On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts

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Abbreviations: DCT, deoxycholyltaurine; TB, tributyrin.

Abstract The interactions between pancreatic lipase and colipase and the substrate and the effect of bile salts on these interactions have been investigated by the use of kinetic experiments and studies on the semiquantitative phase distribution of lipase and colipase activities. The results suggest that lipase binds to hydrophobic interfaces with partial irreversible inactivation. Bile salts in the range of micellar concentrations and above a pH of about 6.5 displace lipase from this binding, resulting in a reversible inactivation. At pH values below about 6.5, lipase binds strongly to the substrate even in the presence of bile salt, and a low activity peak is seen around pH 5.5. This is the result of the binding of lipase to the "supersubstrate" and the activity of the catalytic site. In the presence of bile salt, colipase promotes the binding of lipase to the "supersubstrate" but not to other hydrophobic interfaces, and catalytic activity is reestablished. Kinetic data indicate that the binding between colipase and lipase in the presence of substrate is strong and occurs in an approximately stoichiometric relationship.

Supplementary key words hexadecane · substrate interface

Recent studies (1, 2) have shown that bile salts above the range of their critical micellar concentration inhibit pancreatic lipase activity toward its substrate and that this inactivation is reversed by colipase, a recently isolated peptide cofactor for lipase. Brockerhoff (3) has discussed the role of bile salts and protein such as albumin for the activity of pancreatic lipase and has suggested that they provide protection for lipase at the substrate–water interface by modifying its structure. The hypothesis was put forward that lipase requires isolation from rather than binding to the hydrophobic part of the substrate to prevent unfolding and inactivation at the interface. It was suggested by Brockerhoff (3) that colipase acts in a similar rather nonspecific way and not primarily on the enzyme itself.

In the present investigation, a series of semiquantitative experiments was undertaken to shed additional light on the possible role of bile salts and colipase on the action of lipase on insoluble substrates.

MATERIALS

Chemicals

Tributyrin (TB) used as substrate was a product of BDH Chemicals Ltd., Poole, England, and was purified by fractional distillation (1). Deoxycholyltaurine (DCT) was synthesized in this laboratory (4) and was more than 97% pure as judged by thin-layer chromatography. Hexadecane was a product of Fluka, Bucks, Switzerland (>99% pure by gas–liquid chromatography), and was used as purchased.

Enzymes

Porcine pancreatic lipase was purified as described by Verger et al. (5). Prepared in this way, lipase contains colipase activity corresponding to 20–30% saturation (6) and is less suitable for the kind of experiments discussed here. Lipase essentially free from colipase activity was produced as described by Donnér.[1] The purified enzyme was homogeneous as judged by polyacrylamide disc electrophoresis at pH 8.9. The molecular weight was estimated to be 52,500 by sedimentation equilibrium ultracentrifugation.[1] The enzyme had a molar activity of $5.3 \cdot 10^5$ units/μmole when assayed with TB as substrate at pH 8.0 (3), and its activity in 4 mM DCT at pH 7.0 was 0.1% of the lipase activity in buffer alone.

Colipase (mol wt 11,000) was obtained in pure form from extracts of the porcine pancreatic gland as described by Erlanson, Fernlund, and Borgström (7). The preparation used was a mixture of colipases I and II and gave two

[1] Donnér, J. To be published.

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bands of approximately equal size when separated by polyacrylamide disc electrophoresis at pH 9.3.

EXPERIMENTAL PROCEDURE

Reactions were carried out at 25°C in 2 mM Tris–HCl or Tris–maleate buffer that was 1 mM in $CaCl_2$ and 150 mM in NaCl.

Lipase activity was determined in 10–15 ml of buffer using 500 μl of TB, which was added to the buffer by an automatic pipette. The reactions were carried out in glass vials purchased for use with a Packard liquid scintillation spectrometer. The substrate mixture was stirred by a magnetic stirrer using a Teflon-coated bar. Stirring was performed at conditions that in the absence of the electrode gave a vortex in the solution of approximately the depth of the flask. Automatic titration was performed using a Mettler titration system (Mettler Instrument AG, Zurich, Switzerland). 0.2 M NaOH was delivered to the reaction mixture with a 1-ml burette. Rates of hydrolysis (μmoles/min) were calculated from the titration curves for the first few minutes after achieving a constant reaction rate. Linearity was obtained up to a reaction rate of 5 μmoles/min. Colipase activity was determined under similar conditions in the presence of 4 mM DCT and an excess of lipase, usually 50 pmoles/incubation. In 4 mM DCT, lipase activity was almost completely inhibited, but the increase in reaction rate was directly proportional to the amount of colipase added up to approximately 20 pmoles. In some of the experiments in which lipase activity was measured in bile salt solution, an excess of colipase was added and the reaction rates became dependent on the amount of lipase present.

In the present experiments, mostly relative lipase and colipase activities are reported.

Binding of lipase and colipase to the substrate

Lipase, 10 pmoles, or colipase, 50 pmoles, or both, and 500 μl of TB were added to 15 ml of buffer solution with or without 4 mM DCT. TB was excluded from the control incubations. The vial, containing a magnetic stirring bar, was placed on the automatic titrator, and the pH was adjusted and kept constant if fatty acids were released. After 5 min the contents of the vial were transferred to a 20-ml glass centrifuge tube and centrifuged for 10 min at 1200 rpm. 10 ml of the clear aqueous solution was transferred to another vial and 500 μl of TB was added. When applicable, bile salts, colipase, and lipase were added alone or in combination and the activity was determined by titration. Except for the direct determination of lipase in buffer solution, three different special cases were encountered: (*1*) If lipase only had been added in a bile salt-containing system, colipase had to be added to measure remaining lipase activity; (*2*) if colipase only had been used in the preincubation, lipase and bile salt had to be added to determine colipase activity; (*3*) if both lipase and colipase were present in the preincubation, the amount of activity after the addition of new substrate was dependent on the relative proportions of lipase and colipase present in the aqueous phase. The results obtained were calculated as percentages of the appropriate controls preincubated without TB. Similar experiments were also carried out with 500 μl of hexadecane, instead of TB, in the preincubation. In these experiments the hydrocarbon accumulates at the top of the tube and must be carefully penetrated to obtain a clean aqueous phase.

The lipase or colipase activity in the 5 ml of buffer and TB remaining in the centrifuge tube after sampling the aqueous phase was determined by titration after transfer to another glass vial and addition of 10 ml of fresh aqueous phase.

The effect of pH on the binding of lipase and colipase to its substrate was studied in the range of 4.5–9.0. The buffer was adjusted to the desired pH. TB was added followed by lipase or colipase, or both. The mixture was stirred and the pH was kept constant. After approximately 5 min the contents of the vial were transferred to a centrifuge tube and the oil phase was separated as previously described. 10 ml of the aqueous phase was transferred to a vial, the pH was adjusted to 7.0, and TB was added.

Kinetic studies of the binding of lipase to its substrate

In these experiments the amounts of enzyme (10 pmoles) and substrate (500 μl of TB) were kept constant but the volume of the aqueous phase was 15–100 ml. Different vessels had to be used with the different volumes, and the extent of stirring was changed by the speed of the motor; stirring was kept at the highest possible rate that was consistent with the vessel used.

Kinetic studies undertaken to study the interaction between lipase and colipase in the presence of substrate

These experiments were performed in 4 mM DCT solution in pH 7 buffer at a saturating substrate level of 500 μl of TB in a total aqueous volume of 15 ml. The colipase concentration was varied between 6.7 and $33 \cdot 10^{-10}$ M, and the lipase concentration was $6.7 \cdot 10^{-8}$ M. Identical experiments were performed in which colipase was kept constant at $6.7 \cdot 10^{-8}$ M and lipase was varied in the range of 6.7 to $33 \cdot 10^{-10}$ M. Initial rates of hydrolysis were measured.

RESULTS

Results of the binding studies performed at pH 7.0 with the procedure outlined above are summarized in **Table 1.** Lipase, colipase, or both were preincubated with the sub-

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TABLE 1. Presence of lipase and colipase in the aqueous phase after incubation with TB as substrate in the absence or presence of bile salts

A[a]	B[b]	Activity in Aqueous Phase μmoles/min	% Activity in Aqueous Phase
I Lipase			Lipase, 6.5%
– TB		2.15 ± 0.03 (7)[c]	
+ TB		0.14 ± 0.02 (6)	
II Colipase			Colipase, 51%
– TB	+ DCT	3.33 (2)	
+ TB	+ Lipase	1.70 (2)	
III Colipase + lipase			Lipase, 23%; colipase, 29%
– TB		2.77 (3)	
– TB	+ DCT + lipase	2.97 (2)	
+ TB		0.65 (3)	
+ TB	+ DCT + lipase	1.75 (2)	
IV Lipase			Lipase, 98%
– TB	+ Colipase	2.97 (2)	
+ TB	+ Colipase	2.90 (2)	
V Colipase			Colipase, 38%
– TB	+ Lipase	3.60 (3)	
+ TB	+ Lipase	1.37 (2)	
VI Colipase + lipase			Lipase, 5%; colipase, 36%
– TB		2.15 ± 0.04 (7)	
+ TB		0.11 ± 0.01 (6)	
+ TB	+ Lipase	1.30 ± 0.05 (7)	

I–III, experiments in the absence of bile salts; IV–VI, experiments in the presence of 4 mM DCT.

[a] Conditions during preincubation.

[b] Conditions during reincubation of aqueous phase.

[c] Number of determinations is in parentheses.

strate in pH 7.0 buffer (I–III) or in the same buffer in the presence of 4 mM DCT (IV–VI).

Lipase when added alone (I) to the substrate dispersed in buffer was recovered in the aqueous phase only to the extent of a few percent. When attempts were made to demonstrate the presence of lipase in the oil phase, the recovery was on the order of 30%. Incubation of lipase with the substrate in buffer for 30 min or longer resulted in complete loss of lipase activity. This was demonstrated by adding DCT to the incubations at different times followed by centrifugation and analysis of lipase activity in the aqueous phase after the addition of colipase.

In the presence of 4 mM DCT (IV), on the other hand, more than 98% of the lipase activity added and assayed under the conditions reported was found in the aqueous phase. No activity above that calculated to be present in the aqueous phase left behind could be demonstrated in the substrate plus interface in these experiments.

Under the same experimental conditions, colipase was distributed equally between the aqueous phase and the oil interface (II). This distribution was not appreciably affected by the presence of bile salt (V) and was independent of colipase concentration over a wide range. With both lipase and colipase (III), it was found that the presence of colipase to some extent releases lipase from the substrate–water interface.

In bile salt solution, colipase affects binding of lipase to the substrate interface (VI). Lipase in the aqueous phase was found to be 5% of the activity added, and about 96% of the lipase activity was recovered if the substrate was again dispersed in fresh bile salt solution and titration was resumed.

The results thus clearly demonstrate that at pH 7 bile salts prevent the binding of lipase to its substrate and that colipase present in the substrate–water interface promotes the binding of lipase to this interface. Experiments in which hexadecane was used as oil phase revealed some important differences (**Table 2**). Lipase still had affinity for the interface (I), and the binding of colipase was more important (II). Prolonged incubation of lipase with a hexadecane emulsion led to complete irreversible inactivation of the enzyme. In the presence of bile salt, lipase was cleared from the interface (IV) and, most importantly, in the presence of both colipase and lipase in bile salt solution (VI) the enzyme was found in the aqueous phase to the extent of 98%.

Fig. 1 shows the relationship between reaction rate and the volume of the aqueous phase when lipase was present with a constant amount of its water-insoluble substrate. Under these conditions the efficiency of the stirring became critical. With a constant stirring rate with increasing volumes, the rate of reaction decreased, but this did not neces-

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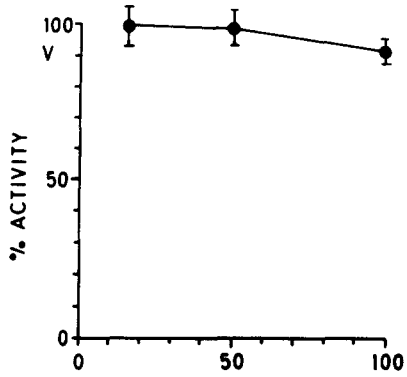

Fig. 1. Lipase activity in relation to the volume of the aqueous phase in which the substrate is dispersed. Porcine pancreatic lipase, 10 pmoles, was added to 15, 50, and 100 ml of buffer solution (150 mM in NaCl, 1 mM in $CaCl_2$, and 2 mM in Tris-HCl, pH 7.0) in glass beakers (50, 100, and 150 ml, respectively, in volume). 500 μl of TB was added and the mixture was stirred with a magnetic bar. Means of seven determinations ± SD.

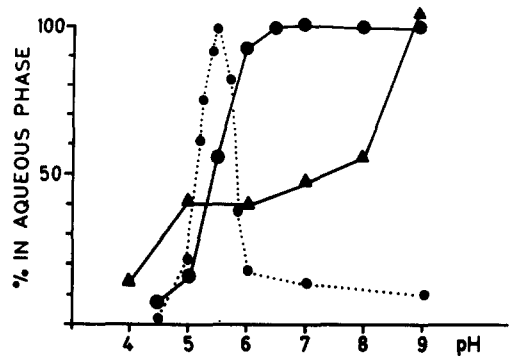

Fig. 2. Percentages of lipase, ●—●, and colipase, ▲—▲, in the aqueous phase after incubation with 500 μl of TB in 15 ml of buffer containing 4 mM DCT at various pH values. The aqueous phase was sampled after centrifugation at 1200 cpm for 10 min. ●- - -●, lipase activity in 4 mM DCT in the absence of colipase; maximum activity = 100%.

sarily mean a decrease in lipase activity. By increasing the rate of stirring from the standard level to the highest rates that could be achieved under the conditions, it was found that the reaction rates showed only a slight decrease when the volume of the aqueous phase was increased from 15 to 100 ml, the total amount of enzyme being kept constant. These results, which indicate a tight binding of lipase to the substrate interface with little enzyme partitioned to the aqueous phase, conform to those from the binding studies already discussed.

Fig. 2 shows the effect of pH on the binding of lipase and colipase to the substrate when present alone in 4 mM DCT solution. Also included is the pH activity curve for lipase in this system. The results show that the distribution of colipase between the aqueous phase and the substrate interface is almost constant over the pH range 5–7; it binds more to the substrate below this range, and at pH 9 it is almost completely in the aqueous phase. Lipase binding to the substrate in 4 mM DCT is markedly influenced by the pH of the system. At acid pH values (<5) it is tightly bound to the substrate; at pH 6.5 and higher the enzyme is found almost completely in the aqueous phase. The transition between the substrate interface and the aqueous phase takes place between pH 5 and 6. The activity of lipase (percentage of the maximal activity) in the presence of 4 mM DCT is low, about 4%, relative to lipase activity in bile salt solution in the presence of colipase or in the absence of bile salts, as shown in **Fig. 3.** In bile salt solution in the presence of both lipase and colipase (**Fig. 4**), lipase binds tightly to the substrate in the pH range 5–7. Above pH 7, the concentration of lipase in the aqueous phase in-

TABLE 2. Presence of lipase and colipase in the aqueous phase after incubation with hexadecane in the presence or absence of bile salts

A	B	% Activity in Aqueous Phase + HD[a]/−HD × 100	
I Lipase		Lipase	40
II Colipase	+DCT + lipase	Colipase	12
IV Lipase	+Colipase	Lipase	98
V Colipase	+Lipase	Colipase	84
VI Lipase + colipase		Lipase	96

I–II, experiments in the absence of bile salts; IV–VI, experiments in the presence of 4 mM DCT. Values are means of two experiments. See Table 1 for explanation of A and B.

[a] HD, hexadecane.

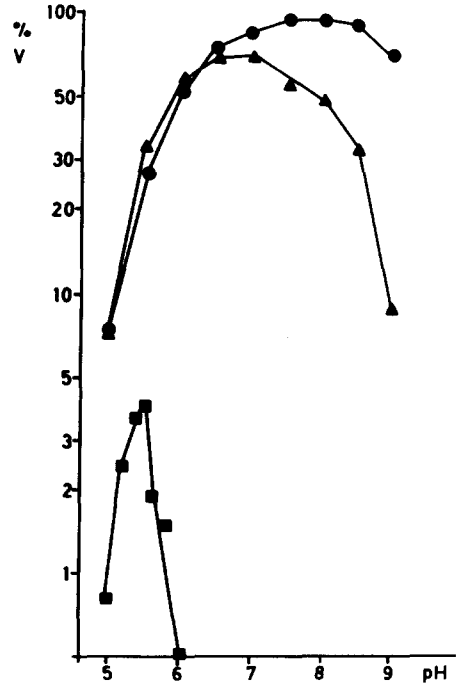

Fig. 3. pH activity curves for pancreatic lipase (10^{-9} M) in buffer (●) containing 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM Tris-maleate or Tris-HCl or in buffer containing 4 mM DCT in the absence (■) or presence (▲) of $5{\cdot}10^{-9}$ M colipase. Substrate, 500 μl of TB, was dispersed in 15 ml of aqueous phase.

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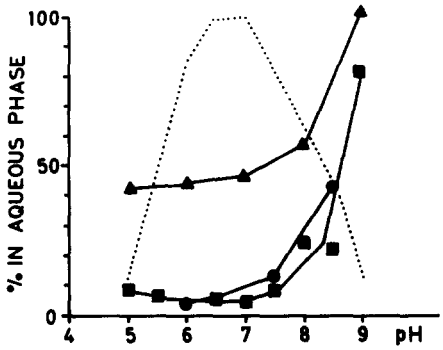

Fig. 4. Percentages of lipase (●) and colipase (▲) in the aqueous phase after incubation with 500 μl of TB in 15 ml of buffer, 4 mM DCT, at various pH values. The aqueous phase was sampled after centrifugation at 1200 cpm for 10 min. ■, lipase activity in the aqueous phase determined after incubation with lipase and colipase in combination. The dashed line is the pH activity curve for lipase plus colipase. Other conditions as in Fig. 3.

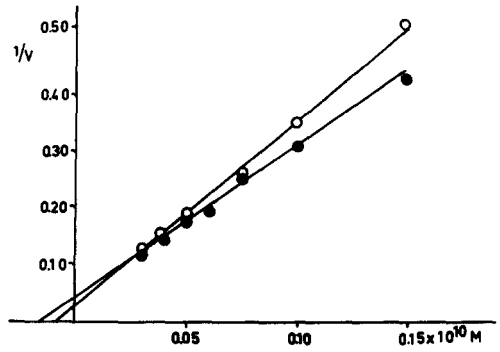

Fig. 5. Plot of $1/V$ vs. 1/colipase (●) with 500 μl of TB in 15 ml of buffer, 150 mM inNaCl, pH 7.0, and 4 mM in DCT. The concentration of lipase was held constant at $6.7 \cdot 10^{-8}$ M, and the colipase concentration was varied between 6.7 and $33 \cdot 10^{-10}$ M. The figure also includes a similar plot (○) for when the concentration of colipase was constant at $6.7 \cdot 10^{-8}$ M and the colipase concentration was varied between 6.7 and $33 \cdot 10^{-10}$ M. Equations for lines and intercepts were calculated by the least squares method. For line ○—○, $y = 3.22x + 0.0277$; for line ●—●, $y = 2.60x + 0.0422$.

creases parallel to a decreased binding of colipase. The lipase activity of the aqueous phase of the complete system (bile salt, lipase, colipase, and substrate) after the addition of new substrate follows that of the lipase concentration, indicating that lipase is the limiting factor under these conditions. It should be pointed out that colipase in these experiments is present in a fivefold molar excess over lipase.

Results of the kinetic studies are given in **Fig. 5,** which shows a plot of the reciprocal of V vs. colipase concentration when lipase was kept constant at a saturating level of substrate and lipase. The system was 4 mM in DCT and the reaction rate was therefore low in the absence of colipase. The K_m calculated from the intercept $-1/K_m$ represents the concentration of colipase at which half-maximal activity of lipase is observed. The value obtained for K_m was $0.6 \cdot 10^{-8}$ M and would indicate a tight binding of colipase to lipase. When the conditions were reversed, i.e., when colipase was kept constant at a saturating level and lipase concentration was varied, the reciprocal curve for V vs. enzyme concentration gave a K_m of $1.2 \cdot 10^{-8}$ M. Thus, the concentration of lipase that gave half the maximal activity with a saturating level of colipase was twice that for colipase when lipase was present in excess. This difference may not be significant and may be caused by the intrinsic colipase activity of the lipase added in a 100-fold molar excess in the one series of experiments in which lipase concentration was kept constant. It may be enough at the present time to conclude that the K_m is on the order of 10^{-8} M.

DISCUSSION

The results of the present investigation have been obtained from a series of experiments of which most can give only qualitative information. The reason for this is, obviously, that the interaction studied occurs at water–oil interfaces, the structure of which is not defined. Furthermore, lipase has been determined as lipase activity, and inactive enzyme may well be present, for example, bound to the substrate but not detected with the methods used.

Nevertheless, the results provide some information of interest. It seems clear that pancreatic lipase has an affinity for hydrophobic interactions. It accumulates not only at the interface between water and substrate but at hydrophobic interfaces in general, as demonstrated in the studies with hexadecane. The orientation of lipase to the water–oil interface also results in the disappearance of lipase activity that cannot be recovered by the addition of colipase and bile salts. The inactivation therefore seems to be irreversible as previously suggested by Brockerhoff (3), who described it as an effect of unfolding of the enzyme molecule. Colipase also has an affinity for hydrophobic interfaces but its biological activity is not affected, probably due to the highly rigid molecular structure (8). For lipase the hydrophobic interaction in binding experiments seems strong, but it is difficult to say how much is adsorption to the interface and how much is inactivation as long as the results are based on measurements of biological activity. The kinetic experiments in which the volume of the aqueous phase was varied, however, indicate that the binding between lipase and its substrate is strong and that the concentration of lipase in the aqueous phase is low.

The effect of bile salt in displacing lipase from the substrate interface parallels the loss of lipase catalytic activity. In Brockerhoff's experiments (3) this inactivation was not seen because of the presence of colipase in the lipase preparation used (6). As previously shown (1), the inactivation of lipase by bile salt is related to its critical micellar concentration. The simplest explanation of this effect is that the inactivation is due to the accumulation of bile salt in the interface, thus giving it a negative charge. This hinders the

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binding of the likewise negatively charged lipase to what Brockerhoff (9) terms the "supersubstrate," thereby also preventing the formation of an enzyme–substrate complex at the catalytic site.

The finding in this investigation that the binding of lipase to the substrate in bile salt solution is influenced by pH may indicate that lipase is prevented from binding to the bile salt-saturated substrate interface by electrostatic repulsion. The results indicate the presence of an ionizing group(s) in lipase with a pK_a on the order of 5.3 and that lipase at a pH of 5 or lower binds to the substrate surface in the presence of DCT. The pI for porcine lipase has been determined to be 5.2,[1] and the ionization of DCT, with a pK_a of around 1.5, cannot be greatly influenced in this region.

It has been previously shown that the lipase-catalyzed hydrolysis of TB is under the control of two ionizable groups in the enzyme with pK values of 5.8 and 10.1 (10), most probably representing the ionization of a histidine residue. It has been suggested that lipase is a "serine enzyme" and that the serine hydroxyl, in analogy with chymotrypsin, is activated by a histidine–aspartic acid charge relay system (11).

The pH activity curve for lipase in the presence of bile salt is most probably the resultant of the activity of the catalytic site on the enzyme and the binding of the enzyme to the substrate interface. The results of the present investigation indicate clearly that in the presence of bile salts the binding to the substrate interface is pH dependent and is possibly dependent on the ionization of the enzyme. The effect of increasing concentration of bile salt in displacing the pH optimum for lipase to the left (1) may be explained entirely by the repulsion of the enzyme from the substrate interface. This can be expected to be most marked at high pH values at low bile salt concentration and may have nothing to do with the ionization of the active site. For activity, lipase thus needs firstly to be bound to its substrate superstructure, a binding that is prevented by bile salts by electrostatic repulsion in the pH region above 6, and secondly to be ionized at the catalytic site.

In the presence of bile salts the aqueous phase contains mixed TB–DCT micelles, and the possibility cannot be excluded that lipase has a stronger affinity for these micelles than for the interface.

Under the conditions of the experiments, colipase was approximately equally distributed between the aqueous and substrate phases. The addition of colipase to the bile salt–lipase substrate system has, however, a dramatic effect on the binding of lipase to the substrate interface that is parallel to a reversal of the inhibition of the catalytic activity. In this effect a specificity for the substrate interface is obvious: colipase does not bind to a pure hydrocarbon interface nor does it affect the binding of lipase to such an interface. It appears that the colipase structure provides a more specific binding to a substrate interface and thus directs lipase to its substrate superstructure.

Indications for strong binding of colipase to lipase has been obtained by measuring the concentration of colipase that gives half-maximal velocity at a saturating amount of enzyme and substrate. The value obtained is on the order of 10^{-8} M. It is not clear if this value relates to the binding of colipase or lipase or to the binding of a lipase–colipase complex to the substrate interface, or to both. The value most probably includes both types of binding. The situation is to some extent equivalent to that earlier discussed for the action of phospholipase A_2 at interfaces (12). A first prerequisite for activity is binding of the enzyme to the substrate interface.

What is the biological importance of the lipase–colipase–bile salt system? The answer can only be speculative. The structure of lipase enables it to act on water-insoluble substrates having a hydrophobic superstructure. For this reason, a part of the lipase other than the active site most probably carries hydrophobic structures to interact with the substrate. These interactions seem to be rather unspecific, as lipase is adsorbed to most hydrophobic interfaces and also becomes unfolded in the interface of its own substrate leading to irreversible inactivation. This is also the case of its interface with its own substrate. Bile salts prevent lipase from binding to hydrophobic interfaces most probably by electrostatic repulsion. Lipase is thus protected from irreversible inactivation, but its catalytic activity against the substrate is lost. Colipase, although having a negative charge in the pH range of lipase activity (8), can displace bile salt from the substrate interface and bind lipase to the structure of the substrate and stabilize its conformation.

Brockerhoff (11) has suggested a model for the binding of lipase to colipase and the substrate interface. The present results provide experimental evidence for such a binding. The orientation of lipase and colipase at the substrate–water interface, however, is hypothetical, and it seems clear that other than hydrophobic forces will be necessary to explain the relative specificity of the binding of the lipase–colipase complex to the substrate compared with a hydrocarbon interface.

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