537 Da which agrees with the predicted weight of 9537 D for the double mutant. The calculated weight is for non-reduced colipase indicating that all 5 disulfide bonds formed in the mutants. Based on these findings, we concluded the proteins were colipase, they contained the aspartate for tyrosine substitutions, and nonspecific proteolytic cleavage did not occur during the expression or purification.

Activity of tyrosine mutants with tributyrin and triolein

We first screened the activity of the tyrosine mutants against a short-chain triglyceride, tributyrin, and a long-chain triglyceride, triolein, in the standard 5-min assay with a 2-fold molar excess of colipase over PTL. All three single mutants activated bile salt-inhibited pancreatic triglyceride lipase to the same extent as wild-type colipase with tributyrin as the substrate. The double mutant had no detectable activity with tributyrin (**Table 1**). Only the Y58D mutant had activity with triolein. The other two single mutants and the double mutant had no activity with triolein. These findings suggest that Tyr55 and Tyr59, but not Tyr58, influence the activity of colipase. The results did not eliminate the possibility the mutants had decreased activity against tributyrin at lower molar ratios of colipase to lipase or activity against triolein at higher molar ratios.

To more precisely determine the activity of Y55D, Y59D, and Y55D/Y59D mutants against tributyrin, we measured their ability to reactivate bile salt-inhibited lipase over a range of colipase concentrations. The lipase and tributyrin concentrations were kept constant. Increasing amounts of wild-type colipase caused a rise in lipase activity with a plateau occurring at a molar ratio of 1:1 to 2:1 (colipase:lipase) (**Fig. 2A**). The two, single tyrosine mutants produced curves indistinguishable from the wild-type colipase curve (Fig. 2A). The double tyrosine mutant had greatly decreased activity against tributyrin (Fig. 2B). Low levels of activity were detected only at 200-fold molar excess of the Y55D/Y59D mutant. Thus, introducing a single, charged residue into the putative lipid interacting surface of colipase did not alter the ability of colipase to reactivate lipase in the presence of a tributyrin and taurodeoxycholate emulsion. Introducing a second charged residue into the hydrophobic surface of colipase did affect activity and demonstrated the importance of the hydrophobic residues in the tyrosine loop of colipase.

The preserved activity of the two single tyrosine mutants argued that the mutants folded correctly and provided the means to further evaluate the conformational stability of the two mutants. To determine whether the mutants retained structural stability, we took advantage of native colipase's stability to low pH and high temperature. We incubated both mutants at pH 2.0 for 30 min on ice and compared their activity to native colipase incubated under the same conditions. Both mutants and native colipase re-

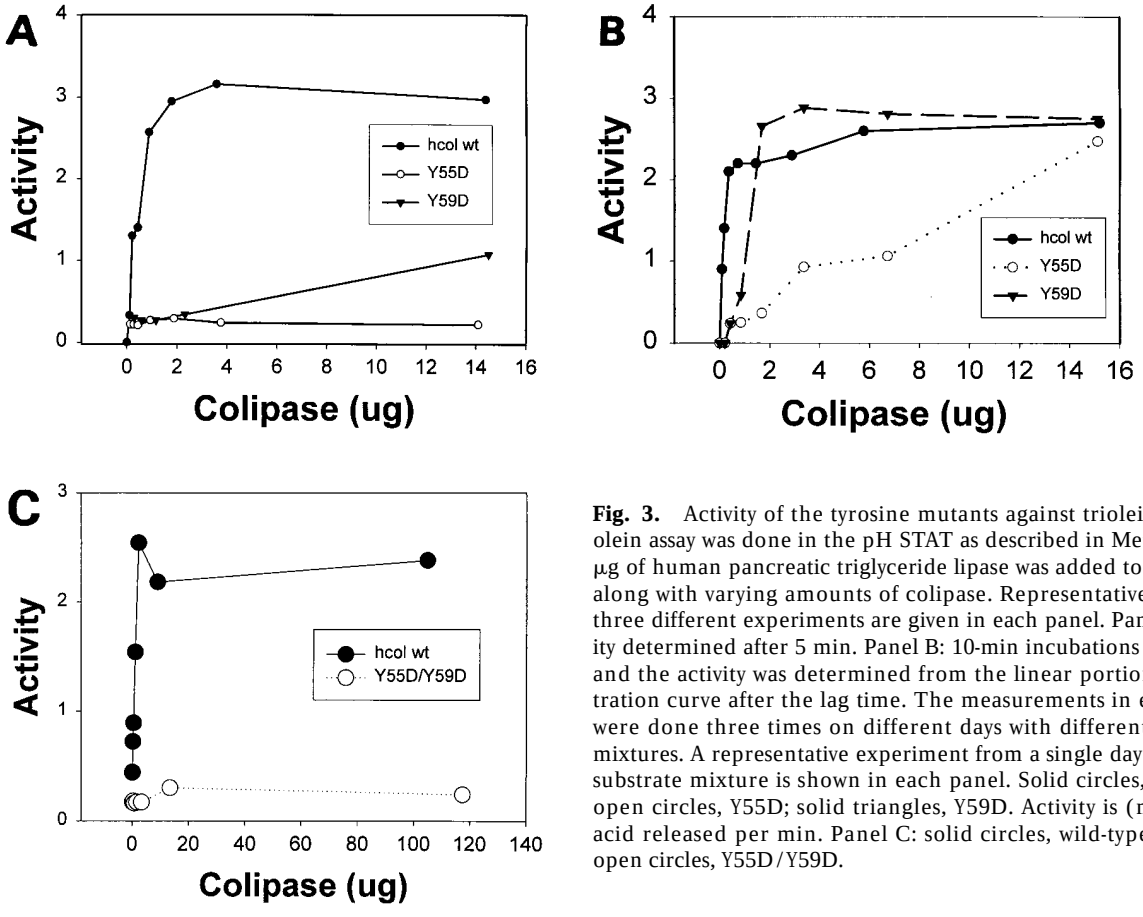

Fig. 3. Activity of the tyrosine mutants against triolein. The triolein assay was done in the pH STAT as described in Methods. Two μg of human pancreatic triglyceride lipase was added to each assay along with varying amounts of colipase. Representative results of three different experiments are given in each panel. Panel A: activity determined after 5 min. Panel B: 10-min incubations were done and the activity was determined from the linear portion of the titration curve after the lag time. The measurements in each panel were done three times on different days with different substrate mixtures. A representative experiment from a single day and single substrate mixture is shown in each panel. Solid circles, wild-type; open circles, Y55D; solid triangles, Y59D. Activity is (moles fatty acid released per min. Panel C: solid circles, wild-type colipase; open circles, Y55D/Y59D.

tained full activity against tributyrin. Next, we heated the mutant proteins for 10 min at 75°C. The mutants and native colipase had 90% activity remaining after this treatment. These two studies proved the conformational stability of the mutants.

Activity of the tyrosine mutants against triolein

We next evaluated the ability of the tyrosine mutants to restore bile salt-inhibited lipase activity against a long-chain substrate, triolein. Triolein, an 18 carbon, monounsaturated triglyceride, has no solubility in water and presents a more hydrophobic interface than tributyrin. In the standard 5-min assay, only Y58D showed normal activity against triolein (Table 1). The activities of Y55D, Y59D, and Y55D/Y59D were examined over a broader concentration range. As seen with tributyrin hydrolysis, triolein hydrolysis varied with the concentration of wild-type colipase until saturation of lipase (**Fig. 3A**). Y59D restored lipase activity against triolein–taurodeoxycholate emulsions, but the activity was at least 20-fold lower than wild-type colipase activity. Y55D had no detectable activity even at a molar ratio of 35 (colipase: lipase). These findings indicated that Y55D and Y59D, but not Y58D, influenced the activity of colipase.

Longer incubations revealed that both single tyrosine mutants showed a delay in the onset of maximal activity, a lag time, not present with wild-type colipase. We incubated the reactions up to 20 min and determined the activity from the linear reaction rate after the onset of hydrolysis. The activity after the lag time was near normal for Y59D, but was still significantly impaired for Y55D, suggesting that the interaction of Tyr55 with the interface differs from that of Tyr59 (Fig. 3B). Even with longer incubation times and high molar excess, the double tyrosine mutant did not reactivate PTL activity against mixed micelles of triolein and taurodeoxycholate (Fig. 3C). Clearly, the efficient restoration of lipase activity against bile salt emulsions of triolein requires the two neighboring tyrosines. The different behavior of these two mutants with triolein and tributyrin indicated that the tyrosines interact with the interface and colipase recognizes differences in the properties of interfaces.

Activity of the tyrosine mutants against intralipid

We then examined the activity of the tyrosine mutants against Intralipid, a substrate with a more complex interface containing phospholipids and long-chain triglycerides. Pancreatic triglyceride lipase does not hydrolyze this lipid substrate unless colipase is present. Even with colipase, there is a delay until the onset of maximum activity, a lag time. The length of the lag time depends on the colipase concentration. We measured the lag times for Y55D and Y59D and compared them to those of wild-type colipase (**Fig. 4A**). Wild-type colipase had a short lag time, about 5 min, at a 1:1 molar ratio of colipase to lipase. The lag time disappeared as the ratio increased. In contrast, Y55D and Y59D had appreciable lag times throughout the concentration range tested. The lag time for Y59D decreased with increasing colipase concentration, but never disappeared even at a 50-fold molar ratio

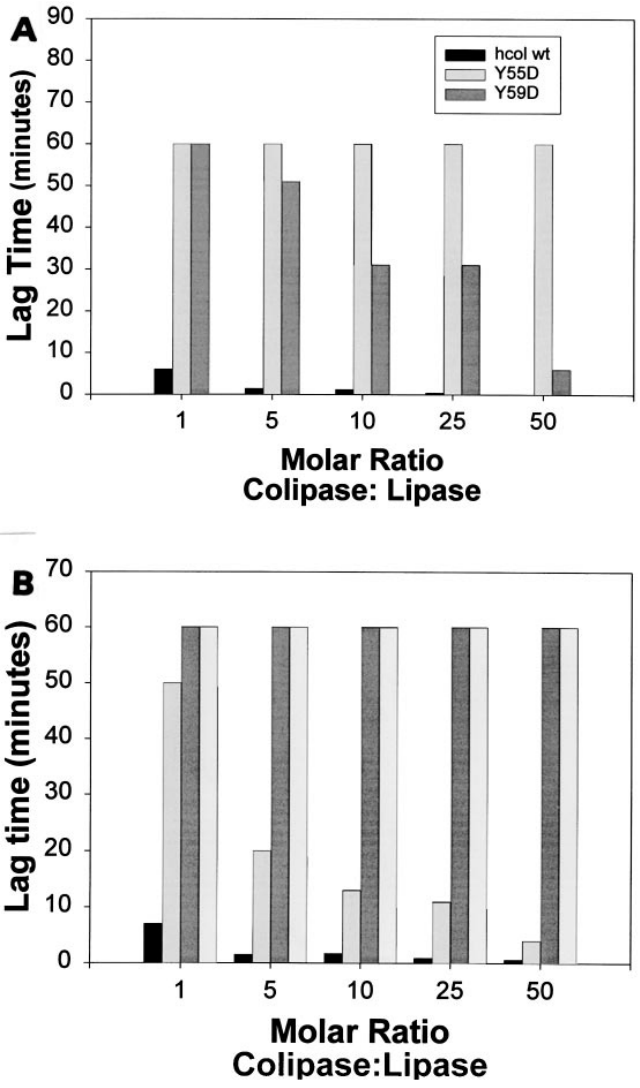

Fig. 4. Lag times of the tyrosine mutants with Intralipid. The tyrosine mutants were incubated with Intralipid and 2.0 μg of human pancreatic triglyceride lipase as described in the Methods. The lag time was extrapolated from the slope of the titration curve. A lag time of 60 min means that no activity was detected after a 60-min incubation. Panel A: black bar, wild-type colipase, medium gray bar, Y55D; dark gray bar, Y59D. Panel B: 1st black bar, wild-type colipase; 2nd medium gray bar, wild-type procolipase; 3rd dark gray bar, Y55D/Y59D colipase; 4th light gray bar, Y55D/Y59D procolipase.

of colipase to lipase. Y55D did not restore activity after 60-min incubations at all molar ratios tested, a result consistent with its poor activity against long-chain triglycerides. Neither the proform of Y55D/Y59D nor the activated form showed any activity extending to 60 min, suggesting that the double mutant was inactive against Intralipid (Fig. 4B). Wild-type colipase had a short lag time at all molar ratios and wild-type procolipase had a 50 min lag time at a 1:1 molar ratio decreasing to 3 min at a 50:1 molar ratio (Fig. 4B). These results confirmed the findings with bile salt–triolein emulsions and demonstrated that the proper interaction of colipase with interfaces of long-chain, water-insoluble triglycerides requires Tyr55 and Tyr59.

Ability of the tyrosine mutants to anchor lipase to substrates

Current models of colipase function hypothesize that colipase restores activity to bile salt-inhibited lipase by anchoring lipase to the substrate. We measured the ability of the tyrosine mutants to anchor lipase to both tributyrin and Intralipid. At all molar ratios of colipase to lipase, the ability of Y55D and Y59D to anchor lipase was indistinguishable from that of wild-type (**Fig. 5A**). In contrast, the Y55D/Y59D mutant did not anchor lipase to tributyrin even at 10-fold molar excess over lipase (Fig. 5C). These findings corresponded with the activity of the mutants against tributyrin.

Next, we determined whether the differences in lag times against Intralipid among the mutants and wild-type colipase resulted from decreased binding or from other properties of colipase. The design of the binding assay was analogous to the tributyrin binding assay in that both measured the ability of colipase to anchor lipase to the interface. In the Intralipid assay, the phases could not be separated by centrifugation as in the tributyrin assay so the phases were separated over an ultrafiltration membrane. Anchoring of lipase to the lipid phase was proportional to the colipase concentration until all of the lipase was bound, demonstrating the colipase dependence of binding (Fig. 5B). Both of the single, tyrosine mutants also showed concentration-dependent anchoring of lipase, but maximum binding occurred at higher molar ratios of the mutants to lipase (Fig. 5B). A low level of binding to Intralipid, less than 10%, was detected for the double mutant. The binding was independent of colipase concentrations suggesting that non-specific binding occurred (Fig. 5D). The inability of the double mutant to anchor PTL to Intralipid explains the low activity of the double mutant against that substrate, but the results with the single tyrosine mutants suggest another mechanism. Even though

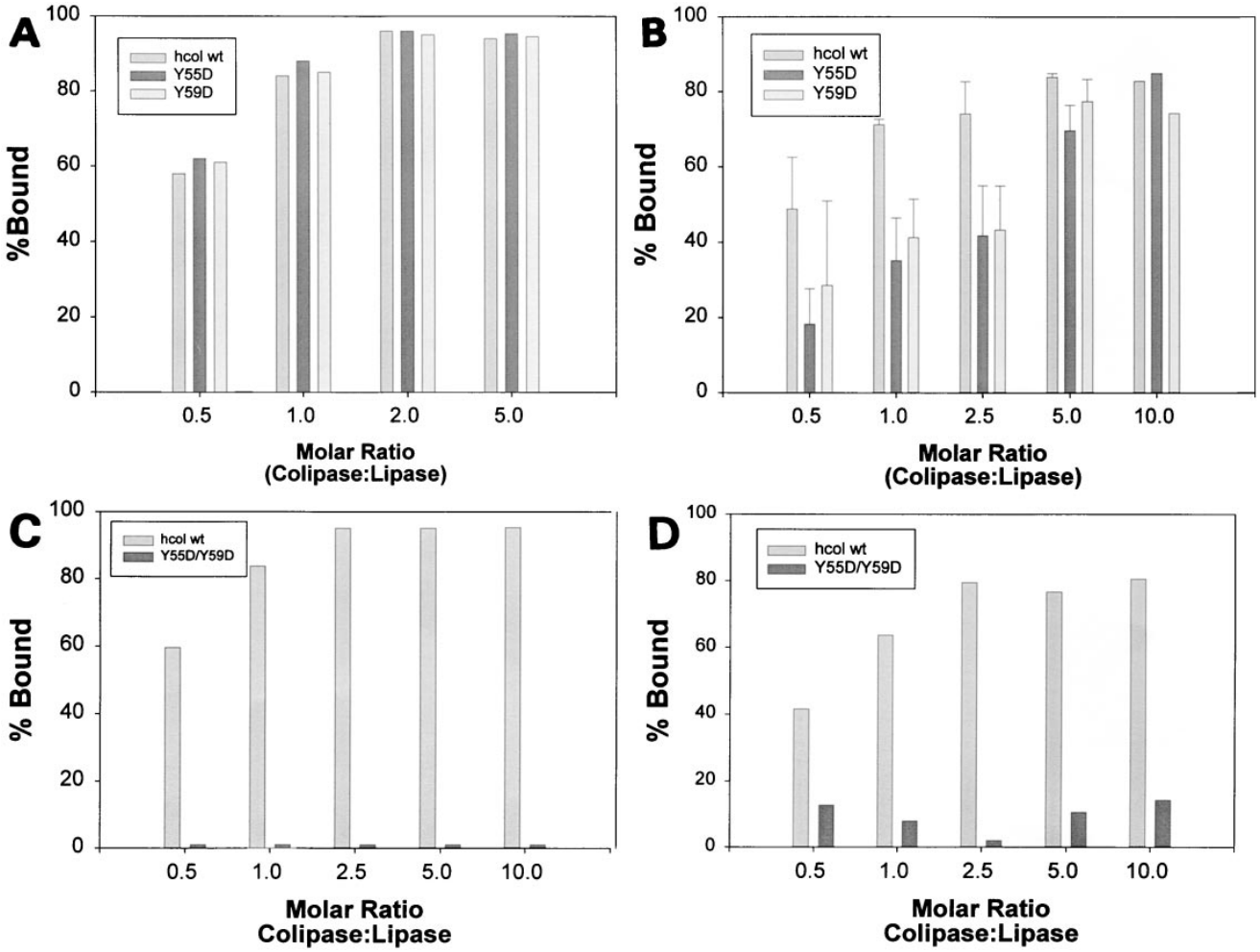

Fig. 5. Binding of the tyrosine mutants to tributyrin and Intralipid. Binding was determined with 4.0 μg of human pancreatic triglyceride lipase as described in the Methods. Panel A: binding of single tyrosine mutants to tributyrin expressed as the percentage of the total added lipase activity bound to the lipid phase after centrifugation; medium gray bar, wild-type colipase; dark gray bar, Y55D; light gray bar, Y59D. Panel B: binding of single tyrosine mutants to Intralipid expressed as the percentage of the total added lipase activity bound to the lipid phase; medium gray bar, wild-type colipase; dark gray bar, Y55D; light gray bar, Y59D. Panel C: binding of double mutant to tributyrin expressed as the percentage of the total added lipase activity bound to the lipid phase after centrifugation; light gray bar, wild-type colipase; dark gray bar, Y55D/Y59D colipase. Panel D: binding of double mutant to Intralipid expressed as the percentage of the total added lipase activity bound to the lipid phase; light gray bar, wild-type colipase; dark gray bar, Y55D/Y59D colipase.

the ability of the single tyrosine mutants to anchor lipase was diminished, the differences could not account for the large differences in lag times. Perhaps, the lag times with Intralipid reflect a function of colipase other than binding.

DISCUSSION

The three-dimensional structure of colipase complexed with pancreatic triglyceride lipase and the recent two- and three-dimensional NMR structure of colipase revealed that colipase has two surfaces, a surface facing lipase and another directed away from lipase (13, 14). Hydrophobic residues predominate on the surface oriented away from lipase. Because of this topology, several groups have postulated that the hydrophobic surface comprises a substrate binding domain. In this study, we successfully introduced charged residues into the hydrophobic surface by replacing Tyr55, Tyr58, and Tyr59 with aspartic acid either as single or double mutants. These mutant proteins allowed us to define the importance of these residues and of the hydrophobic surface in the function of colipase.

One mutant, Y58D, could only be expressed in small quantities. An explanation for the poor expression of this mutant was provided by examining the three dimensional structure of colipase (13, 14). The side chain of Tyr58 extends into the core of colipase where the residues may interact with other amino acids (**Fig. 6**). The introduction of a charge at this position could potentially alter those interactions and change the folding of the protein. If improperly folded, the protein will then be targeted for degradation. Even in this situation, a small amount of properly folded protein may be secreted as we found with the Y58D mutant. The limited amount of mutant isolated from the medium had normal activity against tributyrin and triolein. Based on the preserved activity of Y58D and the central location of Tyr58, it is unlikely that Tyr58 participates in the hydrophobic surface of the tyrosine loop or in substrate binding.

In contrast, our results clearly identified Tyr55 and Tyr59 as the important tyrosines in colipase. Early studies of the tyrosines implicated two of three tyrosines in the function of colipase, but did not identify the involved residues (15, 16). Later studies of dansyl–tyrosine colipase concluded that Tyr55, and not Tyr59, interacted with bile salt micelles (17–19). There are several possible explanations for why this study found a significant role for Tyr59 and the chemical modification study did not. Danylation introduces a large, hydrophobic group onto the tyrosine and this group may alter the orientation of the residue by introducing new interactions with the protein core. Tyr59 may interact with lipids but not with bile salt micelles. A recent study raised questions about where bile salt micelles bind. Examination of the pancreatic–colipase complex bound to a bile salt micelle showed binding of the micelle to the C-terminus of colipase and lipase away from both Tyr55 and Tyr59 (20, 21). These findings suggest that the interaction of Tyr55 with bile salt micelles in the absence of lipase may not be physiologically relevant. Finally, placing a charged group at position 59 could affect other

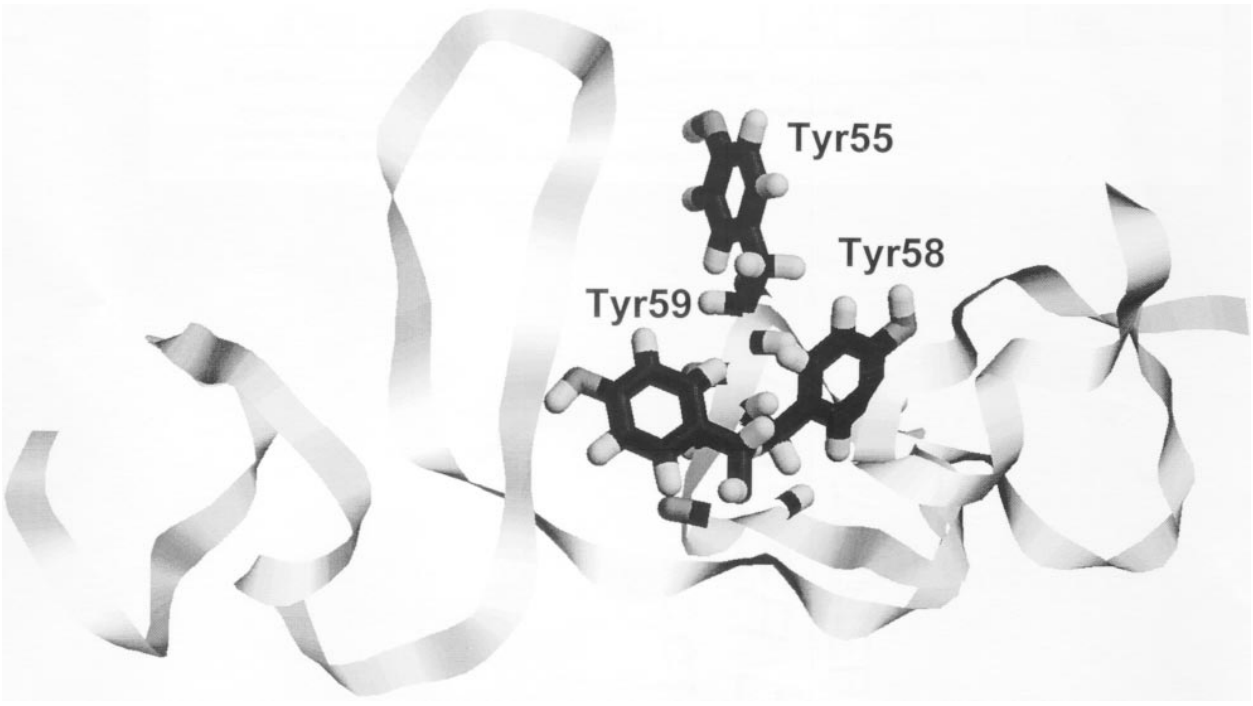

Fig. 6. The three dimensional structure of human colipase. The α-carbon backbone of human colipase is presented as a ribbon. The side chains of the neighboring tyrosines, Tyr55, Tyr58, and Tyr59, are shown as sticks. The surface toward the top is the hydrophobic side and the surface at the bottom is the lipase interacting side. The coordinates were obtained from the Brookhaven data base and the figure was created with Alchemy 2000 (Tripos, St. Louis, MO).

regions of the protein in ways that decrease ability of colipase to function properly. Even though it is difficult to completely eliminate the possibility that a mutation has indirect affects, our data on conformational stability and activity argue against any significant changes in conformation due to the mutation.

The different capacities of the tyrosine mutants to restore lipase activity against tributyrin as compared to triolein may be explained by differences in the physical properties of tributyrin and triolein. For instance, tributyrin has higher water solubility than triolein and the emulsions of tributyrin may contain more water than the emulsions of triolein. The increased hydration of tributyrin emulsions may accommodate a more hydrophilic protein surface than the triolein emulsions. Alternatively, the packing of the acyl chains and the orientation of the glycerol head groups may differ. These differences can alter the hydrophobicity of the interface and allow or prevent penetration of a more hydrophilic protein surface (22). With either explanation, the charged group would remain in the aqueous phase of a triolein–water interface. This position could alter the conformation of colipase or alter its relationship to the interface and prevent proper positioning of lipase for efficient catalysis.

Despite the mechanism, the hypothesis that colipase recognizes differences in substrate interfaces carries important implications. Because pancreatic triglyceride lipase depends on colipase for activity against complex lipid substrates and in the presence of bile salts, the ability or inability of colipase to interact with substrates will influence the substrate specificity of pancreatic triglyceride lipase in the duodenum. Additionally, the modulation of lipase by colipase is influenced by the interface. Thus, an oil–water interface or biological membrane could mediate the function of a protein, lipase in this case, through effects of the interface on the properties of another protein, colipase. This hypothesis predicts that the conformation of colipase may be altered by interactions with a lipid–water interface.

Although colipase binding to the substrate is important to colipase function, other actions of colipase may also influence lipase activity. Our results with Intralipid cannot be adequately explained by a model invoking only colipase anchoring of lipase at the interface. Y55D and Y59D had poor to no activity against Intralipid, yet they retained near normal ability to anchor lipase to Intralipid. The 1.5- to 2-fold decrease in binding did not fully account for the differences in lag times. Thus, the mechanism of colipase action must not be limited to anchoring lipase at the interface.

Other studies of colipase have also suggested that the mechanism of colipase in lipolysis involves multiple steps. The three-dimensional structure of the colipase–lipase complex demonstrated interactions of colipase with the open lid domain of pancreatic triglyceride lipase and led to the hypothesis that colipase stabilizes the lid in the open, active conformation (4, 5). In a subsequent study, we mutated the interacting residue, Glu15, in colipase and showed that colipase activity depended on having a glutamic acid at position 15 (23). This finding confirmed that lipase activity depended on the interaction of colipase with the lid domain. A recent analysis of the catalytic properties of lipase with an oil-drop technique concluded that colipase increased enzyme turnover (24). Another series of studies provided evidence that colipase changed the lateral distribution of lipids in a monolayer by recruiting substrate and excluding poor substrates around its binding site (25, 26). Any model of colipase action will have to account for these functions of colipase as well as include a role for the tyrosine loop in the hydrophobic surface of colipase.

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