On the Function of Bile Salts and Proteins as Cofactors of Lipase

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SUMMARY

Pancreatic lipase is rapidly and irreversibly inactivated at a hexadecane-water interphase. The unfolded protein is only very slowly, in the course of hours or days, released into the aqueous phase. Bile salts prevent the denaturation of the lipase. Bovine albumin also protects the enzyme. Denaturation of lipase occurs also at the interphase of the substrates, tributyrin or olive oil, and water. Kinetic studies show that taurocholate and albumin prevent but cannot reverse the unfolding of the enzyme. The accelerating effect that these agents have on lipolysis can be explained on this basis.

It is concluded that it is one of the functions of bile salts, and of protein cofactors, to protect the native structure of lipase and to keep the oil-water interphase free from blockage by unfolded proteins. Despite its preferential specificity for insoluble triglycerides, lipase is not better adapted to an existence at the oil-water interphase than other enzymes. The enzyme requires isolation from rather than binding to the hydrophobic moieties of its substrates, and it relies on the help of surfactants for the prevention of hydrophobic bonding and unfolding. This interpretation is in harmony with previous evidence for the lack of lipophilic bonding in the substrate-enzyme complex.

Proteins too are known to stimulate lipolysis. Bovine albumin accelerates the action of microbial lipases (9), and a porcine pancreatic lipase recently isolated by Maylie et al. (10), is a protein with the molecular weight of 10,000. We found (unpublished) that the digestive lipase of lobster, in a purified form, will not hydrolyze tributyrin unless a protein cofactor is present. Bovine albumin can replace this cofactor.

It has been generally assumed and often stated (1, 11, 12) that pancreatic lipase is an enzyme specifically acting at an oil-water interphase, and it has been implied that it is uniquely adapted for this function. The report of Entressangles and Desnuelle (13) on the lipolysis of micellar triacetin has thrown doubt on this concept, and I have been able to show (14) that lipase requires esters that are electrophilically activated, sterically unhindered, and hydrophobic, but not necessarily insoluble, and that there is no requirement for lipophilic bonding (i.e. hydrophobic bonding of paraffin chains) between substrate and enzyme (15).

The present study starts from the well known fact that enzymes (and proteins in general) absorb to interphases and are unfolded and denatured; this effect is a function of the interfacial tension (12, 10, 17). The experiments reported here show that porcine pancreatic lipase is irreversibly inactivated at a hexadecane-water interphase, and, more important, also at the interphase of its substrates, tributyrin and olive oil, and water, but that it can be efficiently protected by bile salts, even far below their physiological concentration, as well as by bovine albumin.

METHODS

Reagents—Tributyrin and hexadecane 99%, were obtained from Matheson Coleman and Bell, Cincinnati, Ohio; sodium deoxycholate by neutralising deoxycholic acid, purest grade, from Aldrich, Milwaukee, Wisconsin; sodium taurocholate, 95% pure by thin layer chromatography, from Calbiochem, Los Angeles, California; bovine crystalline albumin from Mann Research Laboratories, New York; and sodium dodecyl sulfate, purest grade, from Aldrich. Olive oil was purified by chromatography on neutral alumina oxide (18).

Porcine Pancreatic Lipase—This was prepared according to the method of Verger et al. (19), and was pure as judged by disc electrophoresis (two bands, lipase A and B). The specific activity when assayed against tributyrin was 10,500 μmoles of fatty acid released per min per mg of protein.

Assay of Lipase—The assay was carried out with tributyrin, 0.5 ml, in 15 ml of aqueous 0.1 M NaCl-0.005 M CaCl₂, at 37° and pH 8 by continuous titration of the liberated butyric acid
with 0.02 n NaOH with a Radiometer (Copenhagen) titrimeter and recorder at maximal stirring. Assays were reproducible within ±7%.

Incubations: Influence of Sodium Deoxycholate (Fig. 1)—Incubations were carried out in test tubes (18 × 150 mm), in 0.1 M NaCl, 0.005 M CaCl₂, 0.05 M Tris, pH 8; the total volume was 2.1 ml. If required, 0.2 ml of hexadecane was added and the contents were sonically emulsified (20 kc, 70 watts 1 min). Lipase was present in the amount of 100 to 500 units. Sodium deoxycholate was added as required. The tubes were shaken by a rotary shaker (Palo multipurpose shaker, Greiner Scientific, New York), stroke 1 cm, 300 times per min, at room temperature. Samples of 0.05 or 0.1 ml were taken for assays when desired.

Incubations: Influence of Sodium Taurocholate, Albumin, and Sodium Dodecyl Sulfonate (Figs. 2 to 5)—In these incubations, gum arabic was added to stabilize the emulsion, and the samples were less vehemently shaken. The test tubes (18 × 150 mm) contained 2 ml of 0.1 M NaCl, 0.02 M Tris, pH 8, 0.05 ml of 10% aqueous gum arabic, 0.1 ml of hexadecane. The mixture was sonically emulsified. Sodium taurocholate or albumin was added as a 1% solution after sonification; the volume of the NaCl-Tris solution had been correspondingly reduced. The tubes were shaken at room temperature in a horizontal shaker at 130 strokes of 2.7 cm per min.

Lipolysis of Tributyrin and Olive Oil (Figs. 6 to 9)—Lipolysis was performed as described in “Assay of Lipase”, above. Olive oil was added as 0.5 ml of a sonic emulsion of the oil (1 part) in 10% aqueous gum arabic (2 parts). The substrate concentrations are so large that the slopes of the titration curves very nearly represent the maximal velocities, \( V_{\text{max}} \).

**RESULTS**

Denaturation of Lipase and Protection by Deoxycholate—Fig. 1A shows the destruction of the lipase by shaking of its aqueous solution. The mechanism is the unfolding of the enzyme at the air-water interphase; denaturation at the glass-water interphase is probably not significant (12). Fig. 1B shows that denaturation at the oil-water interphase is much more rapid (because the surface is larger). After 2 min (the time passed between the mixing and the assay) an appreciable fraction of the enzymic activity is already lost. An equal fraction is lost during the next 1 to 2 hours; this is the turnover time of desorption of the unfolded protein as it is replaced by native enzyme at the interphase. Deoxycholate, at a concentration of \( 4.5 \times 10^{-3} \) M, effectively reduces denaturation (Fig. 1C) at the oil-water and also at the air-water interphase. After 2 min a slight apparent activation of the enzyme is noted.

Protection of Lipase by Taurocholate—Since the incubation tubes were shaken much less vigorously in these experiments, the denaturation at the air-water interphase (Fig. 2A) is much less severe. The unfolding at the oil-water interphase (Fig. 2B), on the other hand, is more dramatic because a much finer emulsion, stabilized with gum arabic, has been used. The loss of activity within the first 2 min is large; the further course of denaturation indicates that the turnover time of desorption is about 1 day in these experiments.

Taurocholate, at \( 3.6 \times 10^{-3} \) molarity, gives nearly complete protection. The apparent activation of the enzyme after 2 min is quite large. It must be remembered, however, that when the enzymic activity was determined some taurocholate was transferred from the incubation mixture to the assay mixture. Later experiments (Fig. 6) show how taurocholate accelerates lipolysis under the conditions of the assay. The ostensible activation disappears if lower bile salt concentrations are used, even though the enzyme is still completely protected (Fig. 3).

Fig. 3 shows that taurocholate becomes effective at concen-
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In incubation of lipase. Protecting effect of taurocholate (TC) at different concentrations. See “Methods.”

In incubation of lipase. Protecting effect of albumin on lipase. See “Methods.”


In incubation of lipase. Lipolysis, continuous titration of liberated fatty acids. Effect of taurocholate on the lipolysis of tributyrin. See “Methods.” One milliliter of 0.02 M taurocholate (TC), pH 8, added at TC.

In incubation of lipase. Effect of taurocholate (TC) on the lipolysis of olive oil. One milliliter of 0.02 M taurocholate, pH 8, added at TC.

Influence of Taurocholate on Hydrolysis of Tributyrin—The titration Curve A in Fig. 6 demonstrates the continuous decrease in the velocity of hydrolysis. If taurocholate (1.3 × 10⁻⁴) is present (c), the progressive loss of activity is appreciably retarded, so that the kinetics become almost linear for several minutes. (This is the reason for the apparently higher activity of lipase in the bile salt system, although the velocities at zero time are the same (5).) The essential result of these experiments appears in Curve B: if the deactivation is allowed to progress to a point (a), and taurocholate is then added, the activity proper for the taurocholate system at this time (as in Curve C) is not restored; enzymic activity has been irreversibly lost. However, further deactivation is for a time halted, and the velocity becomes linear. If taurocholate is added at a later stage (b), linearity is again achieved, but at a lower level of activity.

Olive Oil, Bile Salt, and Lipase—Deactivation proceeds faster with olive oil (Fig. 7A) than with tributyrin, probably because the olive oil has been sonically emulsified and stabilized with gum arabic and thus provides a much larger surface area than the tributyrin system. The sooner taurocholate is added (Fig. 7, B and C), the more activity is recovered and linearity achieved, but the activity is not restored to the level of the system that contained taurocholate from the start (Fig. 7, D).

Two minor features of the curves should be noticed. First, addition of taurocholate to B and C leads to a short burst (humps after TC) in the appearance of titratable fatty acids, a burst that is not found in the tributyrin system. This burst of about 1 × 10⁻⁴ moles cannot be due (as could be suspected) to an enzyme-bile salt interaction with release of titratable groups on the protein, since only about 10⁻⁴ moles of enzyme are present. A plausible explanation is the following. In an oil-water emulsion not all the long chain fatty acid is titratable; the apparent pK of the acids is much higher than that of soluble acids (5). If bile salts and fatty acids meet, the acids will be complexed and mixed micelles will be formed, the acids will become quasisoluble, and a change in the apparent pK will result; therefore the apparent burst of fatty acid release. There is no such effect with the soluble, short chain butyric acid.

The second feature is also peculiar to the olive oil and lacking in the tributyrin system. When taurocholate is added to the partially deactivated system (Fig. 7, B and C) a linear velocity is eventually reached which is somewhat higher than that...
expected from tangential extrapolation at the point of addition. There seems to be some genuine reactivation of the enzyme. This effect is best explained by the hypothesis of Benzonana (5) that long chain acids at the interphase inhibit the lipase but are removed by complex formation with bile salts. The gradual approach to linearity in Curves B and C (Fig. 7) fits in well with this proposal. However, the denaturation of the enzyme, which can be read as D minus C and D minus B at 5 min, is obviously much more important than the inhibition by long chain acids.

**Albumin as Cofactor in Lipolysis—**Albumin, 0.1 mg, added to an already partly deactivated tributyrin-lipase system (Fig. 8A) does not restore the original activity (as it shows in C) but conserves the remaining activity, as shown by the linear velocity after the addition.

A 10 times larger amount, 1 mg of albumin, has an inhibiting effect on lipolysis (Fig. 8B). Since albumin itself unfolds and blocks the interphase (12, 17), the experiment needs no further interpretation.

**Unfolding or Inhibition—**The titration curves of Fig. 9 demonstrate again that denaturation rather than inhibition leads to the decrease of activity with time in the olive oil system. In this interpretation lipolysis loses much of its mystique as an “interfacial reaction.” The interphase becomes a merely circumstantial, and incommodious, arena of performance for the enzyme. The distinction of lipase is not an affinity for lipids or interphases but its inability (contrary to other esterases or proteinases) (23) to penetrate the shield of organized water interphases.

The recently isolated “colipase” (10) invites some speculations. We find that the protein, albumin, protects lipase (Fig. 4) and stimulates lipolysis (Fig. 8) but blocks the interphase at higher concentrations (Fig. 8B). The colipase is reported to have a low molecular weight (10,000) and five disulfide bridges, and to be heat resistant. Such a protein may well be resistant to unfolding and provide protection for the lipase without ever blocking the interphase. I suggest, therefore, that the colipase acts primarily not on the enzyme but, like bile salts or albumin, on the interphase.

The present results and previous ones (14, 15) can be summed up to a consistent characterization of pancreatic lipase. The enzyme requires activated, unhindered, and hydrophobic esters for substrates, but no lipophilic bonding in the substrate-enzyme complex. Lipophilic bonding, in fact, unfolds and destroys lipase just like other enzymes. Since lipase normally acts at oil-water interphases, it must be protected by surface active agents that do not themselves denature the enzyme or block the interphase. Bile salts and proteid cofactors fulfill these conditions.

**REFERENCES**

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