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Identification of amino acids in human colipase that mediate adsorption to lipid emulsions and mixed micelles

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ABSTRACT

The adsorption of colipase is essential for pancreatic triglyceride lipase activity and efficient dietary fat digestion. Yet, little is known about which specific amino acids in the hydrophobic surface of colipase influence adsorption. In this study, we systematically substituted alanine or tryptophan at residues implicated in adsorption of colipase to an interface. We expressed, purified recombinant colipase mutants and characterized the ability of each alanine mutant to restore activity to lipase in the presence of bile salts. The functions of L16A, Y55A, I79A and F84A colipase were most impaired with activities ranging from 20 to 60% of wild-type colipase. We next characterized the fluorescence properties of the tryptophan mutants in the absence and presence of bile–salt–oleic acid mixed micelles. We performed steady-state emission spectra to determine peak shift and I_{330}/I_{350} ratio and acrylamide quenching curves to characterize the environment of the residues. The analysis supports a model of adsorption that includes residues Leu 34 and Leu 36 on the 2nd loop, Tyr 55 and Tyr 59 on the 3rd loop and Ile 75 and Ile 79 on the 4th loop. The analysis confirms that Phe 84 is not part of the adsorption surface and likely stabilizes the conformation of colipase. Contrary to the predictions of computer modeling, the results provide strong support for an essential role of Tyr 55 in colipase adsorption to mixed micelles. The results indicate that the adsorption of colipase to mixed micelles is mediated by specific residues residing in a defined surface of colipase.

1. Introduction

Fatty acids must be released from dietary triglycerides before they are absorbed by intestinal enterocytes and distributed throughout the body. In the duodenum, lipases catalyze the efficient release of fatty acids from dietary lipid emulsions. Prior to hydrolysis, lipases must first bind to the surface of the lipid particles and hydrolysis occurs at that interface. *In vivo*, dietary lipids form multilamellar emulsion particles with phospholipids, cholesterol and cholesterol esters located in the surface layers and a bulk phase of triglyceride inside the outer layers [1]. Dietary proteins and carbohydrates and bile salts cover the surface of the emulsion particles. By itself, the predominant duodenal lipase, pancreatic triglyceride lipase (PTL)[1] cannot bind to the surface of the particles and is effectively inactive [2]. Another pancreatic protein, colipase, acts in a complex with PTL to restore activity.

Despite years of study, the molecular details of the interactions of colipase or the colipase–PTL complex with substrate and bile salt micelles remain obscure. X-ray crystallography of the porcine colipase–human PTL complex revealed the orientation of colipase to PTL in the complex [3,4]. Colipase, a flat and rectangular molecule with four loops or fingers, interacts with the C-terminal domain of PTL. The region of colipase opposite the PTL binding site forms a large hydrophobic plateau with residues in the open lid domain of PTL. It was proposed that residues in the hydrophobic plateau interact with the lipid substrate or bile salt micelles or both [3–5]. Hydrophobic residues reside in the first finger (Leu 16 and Met 18 (Leu 18 in the pig)), the second finger (Ala 33 (Ile 33 in the pig), Leu 34 and Leu 36), the third finger (Leu 54, Tyr 55, Ile 57 (Val 57 in the pig) and Tyr 59) and the fourth finger (Leu 75, Val 76, Ile 79 and Phe 84) [5].

Of these residues, only the tyrosines have experimental evidence for a role in binding lipid emulsions or bile salt micelles. NMR studies implicated the tyrosine-rich region in the binding of porcine colipase to bile salt micelles [6–8]. Fluorescence studies suggested Tyr 55 can insert into the interior of bile salt micelles [9–11]. Neutron crystallography of the porcine colipase–PTL complex also provided evidence that Tyr 55 interacts with bile salt micelles [12]. Site directed mutagenesis of Tyr 55 and Tyr 59 in human colipase to aspartic acid decreased the ability of colipase to restore lipase activity against long-chain triglycerides emulsified in bile salts [13]. No direct evidence for the interaction of Tyr 55 and Tyr 59 with the substrate interface was presented in this study.

In silico modeling of potential interactions between porcine colipase and bile salt micelles or lipid droplets argued against a role for Tyr 55and Tyr 59 in the interaction with either interface [14]. The predicted

Abbreviations: NMR, nuclear magnetic resonance; PTL, pancreatic triglyceride lipase; NaTDC, sodium taurodeoxycholate

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orientations of colipase on the surface of bile salt micelles or lipid droplets placed these tyrosines near but not in the interface. The authors suggested this proximity to the interface could explain earlier experimental results implicating Tyr 55 and Tyr 59 in the absorption of colipase to bile salt micelles or lipid droplets. The modeling also did not identify Phe 84 as a residue that might interact with an interface. The modeling suggested that the tyrosine cluster and Phe 84 influence the function of colipase by stabilizing the conformation of colipase rather than by inserting into a hydrophobic interface.

To directly identify residues that mediate colipase absorption to interfaces, we substituted alanine for each of the putative residues in the hydrophobic plateau, expressed and purified the recombinant human colipase variants and characterized the ability of the variants to restore lipase activity and to mediate binding of lipase to various substrates. We then took advantage of the absence of tryptophan residues in human colipase and made tryptophan substitution mutants in selected residues. We measured the interactions of the tryptophan mutants with bile salt micelles and mixed micelles of bile salt and oleic acid by tryptophan fluorescence.

2. Materials and methods

2.1. Construction of colipase mutants

All manipulations of DNA were done by standard methods unless otherwise noted [15]. Substitution of alanine or tryptophan was accomplished by site-directed mutagenesis using the QuickChange II XL Site-directed Mutagenesis Kit according to the manufacturer's instructions. Synthetic oligonucleotide primers were designed with the desired mutation and were generally 27-bp long. The template was human colipase cDNA cloned into pPIC9 as previously described [16]. Amplification was with 10 cycles with 15 m extension times in an Applied Biosystems 9800 Fast Thermal Cycler. The amplified product was transformed into *Escherichia coli* as described by the manufacturer. Plasmid DNA was prepared from single colonies with the Qiagen Spin MiniPrep Kit according to the directions. The presence of the desired base changes and the absence of unwanted mutations were confirmed by dideoxynucleotide sequencing of both strands of the entire cDNA insert.

2.2. Production and purification of colipase

The plasmids containing the desired colipase construct were transformed into *Pichia pastoris* strain GS115 and expressed as previously described [16]. The cells were removed by centrifugation at 3000 *g* for 5 min and the medium was concentrated using a Labscale TFF System over a Pellicon XL Biomax 5 membrane (Millipore, Bedford, MA). The sample was diluted 1:1 with 0.1 M $NaPO_4$ containing 2 M ammonium sulfate and the pH adjusted to 6.0. The sample was applied to two 5-ml HiTrap Phenyl HP columns (Amersham Biosciences) connected in tandem and equilibrated in 50 mM $NaPO_4$ containing 1 M ammonium sulfate pH 6.0. Chromatography was controlled with an AktaExplorer system (Amersham Biosciences). The column was washed with the equilibration buffer until the OD_{280} returned to baseline and the bound colipase was eluted with a 10 column volume gradient from 1 M to 0 M ammonium sulfate in the phosphate buffer. Fractions containing colipase were identified by monitoring the OD_{280} and by activity assay. The fractions were pooled and concentrated over an Amicon Ultra-15 5000 MWCO centrifuge filter according to the parameters described by the manufacturer (Millipore). The buffer was exchanged by gel filtration over a Superdex 75 HR 10/30 column equilibrated in 50 mM Tris–Cl, pH 8.0 and 150 mM NaCl. Fractions containing colipase were pooled and concentrated over the centrifugation filter as above.

The concentration of wild-type colipase was determined by ultraviolet spectrophotometry at OD_{280} and an extinction coefficient of $E = 3.0\ (g/100\ ml)^{-1}\ cm^{-1}$. The concentration of the mutants was determined by ELISA with wild-type colipase as the standard. 96-well plates were coated with a mouse monoclonal antibody against human colipase by incubating each well with 50 μl of a 1:6400 dilution of the antibody in PBS overnight at 4 °C. The wells were rinsed 3 times with 400 μl distilled water and 3 times with 400 μl SuperBlock buffer (Pierce). Next, diluted colipase in 100 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1% BSA. The colipase standard contained 10 to 200 pg/50 μl. The amount of unknown was estimated by spectrophotometry and two different dilutions were prepared. All assays were done in triplicate. 50 μl was placed in each well and the plate was incubated at room temperature for 2 h. The wells were rinsed 3 times with 400 μl of PBS with 0.5% Tween-20. Then, 50 μl of a rabbit polyclonal antibody against human colipase diluted 1:6400 in SuperBlock buffer was added to each well and incubated 2 h at room temperature. After rinsing the wells 3 times with 400 μl of PBS with 0.5% Tween-20, 50 μl of a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Pierce) diluted 1:12,500 in SuperBlock buffer was added to each well. After 1 h incubation at room temperature, the wells were washed 3 times with 400 μl distilled water. 50 μl of substrate (1-Step Ultra TMB-ELISA, Pierce) was added to each well. The reaction was allowed to proceed for 10–30 min and stopped by adding 50 μl $2MH_2SO_4$ to each well. The plate was read at 450 nm. The homogeneity of the purified colipase was confirmed by SDS–PAGE and stained with GELCODE Blue according to the manufacturer's instructions (Pierce) [17].

2.3. Activity and adsorption assays

Activity against tributyrin, tricaprylin and triolein was done in the pH stat as described previously [13]. For each assay, 0.5 ml of the substrate was added to 14.5 ml of 2 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$, 150 mM NaCl and 4 mM NaTDC. The final concentration of the substrate was 310 mM for tributyrin and 100 mM for tricaprylin or triolein. Adsorption to tributyrin was measured by a centrifugation assay as previously described [13,16]. For these assays, 0.5 ml of substrate was added to 14.5 ml of 50 mM Tris–Cl, pH 8.0, 1 mM $CaCl_2$, 150 mM NaCl, and 4 mM sodium taurodeoxycholate. Recombinant human pancreatic triglyceride lipase (PTL) was prepared as previously described and used in all assays [18]. Colipase and lipase were added in a 1:1 molar ratio. Under these assay conditions, colipase concentration was the limiting factor for lipase activity. Curves were fitted to a rectangular hyperbola function using SigmaPlot 11. To determine the apparent K_d values we fit the curves assuming the maximum binding or activity was the same for the wild-type and the mutants. Since the PTL concentration is not varied, it will determine the maximum values.

2.4. Steady-state fluorescence spectroscopy

Fluorescence measurements were performed at room temperature on a Jasco FP-6300 spectrofluorimeter using a 1 cm quartz cell. The excitation and emission monochromators were set at 5 and 10 nm slit widths respectively. Excitation was at 295 nm to selectively excite tryptophan residues. Emission was monitored between 300 and 450 nm. The average of five separate scans was analyzed. The buffer was 20 mM Tris–Cl at pH 8.0. When included, the concentration of NaTDC or oleic acid was 4 mM. For quenching experiments, acrylamide was added in 2 μl aliquots of an 8.4 M acrylamide stock to 1 ml of protein solution up to a final concentration of 0.4 M acrylamide. The fluorescence intensity changes were recorded at the maximum emission wavelength for the mutant PTL and corrected for dilution. The Stern–Volmer plots were created with the simplified equation [19,20]:

$$F_0/F = 1 + K_q[Q].$$

F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. Q is the molar concentration