Product Activation of Pancreatic Lipase

LIPOLYTIC ENZYMES AS PROBES FOR LIPID/WATER INTERFACES*

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During the action of pancreatic lipase and colipase on racemic 1,2-didodecanoylglycerol monolayers in the absence of bile salts, biphasic kinetics was observed under conditions of high lipid packing. Similar kinetics has earlier been reported using phospholipid-emulsified triolein droplets (Borgström, B. (1980) Gastroenterology 78, 954–986). These kinetics are characterized by a lag time $\tau_h$ dependent on products accumulation at the substrate/water interface. This lag time is differentiated from the previously described enzyme concentration independent lag time $\tau_i$ in systems of low lipid packing (Verger, R., Mieras, M. C. E., and de Haas, G. H. (1973) J. Biol. Chem. 248, 4023–4034). Both $\tau_i$ and $\tau_h$ reflect a rate-limiting step due to the slow enzyme penetration into the substrate interface.

The variation of $\tau_h$ under different conditions (change in pH and concentration of Ca²⁺, enzyme, bovine serum albumin, and lipolytic products) lead us to propose a model for the product activation during lipolysis. We will discuss the use of the pancreatic lipase-colipase system to probe the lipid packing of emulsified triglyceride particles and lipoproteins using $\tau_h$ as a reference value.

Triacylglycerol lipase (EC 3.1.1.3) catalyzes the hydrolysis of triacylglycerols to fatty acids and 2-monoglycerides (1). In the presence of bile salts, lipase has an absolute requirement for a small protein cofactor, colipase, to exert its catalytic function (for review see Refs. 2 and 3). It has been suggested that one role of colipase is to shift upwards the surface pressure range where lipase can penetrate its substrate (4).

A kinetic model for the penetration and reaction steps of lipolytic enzymes acting at lipid/water interfaces has been proposed (5). The kinetics of lipolysis is simply described by a reversible rate-limiting penetration step of the enzyme into the interface characterized by a lag time, $\tau_h$, before the steady state reaction is reached.

The in vitro hydrolysis of phospholipid-emulsified triolein particles, Intralipid, by pancreatic lipase in the presence of colipase has recently been shown to follow an unusual biphasic kinetics. An initially slow accelerating phase preceded a sudden phase of high hydrolytic rate. The duration of the low activity phase was shown to be dependent, among other factors, on the concentration of enzyme, fatty acids, and calcium ions in the system (6).

In order to get new insights into these unusual kinetics, we have adopted the monolayer technique which is a powerful tool in such type of investigations (7). We will report here kinetic observations of the action of the lipase/colipase system on highly packed lipid surfaces. We will discuss the results in view of earlier proposed kinetic models and also the possibility to probe natural lipid structures by the use of the lipase/colipase system.

MATERIALS AND METHODS

Lipids—Racemic 1,2-didodecanoylglycerol was purchased from Serdary Research Laboratories, Inc. (Ontario, Canada) and showed one major spot by thin layer chromatography. Egg-PC was purified from egg as described (8). Dl-C₂₀:PG was a gift from Dr. A. Slotboom (Utrecht, Holland). Monooeistin was synthesized in the laboratory (9). Oleic acid was from British Drug House and purified on an ion exchanger. Intralipid was a product from Vitrum (Stockholm, Sweden). It is available as an emulsion of 200 g of fractionated soybean triacylglycerol, 12 g of egg PC, and 25 g of glycerol/1000 ml. All other chemicals were of analytical grade.

Proteins—Porcine pancreatic lipase and procolipase were prepared as described previously (10, 11). Porcine pancreatic lipase B was purified according to published methods (12), with an additional Sephadex G-100 filtration at pH 9.2 in order to remove remaining colipase contaminations. Procolipase was radiolabeled with [¹²⁵I] using the triiodide method described earlier in the case of pancreatic PLA₂ (13). Approximately 1 atom of iodine/procolipase molecule was found to be incorporated. ¹²⁵I-Colipase was obtained by trypsin activation of ¹²⁵I-procolipase (11). The iodinated cofactors retained 50% of the original colipase activity at pH 6.5 in a tributyrin assay in the presence of taurodoxycholate.

Surface Barostat Techniques—Assays were performed in a special "zero-order" trough (14) drilled into a Teflon block. The reaction compartment contained 230 ml of solution with a total surface of 123 cm². The subphase of the reaction compartment was thermostatically maintained at 25 ± 0.5°C by circulating water in an immersed glass coil. The subphase was continuously agitated with one magnetic stirrer (250 rpm). 1,2-Dilaurin in CHCl₃ solution (±1 mg/ml) was spread over the entire subphase with a microliter syringe. Several minutes were allowed to pass for solvent evaporation. All injections into the subphase were performed behind a small Teflon bar fixed in the reaction compartment in order to avoid crossing the lipid film with the tip of the pipette.

Kinetics was recorded for 15–60 min. When required the film was then collected, and an aliquot of the bulk phase was sampled as described (15). When not otherwise stated each line of data shown in the figures and tables represents one experiment.

Aqueous Subphase—Before each experiment, the trough was cleaned with ethanol, rinsed several times with tap water, and finally

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§ The abbreviations used are: PC, phosphatidylcholine; di-C₂₀:PG, didodecanoylphosphatidylglycerol; di-C₂₀:G and rac-1,2-dilaurin, racemic 1,2-didodecanoylglycerol; PLA₂, phospholipase A₂ (EC 3.1.1.4).
rinsed with distilled water. The aqueous subphase was composed of 10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, and 1 mM CaCl₂. Distilled water was prepared from alkaline KMnO₄ in an all-glass apparatus. Residual surface-active impurities were removed before each assay by sweeping and suction of the surface.

pH Stat Technique—Lipolysis of the Intralipid emulsion was determined isothermally using a Mettler automatic titration system (Mettler Instruments AG, Zurich, Switzerland) as described (16). The incubation solution was composed of 0.5 ml of Intralipid emulsion in 10 ml of a 2 mM Tris-maleate buffer, pH 8.0, and 150 mM NaCl and 1 mM CaCl₂.

Rat thoracic duct chyle was kindly provided by Dr. Britta Larsson (Dept. of Physiological Chemistry, Lund). It was dialyzed against a 2 mM Tris-maleate buffer, pH 8.0, and 1 mM CaCl₂ overnight. Homogenized commercial cow milk containing 3% fat was from Skåne Mejerier.

RESULTS

Fig. 1 shows a plot of the enzyme activity versus surface pressure of pancreatic lipase acting on rac-1,2-dilaurin monomolecular film. As is seen, a sharp decrease in activity is observed in the pressure range of 30–33 dynes/cm. Such decrease in activity in a narrow surface pressure range is typical for enzymes acting at water/lipid interfaces (17). This surface pressure range is slightly shifted to higher values in the presence of colipase (18). A typical recording of the lipase hydrolysis of rac-1,2-dilaurin at a surface pressure of 28 dynes/cm is shown in Fig. 2A. This kinetic is characterized by a progressive increase in hydrolytic rate according to a first order process until a steady state reaction is reached. From the kinetic recording a lag time τ can be determined by extrapolation. This lag time reflecting the rate-limiting step in the adsorption of the enzyme has been shown to be inde-

![Figure 1](https://via.placeholder.com/150)

**Fig. 1.** The lipase activity as a function of surface pressure on rac-1,2-dilaurin monomolecular film. Experimental conditions: pancreatic lipase (24 pmoles), 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 25 °C.

![Figure 2](https://via.placeholder.com/150)

**Fig. 2.** Kinetic tracings of the hydrolysis of rac-1,2-dilaurin and a triolein emulsion by pancreatic lipase. A, kinetic tracing of the hydrolysis of rac-1,2-dilaurin monomolecular film by pancreatic lipase (24 pmoles) under isoionic conditions, 28 dynes/cm. The continuous curve is the tracing of the barostat recorder. The broken lines are computed according to Verger et al. (5). The intercept between the asymptote and the broken line axis is the lag time τ₀. B, kinetic tracings of the hydrolysis of rac-1,2-dilaurin monomolecular film by pancreatic lipase (0.9 nmoles) in the absence (lower graph) and presence of [125I]-colipase (8 nmoles) under isoionic conditions, 40 dynes/cm. The intercept between the broken lines gives the lag time τ₀. The dots () indicate the amount [125I]-colipase bound to the film. Experimental conditions were as in Fig. 1. C, kinetic tracing of the hydrolysis of a phospholipid-stabilized triolein emulsion by pancreatic lipase (0.2 µmole) in the presence of colipase (0.5 µmole). Experimental conditions: 100 mg of triolein emulsion in 10 ml of 2 mM Tris-maleate buffer, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 37 °C.

dependent of enzyme concentration (5) and will be labeled τ₀, lag time independent of enzyme concentration. Plotting the value (P – P⁰) as defined in Fig. 2A versus time in a semilogarithmic way gives a linear relationship indicative of the first order kinetics of the process (Fig. 3). In contrast, the (P – P⁰) versus time plot (open circles) derived from Fig. 2B is not linear. The linear semilogarithmic plot of the kinetics has been used as a criteria to define τ₀ in our experiments, both in the monolayer and in the emulsion system.

At a surface pressure of 40 dynes/cm the lipase activity is very low (Figs. 1 and 2B). In the presence of colipase, however, the reaction process is different (Fig. 2B) from what was observed previously at 28 dynes/cm (Fig. 2A). After a certain time of low hydrolytic rate a short accelerating phase leads to a high reaction rate. This sudden increase in hydrolytic rate is accompanied by a concomitant increase in colipase binding...
to the interface (Fig. 2B). By extrapolating the pseudo-steady state to the time axis, a lag time can be derived, which varies with pH and decreases with the addition of Ca\(^{2+}\) ions into the subphase (Fig. 4, A and B). Furthermore, a lag time decreases with increasing concentration of lipase, colipase (data not shown), and lipolytic products such as oleic acid and monolein and increases with addition of bovine serum albumin. As this lag time is dependent upon the factors influencing the presence of products in the interface, we will name it \(\tau_d\) lag time (product dependent). \(\tau_d\) has earlier been observed during the hydrolysis of phospholipid-stabilized triolein emulsions by pancreatic lipase (6). For the purpose of comparison the kinetics of such an experiment is shown in Fig. 2C. Table I summarizes the \(\tau_d\) values of the kinetics of lipase hydrolysis of rac-1,2-dilaurin monolayers and Intralipid emulsions in the presence of different additions. The dependence of \(\tau_d\) on the surface pressure of a rac-1,2-dilaurin monolayer substrate is shown in Table II.

Furthermore, biphasic kinetics is also observed during the hydrolysis of chylomicrons and milk fat globules (Table II) by pancreatic lipase.

![Semilogarithmic plot](image)

**Fig. 3.** Semilogarithmic plot of \((P - P^0)\), as defined in Fig. 2A, as a function of time at 28 dynes/cm (○) and at 40 dynes/cm (●). The data were obtained from the tracings in Fig. 2, A and B.

![Diagram](image)

**Fig. 4.** The lag time \(\tau_d\) as a function of pH and Ca\(^{2+}\). A, the lag time, \(\tau_d\), as a function of calcium concentration. In the absence of calcium no activation could be observed during the time course of the experiments. Conditions: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl\(_2\), Temperature, 25 °C. B, the lag time, \(\tau_d\), as a function of pH in the presence (●) and absence (○) of 1 mM CaCl\(_2\). Conditions were as above.

### Table I

| Addition                        | \(\tau_d\) Monolayer* | \(\tau_d\) Emulsion† |
|---------------------------------|------------------------|----------------------|
| 0.5 μM colipase                 | 19 min                 | 22 min               |
| 0.5 μM colipase + 10 mM Ca\(^{2+}\) | 10 min                 | 6 min                |
| 0.5 μM colipase + 4 mol % monolein | 3 min                  | 12 min               |
| 0.5 μM colipase + 4 mol % oleic acid | 0 min                 | 0 min                |
| 0.5 μM colipase + 0.1 μM bovine serum albumin | >120 min | 70 min |
| 0.5 M procolipase               | 50 min                 | 50 min               |

* Experimental conditions as in Fig. 2B.
† Experimental conditions as in Fig. 2C.

### Table II

| Substrate                       | Lag time, \(\tau_d\) |
|---------------------------------|----------------------|
| Monolayer*                      |                      |
| Rac-1,2-dilaurin                |                      |
| 33 dynes/cm                     | 0 min                |
| 35 dynes/cm                     | 9 min                |
| 37 dynes/cm                     | 17 min               |
| 40 dynes/cm                     | 19 ± 2 (n = 5)       |
| 41 dynes/cm                     | 41 min               |
| Emulsions†                      |                      |
| Intralipid                      | 47 min               |
| Chylomicrons                    | 37 min               |
| Milk                            | 30 min               |

* Subphase buffer: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl\(_2\). Temperature, 25 °C. 0.9 nm lipase, 8 nm colipase.
† Incubation buffer: 10 ml of 2 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl\(_2\). Temperature, 37 °C. 0.1 μM lipase, 0.2 μM colipase. The emulsion added was equal to 30 μmol of triglyceride.

On monomolecular films of stearic acid at 35 dynes/cm and in the presence of 1 mM CaCl\(_2\), 1.6 μg of \(^{125}\)I-colipase was bound/cm\(^2\). In contrast under the same conditions but in the presence of 1 mM EDTA no detectable colipase binding was observed.

In order to explain the difference in \(\tau_d\) between procolipase and colipase (Table I), binding studies were performed. The binding of \(^{125}\)I-procolipase and \(^{125}\)I-colipase to monolayers of egg-PC, di-C\(_{12}\)-PG, and rac-1,2-dilaurin (di-C\(_{12}\)-G) is shown in Fig. 5, A, B, and C. A difference in the binding of the two cofactors is observed on charged di-C\(_{12}\)-PG and zwitterionic egg-PC monolayer while on the electrically neutral rac-1,2-dilaurin monolayer film no difference was observed. Using three times less cofactor in the assay three times less was observed to bind to the monolayer film (data not shown).

Both procolipase and colipase are weakly tensioactive increasing the surface pressure less than 1 dyne/cm in the surface pressure range studied. Procolipase and colipase did not differ in this respect.

### DISCUSSION

**Pancreatic Lipase as a Probe for Lipid Interfaces—**The monolayer technique and the bulk titrimentical techniques have been used extensively in the kinetic studies of lipolytic enzymes. Although complementary in many aspects the two methods have some unique features. The fat emulsion is the natural form in which the substrate is found in vivo. This emulsion interface is composed apart from the glycerides of amphiphilic molecules partitioned between the water and lipid phase according to their tensioactive properties. It is,
Product Activation during Lipolysis—The well characterized fatty acid inhibition of lipolysis has been explained as an effect of product accumulation at the interface, making the substrate inaccessible to the enzyme (20). This effect was, however, observed when a substantial amount of substrate had been hydrolyzed. In the situation where such an inhibition was observed usually product acceptors (Ca²⁺, bovine serum albumin, bile salts) were not present. In the present situation of product activation only a small amount of the highly packed substrate has been hydrolyzed before the high hydrolysis rate was observed. As biphasic kinetics was not observed at a low surface pressure, a high lipid packing seems to be essential to observe these effects. We interpret this product activation as a consequence of an increased binding of colipase/lipase to the interface (Fig. 2B). This binding can be described by the use of the partition coefficient of colipase, \( k_p / k_d \), between the bulk and lipid phases. \( k_p / k_d \) is constant for a fixed composition of the interface and is characterized by a lag time, \( \tau_d \), described earlier (5, 14). On highly packed lipid/water interfaces, devoid of lipolytic products, colipase is first partitioned in favor of the bulk phase. However, as small amounts of products are formed and remain transitorily at the interface, the interfacial composition changes. This change in the interface increases the \( k_p \) of colipase, leading to an increase in lipase binding with a subsequent increase in product formation. The kinetics of product activation and thus, \( \tau_d \), is characterized by a time-dependent change of \( k_p \) due to a time-dependent change of the interfacial chemical composition. This change in \( k_p \) does not have to be linear with product concentration at the interface. A certain interfacial concentration level of products might induce a large change in \( k_p \).

The molecular interpretation of \( \tau_d \) can so far only be tentative. Our picture of the events taking place is summarized in Fig. 6. At a highly packed lipid/water interface colipase/lipase is adsorbed following a first order process characterized by a lag time \( \tau \). Time products are formed enhancing the lipase/colipase binding which will be reflected by an increase

\[ k_p \] penetration rate constant; \( k_d \), desorption rate constant. For details see Ref. 5.

However, impossible to determine the lipid composition, lipid packing, or surface pressure of such a lipid/water interface. Such parameters can be obtained with the monolayer technique.

In the present investigation we have observed lag times, \( \tau_d \), with the monolayer technique using rac-1,2-dilaurin at high surface pressure, originally found in the emulsion system of triolein-phospholipid particles. We have confirmed and extended the studies made with these emulsions and compared the effects of different additives on \( \tau_d \). As is seen in Table I there is a qualitative agreement between the two methods. From this correspondence it is reasonable to assume that the molecular basis responsible for the existence of \( \tau_d \) is the same in the two systems.

As \( \tau_d \) is not observed at low surface pressure (Fig. 2A) or in emulsion systems such as dispersed tributyrin in water (11), high lipid packing corresponding to high surface pressure thus seems to be a necessary condition to observe these types of lag times. The high surface pressure in a phospholipid-stabilized triolein emulsion is determined by the packing density of the phospholipid monolayer surrounding the triglyceride droplets. In the monolayer system, the surface pressure is regulated at will by the moving Teflon barrier.

Attempts have been made to determine the surface pressure in biomembranes by comparing the hemolysis of red blood cells by the enzymatic activity of different phospholipases against phospholipid monolayers at different surface pressure (19). By analogy, \( \tau_d \) in the rac-1,2-dilaurin system can be used to probe the surface pressure in emulsion systems using lipase/colipase as analytical tools, as exemplified here using chylomicrons and milk fat globules as lipase substrates (Table III). From the present data it is not possible to evaluate the absolute surface pressure value at the interface of an emulsion. However, a value larger than 33 dynes/cm seems reasonable as \( \tau_d \) is observed above this surface pressure value. As an extension of this study it is possible to use the lipase/colipase monolayer system to further investigate the surface properties of lipoproteins using \( \tau_d \) as a reference.

Product Activation during Lipolysis—The well characterized fatty acid inhibition of lipolysis has been explained as an effect of product accumulation at the interface, making the substrate inaccessible to the enzyme (20). This effect was, however, observed when a substantial amount of substrate had been hydrolyzed. In the situation where such an inhibition was observed usually product acceptors (Ca²⁺, bovine serum albumin, bile salts) were not present. In the present situation of product activation only a small amount of the highly packed substrate has been hydrolyzed before the high hydrolysis rate was observed. As biphasic kinetics was not observed at a low surface pressure, a high lipid packing seems to be essential to observe these effects. We interpret this product activation as a consequence of an increased binding of colipase/lipase to the interface (Fig. 2B). This binding can be described by the use of the partition coefficient of colipase, \( k_p / k_d \), between the bulk and lipid phases. \( k_p / k_d \) is constant for a fixed composition of the interface and is characterized by a lag time, \( \tau_d \), described earlier (5, 14). On highly packed lipid/water interfaces, devoid of lipolytic products, colipase is first partitioned in favor of the bulk phase. However, as small amounts of products are formed and remain transitorily at the interface, the interfacial composition changes. This change in the interface increases the \( k_p \) of colipase, leading to an increase in lipase binding with a subsequent increase in product formation. The kinetics of product activation and thus, \( \tau_d \), is characterized by a time-dependent change of \( k_p \) due to a time-dependent change of the interfacial chemical composition. This change in \( k_p \) does not have to be linear with product concentration at the interface. A certain interfacial concentration level of products might induce a large change in \( k_p \).

The molecular interpretation of \( \tau_d \) can so far only be tentative. Our picture of the events taking place is summarized in Fig. 6. At a highly packed lipid/water interface colipase/lipase is adsorbed following a first order process characterized by a lag time \( \tau \). With time products are formed enhancing the lipase/colipase binding which will be reflected by an increase

\[ k_p \] penetration rate constant; \( k_d \), desorption rate constant. For details see Ref. 5.
in lipolytic activity. It has been reported that fatty acids might form small clusters of 10-20 molecules in lipid bilayers and monolayers (21). Furthermore, fatty acid clusters are stabilized by the presence of calcium ions, and the size of the clusters was found to be 50-60 Å (22). The marked increase in binding of colipase to a monolayer of stearic acid in the presence of calcium as compared to that in the absence of calcium is compatible with the formation of such clusters of fatty acid-calcium soaps at the interface. At the boundaries of these clusters discontinuities might exist toward which colipase might have a higher affinity (Fig. 6). The better penetrating capabilities of colipase as compared to procolipase toward charged and zwitterionic interfaces can explain the observed differences in $\tau_d$ in the presence of either of the two proteins (Table II). Although no difference in binding was observed on pure rac-1,2-dilaurin monolayer film (Fig. 5C), the fatty acid and fatty acid-calcium soaps formed during the hydrolysis might induce a change in the charge properties of the surface and thus increase the binding of the cofactors and preferentially colipase.

Bovine serum albumin which is known to bind fatty acid and monoglycerides will cause a fast desorption of products from the interface and consequently the disappearance of the clusters and thus an increase in $\tau_d$. This effect is not due to competition of albumin with colipase for the interface as no albumin is absorbed to the interface at 40 dynes/cm (34).

The $\tau_d$ is apparently not only observed during the action of pancreatic lipase-colipase on highly packed lipid structures. Quarles and Dawson demonstrated that phospholipase D acted in a similar manner on phosphatidylcholine monolayers at high surface pressure. The long lag times observed could be decreased by addition of phosphatidic acid calcium ions (23). Hirawawa et al. (35) further showed that the activity of phospholipase C from bovine is enhanced by the addition of charged amphiphiles to the substrate.

Roholt and Schlamowitz studied the action of phospholipase A$_2$ from Crotalus durissus terrificus. $\tau_d$ was observed when dimyristoyl lecithin was used as substrate and could be decreased by the presence of myristoyl lysolecithin (24).

More recently Upreti et al. showed that the hydrolysis of phospholipid bilayers by bee venom phospholipase A$_2$ is enhanced by the presence of different alkanols in the bilayer due to the free space introduced in the bilayers where the enzyme acts (25). Upreti and Jain further demonstrated that the hydrolysis of PC bilayers by bee venom PLAs proceeds according to a biphasic kinetic (26).

Tinker and Wei have presented a model to describe the biphasic kinetics of Crotalus atrox PLAs activity on phospholipids in the gel phase. However, in contrast to our explanation the authors introduced the postulate of product-stimulated desorption of the enzyme to account for the dramatic stimulation of the reaction induced by both lyso-PC and fatty acids (27). These effects could, however, as well be explained by our model of product activation developed for the lipase-colipase system. The answer to this contradictory interpretation could be given by direct binding studies of the Crotalus atrox PLAs during the lag phases as previously reported by Pattus et al. in the case of the pancreatic PLAs (28).

The physiological relevance of the product activation of the pancreatic lipase/colipase lipolysis is evident. The dietary
glycerides are already hydrolyzed up to 30% by the action of the lingual lipase in the stomach (29–33). The surface of the triglyceride emulsion is thus "activated" by these fatty acids enhancing the binding of colipase and the initiation of duodenal lipolysis.

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