Binding of bile salts to pancreatic colipase and lipase

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Abstract The binding of conjugated bile salts to pancreatic colipase and lipase has been studied by equilibrium dialysis and gel filtration. The results indicate that at physiological ionic strength and pH, conjugated bile salts bind as micelles to colipase: 12–15 moles/mole of colipase for the dihydroxy conjugates and 2–4 for the trihydroxy conjugates. No binding of bile salt takes place from monomeric solutions. Under the same experimental conditions, only 1–2 moles of conjugated dihydroxy bile salts bind to pancreatic lipase.

Supplementary key words micelles · dodecyl sulfate

Pancreatic lipase is inhibited by conjugated bile salts in concentrations above their CMC (1–3). Addition of colipase, a polypeptide cofactor for lipase (4, 5), to such systems restores the activity of lipase under these conditions (1, 2). Gel filtration (1) and ultracentrifugation (3) have indicated that colipase in bile salt solution is converted to a form with approximately twice the monomer molecular weight, and it has been suggested that in bile salt solution colipase is present as a dimer (1).

The interaction between colipase and bile salt in solution has now been studied by equilibrium dialysis and gel filtration, and the results indicate that colipase binds to bile salt micelles. In contrast, lipase seems to have only a weak affinity for conjugated bile salts.

MATERIALS AND METHODS

Lipase and colipase were purified from porcine pancreas as described previously (6, 7). Labeled bile salts, [^{35}S]TDC, [^{3}H]GDC, and [^{3}H]TC, were synthesized in this laboratory (8, 9). [^{35}S]SDS was obtained from the Radiochemical Centre, Amersham, England. The radiopurity of the labeled compounds was determined by thin-layer chromatography followed by counting of radioactivity. The radiopurity for all compounds used was better than 97%.

Equilibrium dialysis was performed essentially as described by Nelson et al. (10). Dialysis tubing, $^{23}/_{32}$ inch in diameter (Union Carbide), was used. It was boiled for 1 hr in a solution 0.1 M in Na_2CO_3 and 0.01 M in EDTA, exhaustively washed in distilled water, and stored in 0.02% NaN_3 at 4°C. The characteristics of this tubing are constant from batch to batch, and it retains colipase almost completely. The dialysis tubing was knotted at one end and inserted into 5-ml glass vials with snap-on caps. The vials contained 2 ml of outer solution with bile salt and 1 ml of inner solution with lipase (72.5 nmoles) or colipase (200 nmoles) inside the dialysis bag; the open end of the bag was pulled down around the rim and the cap was snapped on. The buffer used for both inner and outer solutions was 150 mM in NaCl, 1 mM in $CaCl_2$, and 2 mM in Tris–HCl, pH 7.0, and contained sodium azide, 0.02%. Bile salt was added to the outer solution or to both solutions at the beginning. For equilibration, the vials were rotated on their long axes at a rate of 10–12 rpm at 25°C for 3–5 days. Duplicate 0.2-ml aliquots were taken from the outer and inner solutions and added to 10 ml of Instagel R (Packard Instrument Co.) for radioactivity determinations.

Radioactivity was converted to detergent concentration, the concentration in the outer solution representing the concentration of free detergent in equilibrium with detergent bound to the protein and the concentration in the inner solution representing the concentration of free plus bound detergent. The amount bound to detergent was calculated as the difference between the concentrations in the inner and outer solutions and was converted to moles of detergent bound per mole of protein. The reproducibility of the method is exemplified by an experiment in which the concentration of labeled dihydroxy bile salt on both sides of the membrane from the beginning was 5 mM and the inner solution contained 0.2 μmole of colipase in 1 ml of solution. The equilibration time was 3 days and the mean number of moles of bile salt bound per mole of colipase for nine determinations was 7.58 ± 0.70 (SD). One flask in which no colipase had been added showed that equilibrium over the

Abbreviations: CMC, critical micellar concentration; TDC, taurodeoxycholate; GDC, glycodeoxycholate; TC, taurocholate; SDS, sodium dodecyl sulfate.

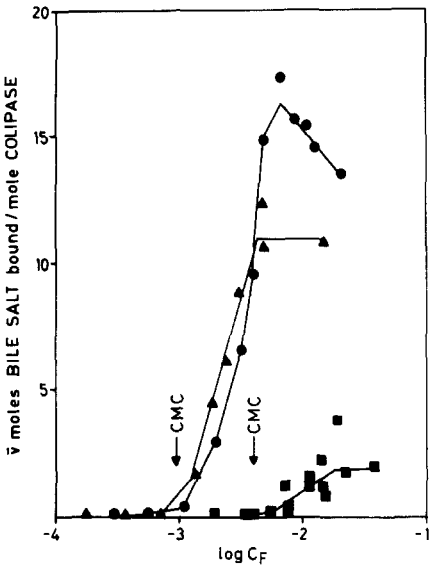

Fig. 1. Binding of TDC (●), GDC (▲), and TC (■) to pancreatic colipase at 25°C in 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM Tris-HCl, pH 7.0, measured by equilibrium dialysis. 0.2 μmole of colipase was dissolved in 1 ml of solution placed in the inside of the dialysis bag. The outer solution (2 ml) contained different bile salt concentrations. Equilibration time, 3 days. C_F, free bile salt concentration.

membrane had been obtained in 24–48 hr. The accuracy of the method, however, is dependent on the total detergent concentration, the protein concentration being constant, for two reasons. First, at detergent concentrations high in relation to the number of moles bound to the protein, the latter figure will be obtained as the difference between two nearly equal figures. Second, at high micellar concentrations, the time needed to achieve equilibrium becomes impracticably long. This is true especially when SDS is used, and it is questionable whether complete equilibration can be obtained with this detergent at high micellar concentrations

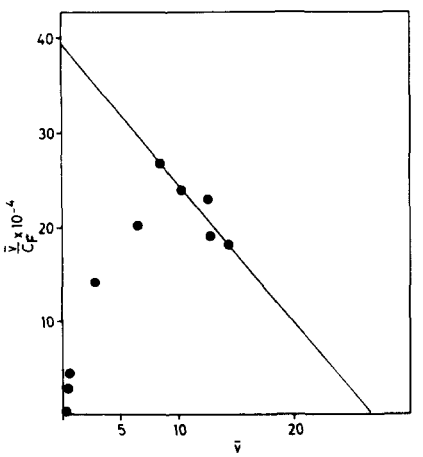

Fig. 2. Scatchard plot of data obtained from equilibrium dialysis of GDC and colipase. Conditions similar to those in Fig. 1.

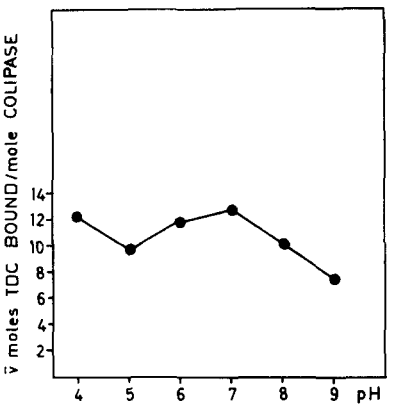

Fig. 3. Effect of pH on the binding of TDC to colipase as determined by equilibrium dialysis. Total concentration of TDC was 6 mM. Buffers used were 2 mM sodium acetate (pH 4), 2 mM Tris-maleate (pH 5 and 6), and 2 mM Tris-HCl (pH 7–9), 150 mM in NaCl and 1 mM in $CaCl_2$. Other conditions as in Fig. 1.

with the method used (11). The figures obtained for low detergent concentrations thus can be obtained with a reasonably high degree of accuracy. At high micellar concentration, the figures are less dependable (as is apparent from the results) and may be obtained only by extrapolation.

Gel filtration experiments were performed using Sephadex G-25 and G-100 gels in K9/30 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) (bed volume = 18 ml) preequilibrated with labeled bile salt solutions of different concentrations below and above the CMC. The available volume of the gel was calculated as $K_{av} = (V_e - V_o)/(V_t - V_o)$ (Ref. 12). V_o is the void volume of the column and was obtained using blue dextran (Pharmacia). V_e is the effluent volume obtained by extrapolating the volume with the maximal concentration of the eluted compound. V_t is the total volume of the gel determined by the elution volume of glucose.

RESULTS

Equilibrium dialysis

Fig. 1 shows the results obtained for the binding of different bile salts. Moles of bile salt bound per mole of colipase are plotted against log free concentration of bile salt. No binding takes place below the CMC for the bile salts, which is approximately 1 mM for the dihydroxy and 4 mM for the trihydroxy conjugates (13). Above the CMC, the binding increases steeply for the dihydroxy conjugates, reaching values of 12–15 moles/mole of colipase. The figures obtained at high bile salt concentrations were somewhat variable from experiment to experiment. This was probably due to the difficulties previously discussed in obtaining equilibrium with high micellar concentrations. The

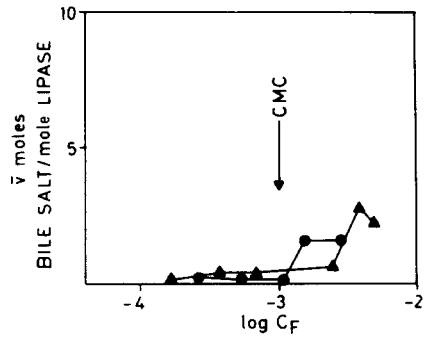

Fig. 4. Binding of TDC (●) and GDC (▲) to pancreatic lipase as determined by equilibrium dialysis. Inner solution contained 72.5 nmoles of lipase in 1 ml of buffer. Other conditions as in Fig. 1.

results, however, seem to indicate that the extent of binding does not increase further with increase in micellar bile salt concentration. A Scatchard plot of the binding of GDC to colipase is shown in **Fig. 2.** $\bar{v}$ is the average molar ratio of bile salt bound to colipase, and C_F is the molar concentration of unbound bile salt in equilibrium with the bile salt–colipase complex. It is evident that the points obtained do not fit a linear curve and that therefore bile salts do not bind to independent and identical sites on the colipase molecule. On the contrary, the Scatchard plot has a convex curvature that is evidence for cooperative binding, as discussed by Sukow and Sandberg (14). At higher values for $\bar{v}$, the data can be fitted to a straight line, and extrapolation gives a value of > 20 for the total number of binding sites. The binding of TDC to colipase does not show any pronounced changes in the pH range of 4–9 (**Fig. 3**). With the trihydroxylated taurocholate, 2–4 moles were bound per mole of colipase, and again binding occurred only over the CMC (Fig. 1). When the bile salt monomer concentration was changed by varying the sodium chloride concentration, the binding was found to decrease with increase in monomer concentration and, conversely, to increase with micellar concentration while the total bile salt concentration remained constant (**Table 1**).

TABLE 1. Binding of TDC to colipase in relation to the monomer concentration of bile salt

NaCl	Monomeric	Micellar	$\bar{v}$
mM	*mM*	*mM*	
0	1.8	1.2	1.9
10	1.7	1.3	3.6
50	1.4	1.6	4.1
75	0.9	2.1	4.5
150	0.8	2.2	5.2

Total bile salt concentration was 3.0 mM. Concentrations of bile salt in monomeric and micellar forms were varied by varying the concentration of NaCl from 0 to 150 mM. CMC was determined by the spectral shift method using rhodamine 6B (13). $\bar{v}$, moles of TDC bound per mole of colipase.

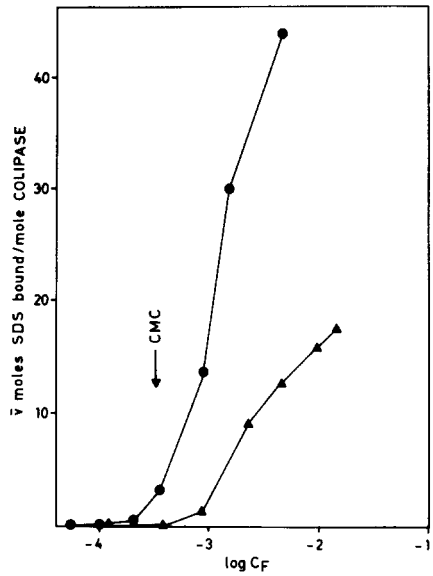

Fig. 5. Binding of SDS to colipase at low and high salt concentrations. ▲, binding in a buffer solution 2 mM in Tris-HCl and 1 mM in $CaCl_2$, pH 7.0, with no NaCl added. ●, binding in the same buffer, 150 mM in NaCl. 0.2 μmole of colipase was in the inner solution. Equilibration time, 5 days. CMC for SDS in 150 mM NaCl was determined by the spectral shift method using rhodamine 6B (13).

The binding of bile salts to pancreatic lipase calculated from the equilibrium dialysis experiments (**Fig. 4**) indicates a binding of, at the most, 1–2 moles/mole of lipase and no binding below the CMC.

The binding of SDS to colipase (**Fig. 5**) was found to be in principle similar to that for bile salt and colipase: no binding at a concentration below the CMC, and a rapid increase above the CMC, reaching extrapolated figures of 40–50 moles bound per mole of colipase. When the sodium chloride concentration was zero, the concentration of SDS at which binding started was higher and the aggregation number lower.

Gel filtration experiments

In these experiments the columns were preequilibrated by the solution containing labeled bile salts at concentrations below and above the CMC. Colipase or lipase dissolved in the same solution as used for equilibration and subsequently for the elution of the columns was then applied to the top of the column. **Fig. 6** shows the results using a G-100 Sephadex column run with 4 mM TDC, with 450 nmoles of colipase applied. Colipase was eluted with a K_{av} of 0.42, with a bile salt peak slightly ahead, followed by a trough. The elution pattern is typical for binding of a ligand to a protein, as first described by Hummel and Dreyer (15). When the concentration of bile salt used was below the CMC (0.6 mM), no indications for any binding were obtained, nor was binding noted when the

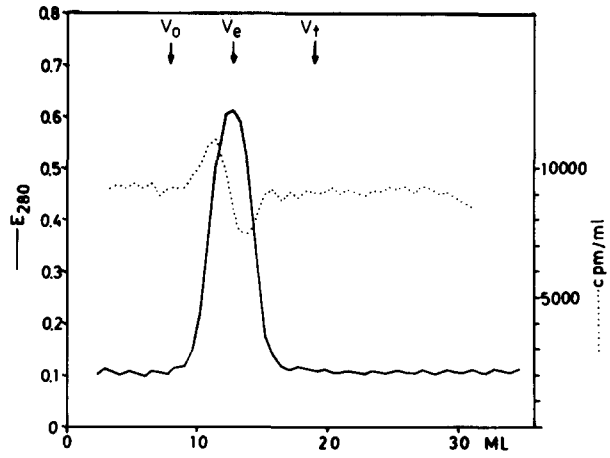

Fig. 6. Gel filtration of colipase in bile salt solution. A Sephadex G-100 column, V_t = 19.2 ml and V_o = 7.9 ml, was equilibrated with a solution 4 mM in ^{35}S-labeled TDC, 150 mM in NaCl, 1 mM in $CaCl_2$, and 2 mM in Tris–maleate, pH 7.0. 450 nmoles of colipase dissolved in 0.5 ml of the above solution, which also was used for elution, was applied to the column. Fractions of approximately 0.6 ml were collected and used for determination of protein ($A_{280\ nm}$) and radioactivity. V_e was 12.7 ml and K_{av} for colipase was 0.42.

bile salt concentration was increased to 12 mM. The interpretation of these results, which deviate from those usually obtained for protein ligand binding, i.e., no apparent binding at low and high ligand concentrations, is suggested to be as follows. Only the monomeric form of the bile salt penetrates the gel fully, and this concentration is constant regardless of the total bile salt concentration when this is above the CMC. Monomeric bile salts are therefore completely (G-25) or partially (G-100) separated from the colipase. Bile salt micelles of dihydroxy conjugated bile salts have approximately the same dimensions as colipase and they cannot be well separated by gel filtration (12). At bile salt concentrations below the CMC, no binding is seen in the gel filtration experiments, indicating no binding of monomeric bile salts to colipase. When the bile salt concentration is above the CMC, binding between colipase and bile salt is indicated by the bile salt peak followed by a trough (16). When the bile salt concentration is further increased to well above the CMC, the binding will be obscured as the protein concentration becomes low relative to the concentration of micellar bile salt. In actual experiments (G-100 gels), the peak concentration of colipase was on the order of 120–140 nmoles/ml compared with a concentration of micellar bile salt of approximately 11 μmoles/ml. Even if 12–15 moles of bile salt bind per mole of colipase, as judged from the equilibrium dialysis experiments, the concentration of bound bile salts will be low compared with that of micellar bile salt at the high total bile salt concentration used.

TABLE 2. K_{av} values and apparent molecular weights for colipase under various conditions

	K_{av}	Apparent Mol Wt
Buffer	0.59–0.61	12,000
TDC		
2 mM	0.50	19,000
4 mM	0.42–0.45	24,000
6 mM	0.41–0.45	24,000
12 mM	0.45	24,000
TC, 6 mM	0.59	12,000
SDS, 6 mM	0.16–0.17	90,000

Colipase was eluted from a Sephadex G-100 column preequilibrated by buffer, bile salts at different concentrations, or SDS. Solutions in all cases contained 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM Tris–HCl, pH 7.0. K_{av}, available gel volume; see text.

In **Table 2** are given the K_{av} values obtained for colipase using a G-100 Sephadex column under various conditions. The values obtained for buffer solution, 0.59–0.61, are similar to those reported earlier (1) and correspond to an apparent molecular weight of about 12,000 for colipase, identical with that calculated from ultracentrifugation and amino acid analysis (5). With bile salt (TDC) concentrations above the CMC, the K_{av} drops to values of 0.42–0.45, corresponding to an apparent molecular weight of 22,000–24,000.

With the gel column equilibrated with TC above the CMC, the K_{av} for colipase did not change significantly from that obtained in buffer solution, which is in agreement with the finding from the equilibrium dialysis experiments that only a small amount of this bile salt binds to colipase. In similar experiments with micellar SDS (4 mM), colipase was eluted from a G-100 column corresponding to an apparent molecular weight of about 90,000.

DISCUSSION

The findings of the present investigation, that no binding between colipase and bile salts occurs at free amphiphilic concentration below the CMC and that the binding number over the CMC per mole of colipase agrees closely with the aggregation numbers given for the di- and trihydroxy conjugated bile salts in micellar solution (17), would indicate that binding occurs between colipase and bile salt micelles. The binding of 15–20 moles of deoxy bile salts to colipase would give an anhydrous particle weight on the order of 19,000–22,000, which would agree with the figures calculated from the gel filtration data of this and a previous investigation (1) and with the ultracentrifugation experiments of Maylie et al. (3). Evidence from both methods used for characterizing the interaction of colipase and bile salt thus indicates a binding of colipase to bile salt micelles and provides a satisfactory explanation for the earlier

reported increase in size of colipase in solution of micellar TDC; this size change had previously been interpreted to indicate dimerization of colipase (1).

The binding of SDS to colipase seems to be of a similar nature and also occurs as a binding to micelles. Indications for this are again the relationship of the binding to the CMC and the finding that the binding is related to the salt concentration similar to the effect of salt concentration on micelle formation, i.e., a decrease in the CMC and an increase in aggregation number with increase in NaCl concentration (18). In this respect, the binding of SDS to colipase is different from that described for other proteins (19), an exception being cytochrome b_5 (20).

The relatively good agreement between the figures obtained for the apparent molecular weight of the colipase–TDC complex by gel filtration and the particle weight calculated from the equilibrium dialysis data would indicate that the colipase–TDC complex is fairly symmetrical. The disproportion between these parameters for the SDS–colipase complex (an apparent molecular weight by gel filtration of about 90,000 and a binding of 50 moles of SDS/mole of colipase, corresponding to a particle weight of about 25,000) would, on the other hand, indicate a high degree of asymmetry. Circular dichroism spectra of colipase in micellar solution of TDC do not reveal any conformational changes in the colipase molecule.[1] As the binding of bile salts to colipase does not show any more profound changes over the pH range of 4–9, ionic interactions most likely are not important (21). The colipase molecule is rich in hydrophobic and hydrophilic amino acids (5) and may have a unique anphiphilic structure with domains for hydrophobic and hydrophilic interactions of importance for its physiological function (2).

The Scatchard plot of the binding of GDC to colipase (Fig. 2) has a convex appearance and is evidence for a cooperative binding especially documented at low values for $\bar{\nu}$. At higher values of $\bar{\nu}$, a straight line may fit the data, which can be extrapolated to a value of about 20 moles of bile salt bound per mole of colipase. This should not, however, be interpreted to mean that the binding sites are independent and identical (14).

The binding of bile salts to colipase over the CMC most probably is a manifestation of the same tendency that results in the aggregation of bile salts to form micelles. Due to its special physicochemical properties, colipase may serve as a nucleus for micellization.

With pancreatic lipase, the glycine and taurine conjugates of deoxycholate were bound as 1–2 moles/mole of protein, with no binding occurring below the CMC of the bile salt. The values, especially for higher concentrations of bile salts under the experimental conditions, were uncertain due to the fact that they were calculated as a difference between two large, almost equal numbers. Gel filtration experiments with lipase and bile salts similar to those with colipase did not show any significant binding.

[1] Donnér, J., and I. Sjöholm. Unpublished results.

This work was supported by the Swedish Medical Research Council (grant B72-74-13X).

Manuscript received 1 October 1974; accepted 14 March 1975.

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