Communication

Colipase Stabilizes the Lid Domain of Pancreatic Triglyceride Lipase

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Pancreatic lipase is characterized by increased activity against water-insoluble substrates and by dependence on another protein, colipase, for binding to the substrate interface. In most models of pancreatic lipase activity, colipase functions to anchor lipase on the substrate interface. Recent studies of the x-ray crystal structure of the complex between colipase and lipase suggest another function for colipase in maintaining the active conformation of lipase. We tested this hypothesis by introducing mutations into colipase at position 15, a residue that contacts the lid domain lipase in the open conformation. Multiple mutant colipases were expressed and shown to have decreased activity. To further investigate the function of the interaction between Glu15 of colipase and lipase, we examined one mutant, E15R, in detail. This mutant had 175-fold less activity compared with wild-type colipase. Although E15R had decreased activity, it was as effective as wild-type lipase in anchoring lipase to mixed emulsions of bile salt and tributyrin. The importance of the interaction with the lid domain was tested by determining the activity of E15R with lid deletion mutants of lipase. E15R was as active as wild-type colipase with these mutant lipases. These results indicate that Glu15 is critical for activity of the colipase-lipase complex at an interface and that colipase has a function in lipolysis in addition to anchoring lipase to an interface. We propose that this function is to stabilize the lid domain of lipase in the open conformation, thereby facilitating lipolysis.

For a number of years, lipases and phospholipases have been models for the interaction of proteins with lipid-water interfaces like those found in cell membranes, in lipoprotein particles, or in emulsions of dietary fats. Interest in the function of lipolytic enzymes has increased in recent years because of the kinetic mechanism of lipolytic enzymes is distinguished by facile diffusion into the active site. No interactions between the lid domain and colipase were present in this conformation. In the presence of mixed micelles, the lid domain moved into a markedly different position that opened the active site. Furthermore, this movement created new interactions between residues in the lid and colipase residues Glu15 and Arg28 (9). Both of these residues are conserved in colipase isolated from many species including dogfish, chicken, and various mammals, implicating a role for these residues in colipase function (10, 11). The formation of these new interactions between colipase and the PTL lid domain indicated that colipase may function to stabilize the lid domain in the open, active conformation, thereby facilitating PTL activity. We directly tested the hypothesis that the interaction between colipase and the lid domain of PTL is functionally important by creating, expressing, and characterizing colipase mutants with amino acid substitutions at position 15.

MATERIALS AND METHODS

Construction of the Mutants—All manipulations of DNA were done by standard methods (12). Mutations were introduced into the cDNA encoding human procolipase by the polymerase chain reaction (PCR) overlap extension method (13). Primers containing a specific mutation were used to produce the E15R and the E15Q mutants. The other mutants were expressed in COS-1 cells transfected by the DEAE method as described previously for pancreatic lipase mutants (13). The production of recombinant baculovirus and expression in Sf9 cells was done as described for wild-type colipase (14). Purification was done on an immunoaffinity column as described (14).

Expression of Recombinant Proteins—All of the mutant colipases were expressed in COS-1 cells transfected by the DEAE method as described previously for pancreatic lipase mutants (13). The production of recombinant baculovirus and expression in Sf9 cells was done as described for wild-type colipase (14). Purification was done on an immunoaffinity column as described (14).

Analysis of Recombinant Protein—The medium from COS-1 cells was homogenized using a dounce homogenizer and clarified by centrifugation at 100,000 × g. The supernatant was applied to a column of a monoclonal antibody against colipase. After washing with wash buffer, bound material was eluted with 1 M glycine, pH 2.5. Fractions were collected and dialyzed against wash buffer at pH 7.0. The eluted material was further purified by electrofocusing on a 7-50% gradient of 8 M urea.

The abbreviations used are: PTL, pancreatic triglyceride lipase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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analyzed by SDS-PAGE followed by immunoblot with a polyclonal antibody to human colipase. Purity of the purified, recombinant colipase was assessed by SDS-PAGE. The CD spectrum was recorded on a Jasco J-600 spectropolarimeter in the far ultraviolet region of the CD spectrum, 190–250 nm. Colipase was dissolved in water at a concentration of 0.01 mM.

**Colipase Assays**—Binding assays were done in 0.1 M Tris-Cl, pH 8.0, 0.1 mM NaCl, 2 mM CaCl₂, and the stated concentration of taurodeoxycholate. Tributyrin was added to the binding buffer in a ratio of 0.5 ml of tributyrin to 14.5 ml of buffer, and the mixture was emulsified by homogenization and sonication. Triplicate assays were done in 1.7-ml microfuge tubes. 1 ml of buffer and the indicated amounts of colipase and lipase were added. The tubes were shacked at room temperature for 1 min, and the phases were separated by centrifugation in a microfuge at high speed for 10 min. 500 μl of the upper, aqueous phase was assayed by the titrametric method. The 100% value was determined in an identical tube containing buffer without tributyrin that was handled in parallel with the other samples.

**RESULTS AND DISCUSSION**

The functional importance of Glu₁⁵ to the activity of PTL was tested by introducing mutations into this position. Initially, six mutants, E15G, E15D, E15Q, E15R, E15T, and E15W, were constructed and expressed in COS-1 cells (13). The medium of cells transfected with each mutant was examined by SDS-PAGE and immunoblot and was assayed for colipase activity. The immunoblot is shown, and the mutation is indicated over each lane. B, E15R colipase and wild-type colipase were expressed in a baculovirus system and purified by immunoaffinity chromatography. 5 μg of purified protein was separated by SDS-PAGE and stained with Coomassie Blue. WT, wild type.

Correctly was confirmed by determining the CD spectrum of the mutant (Fig. 2). The spectrum of E15R colipase was indistinguishable from the spectrum of wild-type colipase consistent with the proper folding of the mutant colipase.

To determine if E15R possessed activity, the mutant was tested over a broad range of concentrations (Fig. 3). At high concentrations, E15R reactivated PTL fully. But the concentration of the mutant to give half-maximal activity was 175-fold greater than for wild-type colipase, 1.7 × 10⁻⁵ M versus 3.0 × 10⁻⁷ M. The molar ratio of wild-type colipase to PTL at this concentration was 0.47, whereas the ratio of E15R to PTL was 83. Longer incubations, 60 min, at low concentrations of E15R did not give any activity, demonstrating that the low activity was not due to a long lag time. These findings suggested that the mutation of Glu₁⁵ to Arg₁⁵ decreased the affinity of the mutant colipase for PTL and implied that Glu₁⁵ is critical for the interaction of colipase with PTL. The decreased interaction could affect PTL function through two possible mechanisms. First, the mutant colipase may interfere with opening of the lid domain. Second, the mutant colipase may not anchor PTL to mixed micelles.

The ability of E15R to anchor PTL to mixed micelles was tested directly. PTL was incubated with an emulsion of tributyrin and taurodeoxycholate in the absence or the presence of varying amounts of colipase, wild type, or E15R (15). The organic phase was separated from the aqueous phase by centrifugation, and the amount of PTL remaining in the aqueous phase was determined by activity measurements. When PTL was incubated with tributyrin alone, 10% or less remained in the aqueous phase. If taurodeoxycholate was also present, the interaction of colipase with PTL was determined by activity measurements. When PTL was incubated with tributyrin alone, 10% or less remained in the aqueous phase. If taurodeoxycholate was also present, greater than 80% of the PTL was found in the aqueous phase (Fig. 4). If either colipase or E15R was included, the inhibition of binding by taurodeoxycholate was overcome in a concentration-dependent manner. The ability of E15R to anchor PTL to mixed micelles was slightly less than that of wild-type colipase, but the difference could not explain the large difference in activity between the two. This result suggested that E15R could interact with PTL at an interface and separated, for the first time, the effect of colipase on PTL activity from its effect on anchoring PTL.

Because Glu₁⁵ was found to interact with a lid domain residue in the crystal structure of the colipase-PTL complex, we tested the activity of E15R with two lid domain mutants we had previously created and characterized (15). These mutants contained deletions in the lid domain. One deleted the α-helix at the end of the surface loop, residues 248–257, retaining Asn²⁴¹, which bonds to Glu¹⁵, and the other deleted the entire lid domain, including Asn²⁴¹ from residue 240 to residue 260. Both of these mutants were active and required colipase for full activity, and importantly, both mutants had good activity...
lipase. The test reactions bound lipase did not inhibit the activity of native colipase with PTL in lipase activity added. The amount of E15R colipase that completely by the pH-STAT method and expressed as a percentage of the total phase was separated from the aqueous phase by centrifugation (15). After a 1-min incubation, the organic phase was incubated with emulsions of tributyrin and taurodeoxycholate and was assayed with varying amounts of wild-type or E15R colipase. The results were expressed as a percentage of the activity obtained with each lipase and wild-type colipase. Open bars, wild-type colipase; closed bars, E15R colipase. The lipase assayed is given below each pair of bars. Standard deviation for four separate experiments is shown. B, the 240–260 lid domain deletion mutant was assayed with varying amounts of wild-type or E15R colipase by the pH-STAT method. Closed circles, E15R colipase; closed squares, wild-type colipase. The error bars give standard deviation for three separate experiments. hPL, human pancreatic lipase.

PTL that is consistent with the hypothesis that Glu15 interacts with the lid domain residues and stabilizes the lid in the open, active conformation.

Pancreatic colipase functions to restore activity to bile salt-inhibited PTL. Abundant experimental evidence has demonstrated that one function of colipase is to anchor PTL to the surface of the substrate (6). Recent structural data suggested an additional role for colipase in the lipolytic mechanism, stabilizing the open, active conformation of PTL (7, 8). Our findings that E15R has decreased affinity for PTL, yet it has preserved the ability to effectively anchor PTL to the substrate, provide direct evidence for this hypothesis, and support a model of lipolysis that includes separate roles for colipase in PTL binding and activity. In this model, colipase-mediated binding of PTL does not require interactions with the lid domain, but for activity, PTL must move into the open conformation, presumably triggered by an interface, and form a stable, high affinity complex of colipase and lipase on the substrate interface. Once a complex is formed, colipase stabilizes the open conformation and both increases the affinity of PTL for the interface and allows catalysis to proceed. If the open conformation does not form, then PTL dissociates from colipase and the substrate. It is in the formation of a high affinity complex of colipase and lipase in the open conformation on an interface that is disrupted by mutations in Glu15.

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