Effects of Colipase and Taurodeoxycholate on the Catalytic and Physical Properties of Pancreatic Lipase B at an Oil-Water Interface*

WILLIAM E. MOMSEN AND HOWARD L. BROCKMAN

From the Hormel Institute, University of Minnesota, Austin, Minnesota 55912

A monolayer reaction system employing tripropionin and siliconized glass beads was used to study the effects of taurodeoxycholate and colipase on the catalytic activity, interfacial stability, and interfacial affinity of porcine pancreatic lipase B (EC 3.1.1.3). The stability and catalytic activity of lipase at the bead-water interface are governed by the same two ionizable groups with pK, values (in the absence of cofactors) of 5.6 and 9.3. Colipase alone or with bile salt caused only a slight perturbation of these values.

At low concentrations, 0 to 0.3 mM, taurodeoxycholate increases the stability of lipase by 5-fold. At higher concentrations, 0.3 to 0.8 mM, but still below its critical micelle concentration, taurodeoxycholate prevents the adsorption of lipase to the bead-water interface. This appears to be the major mechanism by which this bile salt inhibits lipolysis.

Colipase exerts small positive effects on lipase stability and catalytic activity. More importantly, colipase enables the adsorption of lipase in the presence of bile salt, thereby reversing the inhibition.

Many reactions of biological importance occur at phase boundaries. Interest in the control of these reactions has, in recent years, prompted the development of experimental systems for monitoring enzymatic reactions occurring at interfaces. By employing monolayers of substrate, or enzyme, or both, of a known composition and area, it has been possible to measure kinetic and thermodynamic parameters both accurately and reproducibly (1-4). The results of such studies with pancreatic lipase have indicated that in a well defined, monolayer system the metal ions, bile salts, albumin, and other cofactors used in emulsion assays are unnecessary for catalytic activity (1). However in emulsion systems, and presumably in vivo, cofactors are often required or may exert a significant influence on rates of reaction (5).

Two naturally occurring cofactors for the hydrolysis of glycerides by pancreatic lipase are bile salts and the protein, colipase. An extensive description of the effects of these cofactors and their possible modes of action has been presented recently (5). Briefly, bile salts can either stimulate or inhibit rates of lipolysis depending upon the particular conditions under which activity is determined. In addition, bile salts reportedly improve the linearity of hydrolysis with time and shift the pH optimum for hydrolysis downward. Numerous, and sometimes conflicting, explanations for these effects have been suggested. Bile salts reportedly increase the surface area of the substrate, remove reaction products and/or denatured protein from the interface, prevent the denaturation of the enzyme at the interface, and facilitate adsorption and desorption of the enzyme at the interface. In regard to this latter point, it has been proposed recently that bile salts block the adsorption of lipase to the substrate surface by altering the properties of the interface (6).

The most pronounced effect of colipase is to reverse the inhibitory effects of bile salts. In addition, it causes a stimulation of the rate of hydrolysis in the absence or presence of bile salts and it markedly improves the linearity of product formation as a function of time (5). It has been suggested recently that these effects are the result of a stoichiometric binding between lipase and colipase (7) which increases the rate of lipase adsorption onto its substrate and stabilizes the enzyme at the interface (8). Alternatively, it has been proposed that colipase coats the substrate surface and thereby blocks enzyme denaturation, facilitates the binding of enzyme to the interface and/or supports the proper orientation of the bound enzyme (5).

In the foregoing studies emulsion assay systems of varying compositions were routinely employed. Because of the intrinsic ambiguities associated with the interpretation of results obtained in such systems, it is difficult to define precisely the role (or roles) of bile salts and colipase in glyceride hydrolysis. The recent development of well defined, monolayer reaction systems eliminates many of the difficulties encountered in working with emulsions. With such a system we have examined the effects of sodium taurodeoxycholate and pancreatic colipase on the hydrolysis of the partially soluble triglyceride, tripropionin, by pancreatic lipase B adsorbed to hydrophobic, siliconized glass beads. This system (3) was chosen in preference to other

* This investigation was supported by The Hormel Foundation and by Grant HL-08214 from the United States Public Health Service.
Cofactor Effects on Properties of Lipase B at an Interface

EXPERIMENTAL PROCEDURE

Lipase—Porcine pancreatic lipase B was purified as described by Verger et al. (10) as modified by Logocki et al. (1) and was judged homogeneous by polyacrylamide gel electrophoresis at pH 8.9 (11). Protein concentration was determined by absorbance using E1% = 13.3 and a molecular weight of 50,000 (12). As judged by the assay described below the preparation contained no detectable colipase. Using the tripropionin-siliconized glass bead assay system described by Brockman et al. (3) the purified enzyme exhibited a second order rate constant of 1.14 x 10^{-10} cm² mol⁻¹ s⁻¹. The specific activity (expressed as a pseudo-first order rate constant) as measured under standard conditions of 5 mM tripropionin, 400 mg of beads and pH 7.5, was 3200 s⁻¹.

Colipase—Porcine pancreatic colipase was obtained from the Sephadex G-100 column employed in purification of the lipase. As described by Maylite et al. (13) colipase eluted well behind the lipase peak. The active fractions were combined and brought to 90% saturation with ammonium sulfate. The resulting precipitate was dissolved in deionized water and used without further purification. This protein was homogeneous as judged by polyacrylamide gel electrophoresis at pH 8.9 (11).

Colipase activity was measured as the ability to relieve the bile salt-induced inhibition of the hydrolysis of a 0.1 M tributyrin emulsion by pancreatic lipase after the manner of Erlanson and Borgström (14). At pH 6.5 complete inhibition of lipase activity in this emulsion was obtained with 3 mM taurodeoxycholate. Protein concentration was determined from absorbance using E1% = 4.0 and a molecular weight of 10,000 (14). Under these conditions our preparation exhibited a second order rate constant of 1.1 x 10^{-10} s⁻¹.

Tripropionin—The preparation of the tripropionin (glycerol tripropionate) used in this study has been described previously (3). Solutions of tripropionin were prepared by adding the triester to vigorously stirring 1 M potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl at 24°C. Stirring was maintained for at least 10 hours to ensure complete dissolution of the substrate. During this time negligible hydrolysis was observed.

Trubtyrin—Reagent grade tributyrin (glycerol tributanolate, Eastman) was washed with dilute sodium bicarbonate and water to remove traces of butyric acid and was dried over calcium sulfate. Tributyrin emulsions were prepared immediately prior to use by adding the substrate to 2 M Tris buffer, pH 0.5, 1 M CaCl₂, 0.15 M NaCl, and vigorously shaking the mixture.

Other Reagents—Sodium taurodeoxycholate (>98% pure) was obtained from Calbiochem and rhodamine 6G from Gallard-Schlesinger. All other chemicals were reagent grade.

Glass Beads—Class VA unaspheres, ~230 to 270 mesh, 98% true spheres were obtained from Cataphe Division, Ferro Corporation, Jackson, Miss. From the reported density and average radius, the surface area was calculated to be 428 cm². The beads were washed with hot detergent and water and rinsed thoroughly with hot tap water. Small particles of paramagnetic material were removed with a magnet during the rinse cycles. The washed beads were soaked in chromic acid cleaning solution for 15 min and rinsed thoroughly with deionized water until the specific resistance of the rinse solution was approximately 1 megohm cm. After air drying at room temperature the beads were coated with Dri-Film SC-87 siliconizing reagent ( Pierce Chemical Co.) in redistilled hexane to a surface density of 2.4 x 10⁻⁹ g cm⁻² as previously described (3). This procedure was modified in that a much larger batch of beads was coated in a single treatment and that the excess solvent was allowed to evaporate without heating.

Measurement of Hydrolysis—The rate of tripropionin hydrolysis was measured by monitoring proton release with a pH-stat (Radiometer) as described by Brockman et al. (3). A stirring speed of 1000 rpm, a pH of 7.5, and a reaction temperature of 25°C were maintained unless noted otherwise. The air space in the reaction vessel was purged with water-saturated nitrogen. Four hundred milligrams of bead phase. The Vmax was determined in each assay. This corresponds to 171 cm² of surface, an amount calculated to adsorb greater than 93% of the lipase present at pH 7.5 (3). Increasing the surface area by 50% did not affect significantly either the rate of hydrolysis or the rate of lipase inactivation. Prior to addition of enzyme the beads were stirred for 20 min with an aliquot, usually 3.0 ml, of the tripropionin solution described above. By the end of the preincubation, the negative drift rate caused by the beads was relatively small and constant. All initial velocities were corrected for this blank rate. When used, taurodeoxycholate was added at the start of the preincubation and colipase was added after 15 min. The pH was adjusted to the desired value just before the reaction was initiated by the addition of lipase.

Measurement of Lipase in Bulk Phase—An assay was started in the sample vessel as described above. When maximum velocity was attained, an aliquot, usually 1.5 ml, was withdrawn with a pipette and transferred to a second vessel. The tip of the pipette was placed in a nitrogen atmosphere to prevent transfer of beads. In each case, the second vessel contained 400 mg of beads, sufficient substrate solution to give a final volume of 3.0 ml, taurodeoxycholate to give a final concentration of 1.0 mM and an excess of colipase. After the transfer, the rate of proton release (dP/dt) increased to a constant value. By adding known amounts of lipase it was determined that this velocity was proportional to the amount added.

Measurement of Critical Micelle Concentration—The critical micelle concentration of taurodeoxycholate was determined by measuring the shift in the absorbance maximum (λmax) of rhodamine 6G according to the method of Carey and Small (15) with the following changes. The dye was added to the 5 mM tripropionin solution described above to give a final concentration of 9.6 x 10⁻⁴ M. The higher concentration was necessary because a significant amount of the dye was adsorbed to the bead surface. Three milliliters of this dye solution and an aliquot of bile salt were added to 400 mg of beads. After stirring for 20 min at 25°C, an aliquot of the bulk phase was removed and λmax measured with a Beckman DB-G spectrophotometer. In the absence of bile salt, tripropionin had no effect on λmax of the dye. From surface excess values measured at the air-water interface (16), it was calculated that the amount of taurodeoxycholate adsorbed by the beads should be negligible relative to that in the bulk phase. Under these conditions the spectral transition, i.e. micelle formation, occurred over the same range as that observed by Carey and Small (15) in the absence of tripropionin (see Fig. 1, triangles, of Ref. 9 for our data). Using the method which they describe, the critical micelle concentration was determined to be about 1.5 mM.

RESULTS

In the absence of either bile salts or colipase, emulsions of tributyrin are readily hydrolyzed by porcine pancreatic lipase (5). Following the addition of enzyme to the substrate emulsion, the velocity of the reaction is initially high and then falls rapidly to zero (6, 17). Fig. 1 shows that similar kinetic behavior is observed in the hydrolysis of tripropionin by pancreatic lipase in the presence of siliconized glass beads. The short lag, <30 s, at the beginning of the reaction reflects the time required for the enzyme to be adsorbed to the beads and has been described previously in detail (3). In the short time scale of the earlier study the rate of hydrolysis, referred to as a "steady state velocity," was essentially constant following the acceleration. Over the much longer time scale of Fig. 1, this velocity is more properly referred to as an initial velocity, Vc = (dP/dT)ₐ, in mol s⁻¹. It was found previously (3) and verified in this study that this velocity was proportional to Eₐ, the
If Equation 6 is evaluated at $t = \infty$, i.e. $P = P_c$, and Equation 6 is subtracted from this result one obtains

$$P_\infty - P = \frac{k_e E_o}{k_i} e^{-k_i t}$$

Equation 7 predicts that a plot of $\ln (P_m - P)$ versus $t$ should be linear with a slope of $-k_i$ and an intercept, $I$, of $\ln ((k_e/k_i) E_o)$. The inset to Fig. 1 shows that such a plot is linear, indicating the agreement of the data with the proposed model for the inactivation process. In this and all subsequent determinations of $k_i$, the reaction was first order until at least 80% of $E_o$ was consumed.

The dependency of $k_i$ and $e'$ on $E_o$ was also examined. Fig. 2 shows that in agreement with Equation 7, the value of $k_i (3.5 \pm 0.4$ (S.D.) $\times 10^{10}$ s$^{-1}$) is independent of $E_o$, while $e'$ is directly proportional to $E_o$. The slope of this latter line, $k_i/k_o$, was 6.4 $\pm$ 0.3 (S.D.) $\times 10^9$ which, when multiplied by the average value of $k_o$, gives $k_o = 3.5 \pm 0.4$ (S.D.) $\times 10^9$ s$^{-1}$.

For comparison, $k_o$ was directly determined from initial velocity measurements. Because of the acceleration, only an approximate value for $V_o$ can be measured graphically. To make all our $V_o$ data comparable, this measured value for $V_o$ was corrected to $t = 0$ by using the value of $k_o$ determined during the assay. In no case was the correction more than 10%.

All reported velocities obtained under inactivating conditions were corrected in this manner. As predicted from Equation 1 a plot (not shown) of $V_o$ versus $E_o$ over the range of 0 to 3.2 pmol was linear with a coefficient of correlation of 0.99. The slope, $k_o$, was 8.2 $\pm$ 0.1 (S.D.) $\times 10^9$ s$^{-1}$. The agreement of this value with that calculated from the data in Fig. 2, further supports the proposed first order conversion of the adsorbed enzyme to an inactive form.

The results obtained above do not distinguish between the various mechanisms by which loss of activity may occur. However, the first order nature of the process, as well as the observation that inactivation is complete and apparently irreversible, suggests that the inactivation reflects the denaturation of the enzyme at the interface. Since protein denaturation usually involves large changes in entropy, we measured the thermodynamic parameters for the inactivation process. Fig. 3 shows an Arrhenius plot for $k_i$ over the range from 20-40$^\circ$. The linearity of this plot (coefficient of correlation = 0.99) shows that in this temperature range the inactivation is governed by a single rate-determining step. From the slope of the line the energy of activation was calculated to be 33.9 kcal mol$^{-1}$. Assuming the applicability of transition state theory,
values for \( \Delta G^\circ \), \( \Delta H^\circ \), and \( \Delta S^\circ \) can be calculated (18). At 25\(^\circ\)C the values obtained, 21 kcal mol\(^{-1}\), 33 kcal mol\(^{-1}\), and 42 cal mol\(^{-1}\) deg\(^{-1}\), are in good agreement with those from thermal denaturation studies of other proteins with a similar molecular weight (19). This agreement and, in particular, the large entropy change, support the hypothesis that the observed inactivation reflects denaturation of the enzyme at the interface.

The results of the preceding experiments show that the parameters \( V_0 \) and \( k_t \) can be considered measures of the catalytic activity and lability of the surface-bound enzyme. Because previous studies have indicated that both of these parameters may be affected by bile salts, and colipase, the apparent specific activity (i.e. \( V_0/E_0 \)) and \( k_t \) were measured in the presence and absence of these effectors. For each set of conditions a pH profile was determined (Figs. 4 and 5). The level of bile salt, 1 mM, was that which was sufficient to cause complete inhibition of lipolysis in the absence of colipase.

Colipase itself was employed in a 15-fold molar excess and addition of more of this cofactor did not enhance its effects. In the absence of added cofactors (Fig. 4, circles), the apparent specific activity exhibits a bell-shaped pH profile with graphically determined \( pK_a \) values of 5.5 and 9.3. If, as suggested by the data, enzymatic activity is exhibited only by the species containing one protonated (\( pK_a = 9.3 \)) and one deprotonated (\( pK_a = 5.5 \)) group, a theoretical curve (Fig. 4, solid line) can be generated readily. The other curves in this figure, obtained with added colipase (squares) and with colipase plus bile salt (triangles), were treated in the same manner and the resulting parameters are summarized in Table I. Within experimental error, the \( pK_a \) values are similar, although a slight narrowing of the profile may occur when colipase and bile salt are both present. These values are consistent with those obtained using emulsion assay systems (17, 20, 21). Also in agreement with earlier work is the observation that the optimum pH, excess colipase alone stimulates the velocity approximately 1.4-fold whereas colipase plus bile salt gives a value approximately equal to that obtained without cofactors.

In the absence of cofactors, the pH dependence of \( k_t \) shows an inverse relation to that for the apparent specific activity and gives, within error, the same \( pK_a \) values (Table I and Fig. 5, circles). Generation of a theoretical curve (Fig. 5, solid line) required graphical estimation of the maximum and minimum values of \( k_t \) as well as measurement of the \( pK_a \) values. This assumes that all three enzyme species denature unimolecularly but with different intrinsic rate constants.

With colipase, or bile salt, or both, present, \( pK_a \) values could not be measured accurately from inactivation data. In particular, with both cofactors present, we could not obtain \( k_t \) values at high pH because of an unusually long lag period. This phenomenon has been previously observed (8) and is discussed in detail in the accompanying paper. In any case, the available data suggest that the cofactors do not significantly alter the \( pK_a \) values. This fact coupled with the identity of the \( pK_a \) values from Figs. 4 and 5 suggests that both the catalytic activity and the rate of denaturation of the adsorbed active enzyme are dependent upon the same two ionizable groups, i.e. that the active lipase species is also the most stable.

Fig. 5 also shows that the addition of colipase increases the stability of the lipase 2- to 3-fold. If bile salt is also present, the enzyme is, within the limits of our measurement, completely stable between pH 6.5 and 7.5. Because pH 7.5 is also at or near the optimum value of the specific activity (Fig. 4), all subsequent experiments were performed at this pH.

In the preceding experiments, the observed rates of catalysis and inactivation were dependent upon the presence of the siliconized glass surface. This implies that the lipase species being monitored are located at the bead-water interface. However, only in the absence of cofactors and at pH 7.5 is the enzyme known to be adsorbed (3). To determine the relationship between the cofactor effects and the location of the lipase we measured the fraction of lipase in the bulk phase at pH 7.5 in presence and absence of cofactors. The transfer procedures employed are described under "Experimental Procedure." From the amount of lipase added to the sample vessel and the amount transferred to the second vessel, the percentage of the lipase in the bulk phase of the sample vessel was readily calculated. The percentage of lipase on the bead surface was then calculated to be 100 minus the percentage transferred. In each calculation the value of \( E_0 \) employed was corrected to account for any denaturation which occurred between addition of the lipase to the sample vessel and the subsequent transfer. The bile salt concentrations employed were 0, 0.3, and 0.8 mM. At 0.3 mM, the surface excess of bile salt should be near its maximum value (16, 22) and 0.8 mM is just below its critical micelle concentration (9). Values for the apparent specific activity and rate constant for lipase inactivation were also determined at these concentrations.

Table II shows the parameters \( E_0 \), \( V_0/E_0 \), and \( k_t \) expressed as a percentage of the values obtained in the absence of cofactors. The data show that taurodeoxycholate inhibited the apparent

<table>
<thead>
<tr>
<th>Components</th>
<th>Measured parameter</th>
<th>( pK_a ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>( V_0/E_0 )</td>
<td>5.5, 9.3</td>
</tr>
<tr>
<td>Lipase</td>
<td>( k_t )</td>
<td>5.6, 9.2</td>
</tr>
<tr>
<td>Lipase + colipase</td>
<td>( V_0/E_0 )</td>
<td>5.7, 9.5</td>
</tr>
<tr>
<td>Lipase + colipase + bile salt</td>
<td>( V_0/E_0 )</td>
<td>6.2, 8.4</td>
</tr>
</tbody>
</table>
specific activity of the adsorbed enzyme in a concentration-dependent manner, both in the presence and absence of colipase. However, colipase significantly decreased the extent of this inhibition, and in the absence of bile salt, colipase stimulated the specific activity by approximately 50%.

The effect of taurodeoxycholate on \( k_i \) is even greater than on specific activity; at 0.3 mM this rate constant was reduced by \( \beta \)-fold. Alone, colipase causes a smaller, but significant decrease in \( k_i \). When both cofactors were present, \( k_i \) was smaller than with either one alone.

The most interesting of our observations are the effects of cofactors on the adsorption of the enzyme. At 0.3 mM taurodeoxycholate, where large changes in \( V_{Ad} / Eo \) and \( k_i \) are already apparent, the enzyme was essentially all adsorbed. However, increasing the bile salt concentration to 0.8 mM dramatically reduced the amount of enzyme at the interface. Colipase acted to reverse this inhibition. As the table shows, the net result of having excess colipase and 0.8 mM bile salt was to maintain the enzyme in an active, stable configuration at the interface.

**DISCUSSION**

In our system the inhibitory effects of 1.0 mM taurodeoxycholate (Fig. 4) on the apparent specific activity of added enzyme are virtually identical with those observed using emulsions of tributyrin (21). Also, we and others (7, 21) have observed that addition of saturating amounts of colipase stimulates rates of hydrolysis approximately 50% and that colipase is able to reverse the large inhibition produced by bile salt. Furthermore, the effects of the cofactors on our pH profiles for apparent specific activity are similar to those reported elsewhere (17). The \( pK_a \) values show only small shifts in the presence of colipase alone or colipase plus bile salt. These similarities indicate that our system yields results which are both qualitatively and quantitatively comparable to those obtained using emulsions of tributyrin.

Also similar to results obtained using other systems, is the observed inactivation of lipase (6, 17) which occurs under conditions of our assay. In the present case, however, we have been able to quantitate this loss of activity and show that it is a unimolecular reaction with a large positive entropy of activation. Such results strongly support the earlier suggestion that this loss of activity reflects denaturation of the enzyme at the interface (6).

In addition to inactivation and activity of the adsorbed lipase we were also able to measure the distribution of the enzyme between the bulk and surface phases. Taken together the three parameters obtained, \( k_i \), \( B_m \), and \( E_o \), allowed us to delineate and quantitate the effects of bile salts and colipase on lipolysis.

**In vivo**, two recognized functions of bile salts are to aid in the adsorption of fats (23) and to disperse fats for lipolysis (24). In addition, our data show that bile salts serve to maintain the activity of pancreatic lipase by preventing its denaturation at the interface. Since this latter effect occurs predominantly between 0 and 0.3 mM, it is probably related to the formation of a surface excess of charged taurodeoxycholate molecules at the substrate-water interface. The adsorption of bile salt to the surface will also decrease the surface concentration of substrate. Since the velocity of hydrolysis is proportional to this substrate concentration (3), the observed decrease in the apparent specific activity of the adsorbed enzyme is probably a result of simple, substrate dilution.

At higher concentrations, but still below its critical micelle concentration, taurodeoxycholate exerts another effect; it blocks the adsorption of lipase to the interface and hence prevents lipolysis. Colipase has apparently arisen to counteract this latter effect by enabling the enzyme to be adsorbed at high bile salt concentrations. Indeed, many studies have shown that colipase can reverse the bile salt induced "inhibition" of lipolysis even at concentrations of bile salt well above the critical micelle concentration. Our study shows that with both cofactors present in adequate amounts, lipase will remain at the substrate-water interface in an active and stable configuration.

**REFERENCES**

Cofactor Effects on Properties of Lipase B at an Interface

Effects of colipase and taurodeoxycholate on the catalytic and physical properties of pancreatic lipase B at an oil water interface.
W E Momsen and H L Brockman


Access the most updated version of this article at http://www.jbc.org/content/251/2/378

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/2/378.full.html#ref-list-1