Articles

Effects of Colipase and Bile Salts on the Catalytic Activity of Human Pancreatic Lipase. A Study Using the Oil Drop Tensiometer

Sandrine Labourdenne,[‡,§] Olivier Brass,[‡,§] Margarita Ivanova,[‡,||] Alain Cagna,[§] and Robert Verger*[,‡]

UPR 9025 de l'IFR1 du CNRS, Laboratoire de Lipolyse Enzymatique, 31 Chemin Joseph-Aiguier 13402 Marseille Cedex 20, France, Biophysical Chemistry Laboratory, University of Sofia, James Bourchier str. 1, 1126 Sofia, Bulgaria, and Society Interfacial Technology Concept, Parc d'activité Chancolan, 69770 Longessaigne, France

Received June 5, 1996; Revised Manuscript Received December 17, 1996[⊗]

ABSTRACT: Using the oil drop technique, we studied the effects of colipase and bile salts on the rate of hydrolysis of soybean oil by human pancreatic lipase (HPL) as well as on the interfacial binding. Upon continuously recording the decrease in the interfacial tension with time, a 10−15-fold increase in the HPL activity was found to occur in the presence of colipase. The catalytic rate constants of hydrolysis measured at the oil drop surface were found to be of the same order of magnitude as those obtained with monomolecular films spread at the air−water interface. Biotin-labeled HPL (HPL*) was used to determine the amount of adsorbed enzyme using an ELISA test. Less than 1% of the total amount of injected HPL* molecules was found to have adsorbed to the oil−water interface, and no significant effects of colipase on HPL* binding were observed. No significant changes in the hydrolysis rates or the binding of HPL* were detected in the presence of bile salts at concentrations ranging from below their critical micellar concentration (CMC) up to 100 μM. At the oil−water interface, in the absence or presence of bile salts below their CMC, it can be concluded that the colipase is a true lipase cofactor, *i.e*, it increases the enzyme turnover (approximately 10−15-fold) and does not affect the interfacial lipase adsorption.

Human pancreatic lipase (HPL;[1] Triacylglycerol hydrolase; EC 3.1.1.3) is the main lipolytic enzyme involved in the digestion of dietary fat and amounts to 3% of the total proteins secreted by the exocrine pancreas (Verger, 1984; Desnuelle, 1986). HPL is directly secreted in the form of an active 50 kDa enzyme, unlike most of the pancreatic enzymes, which are secreted in the form of proenzymes and are further activated by proteolytic cleavage in the small intestine.

HPL hydrolyzes primary ester bonds of tri- and diglycerides thus generating 2-monoglycerides and fatty acids. This enzyme is active in the neutral and alkaline pH range, in the presence of a nonenzymatic pancreatic cofactor, the colipase which specifically anchors HPL in the presence of bile salts (Chattoraj & Birdi, 1984; Desnuelle, 1986; Erlanson-Albertsson, 1992). Bile salts are tensioactive physiological compounds which are to be found, at concentrations above their CMC, in the small intestine during digestion. They inhibit the action of pure pancreatic lipase *in vitro*. To counteract this effect, colipase facilitates the adsorption of lipase to bile salt-covered lipid−water interfaces and consequently reactivates HPL (Borgström & Erlanson-Albertson, 1971; Maylié et al., 1971; Morgan & Hoffman, 1971; Borgström & Erlanson-Albertsson, 1973; Maylié et al., 1973; Borgström, 1975, 1976; Chapus et al., 1975; Canioni et al., 1976; Momsen & Brockmann, 1976; Vandermeers et al., 1976). Investigations on the structure−function relationships of HPL and colipase have provided evidence that colipase, once bound to lipid, anchors lipase to its substrate by forming a stable protein−protein association in a 1:1 molar ratio. Limited proteolysis studies have shown that only the C-terminal domain of pancreatic lipase may interact with the colipase (Mahe-Gouhier & Leger, 1988; Chaillan et al., 1992). This finding has been confirmed and further developed by determining the 3-D structure of the HPL−colipase complex (van Tilbeurgh et al., 1992; Egloff et al., 1995b). In the absence of lipids, the binding of colipase to the lipase molecule did not induce any conformational changes. The same group of authors have determined the 3-D structure of the lipase−colipase complex cocrystallized with mixed micelles of phosphatidylcholine and bile salts (van Tilbeurgh et al., 1993) or in the presence of phosphonate inhibitors (Egloff et al., 1995a). The lid, a surface helix covering the catalytic triad of lipase in its closed form, adopts a totally different conformation in the presence of lipids. The open lid becomes an essential component of the active site and interacts with colipase forming the lipid−water interface binding site, which is more than 50 Å in length and has an area of approximately 750 Å^2. De la Fournière et al. (1994) have studied the tensioactivity of HPL and colipase and have observed that the HPL exhibited a comparable degree of

[‡] Laboratoire de Lipolyse Enzymatique.
[§] Society Interfacial Technology Concept.
[||] University of Sofia
[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.
[1] Abbreviations: HPL, human pancreatic lipase; HPL*, biotinylated human pancreatic lipase; ELISA, Enzyme Linked Immunosorbent Assay; TBS, Tris Buffer Saline: 150 mM Tris-HCl buffer (pH 7.4), 140 mM NaCl, and 3 mM KCl; NaTDC, Sodium Taurodeoxycholate; $\gamma_{o/w,}$ oil−water interfacial tension (mN m^{-1}); SA, specific activity (μmol min^{-1} mg^{-1} or U mg^{-1}). β-CD, β-cyclodextrin; CMC, critical micellar concentration.

S0006-2960(96)01331-1 CCC: $14.00

tensioactivity whether or not a phospholipid monolayer was present at the interface. These authors have reported that the colipase considerably increased the initial adsorption rate and the final surface pressure reached by HPL in comparison with its adsorption without colipase. The effects of colipase have therefore been attributed to the large hydrophobic plateau formed at the surface of the HPL−colipase complex (van Tilbeurgh et al., 1993).

Lipase activity has usually been determined on substrates organized at lipid−water interfaces such as micelles, emulsions, monomolecular films spread at the air−water interface, or oil drops. The main advantage of the monolayer technique over bulk methods is that it can be used to monitor and control several physicochemical parameters of the interface, such as the surface pressure (interfacial free energy), the molecular area of the substrate, and the surface excess of the water-soluble lipases (Verger, 1980; Ransac et al., 1990; Aoubala et al., 1995b). With the monolayer technique synthetic medium acyl chain glycerides are generally used as the substrate because they can form stable films at the air−water interface and their lipolytic products are rapidly desorbed and solubilized into the aqueous phase (Verger & de Haas, 1973). Recently, Laurent et al. (1994) and Ivanova et al. (1996) described the use of a water soluble nontensioactive cyclic heptaglucose, β-CD, to trap into the water subphase the lipolytic products generated upon the hydrolysis of monomolecular films of long-chain (phospho)glycerides. Another method was the oil drop technique adapted by Nury et al. (1987) for monitoring the lipase hydrolysis of natural long-chain triacylglycerol. The oil−water interfacial tension variations versus time ($d\gamma/dt$) were recorded using the oil drop tensiometer in order to accurately monitor the lipase activity (Labourdenne et al., 1994). The main advantage of the oil drop tensiometer over the monolayer technique is that it can be used to monitor the lipase activity on natural long-chain triacylglycerol, at a closely controlled oil−water interface.

The aims of the present study were three-fold: (1) Determine the amount of adsorbed HPL*, using an ELISA test to estimate the specific activity at the oil−water interface. (2) Study the effects of colipase and bile salts on the HPL activity and HPL* interfacial binding. (3) Compare the enzymatic kinetic constants recorded with the monolayer and the oil drop techniques.

MATERIALS AND METHODS

Lipids. The edible soybean oil used was marketed by the company Lesieur and purified by the company Transbiotec (Axone, 69930 Saint-Clément-Les-Places, France) on a column of silicic acid (Merck) equilibrated in hexane−ethyl ether. NaTDC and β-CD were obtained from Sigma and used with no further purification. Dilaurin (1,2-didodecanoyl-*sn*-glycerol), Diolein (1,2-di[*cis*-9-octadecenoyl]-*sn*-glycerol) were from Sigma. Dicaprin (1,2-didecanoyl-*sn*-glycerol)was from SRL.

Proteins. HPL and porcine pancreatic colipase were purified at the laboratory by Youssef Gargouri and Josiane de Caro, using methods described in the literature (Maylié et al., 1973; de Caro et al., 1977; Chapus et al., 1981). Polyclonal antibodies against native HPL were prepared at the laboratory by Aoubala et al. (1995a).

Oil Drop Method. The kinetics of hydrolysis were recorded with the Oil Drop Tensiometer (ITConcept-France), based on the principle of the method described by Nury et al. (1987). A drop of purified soybean oil (volume 50 μL) was delivered using a microsyringe (Exmire) through a U-shaped stainless steel needle (external diameter 2 mm) into an optical glass cuvette measuring 1 × 2 × 4.3 cm (Hellma-France) thermostated at 25 °C, containing 5 mL of an aqueous phase composed of a 10 mM Tris-HCl buffer (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA, and stirred continuously with a small magnetic bar. The lipase and colipase samples were injected into the aqueous phase (using Hamilton syringe) after the formation of a drop. The interfacial tension was measured by automatically analysing the oil drop profile on-line, using the Laplace equation (Grimaldi et al., 1991; Labourdenne et al., 1994).

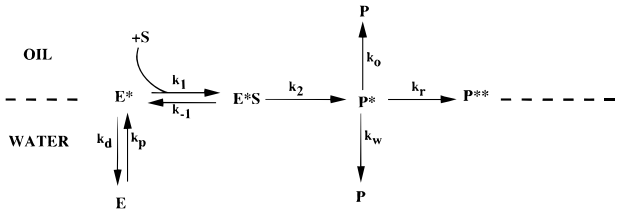

FIGURE 1: Adaptation of the Michaelis−Menten kinetic scheme describing interfacial catalysis at the oil−water interface. For definitions and abbreviations see Materials and Methods.

Monolayer Technique. The monomolecular films were formed by spreading a lipid chloroform solution (approximately 1 mg mL^{-1}) at the air−water interface. The Wilhelmy method (Wilhelmy, 1863) was used to measure the surface pressure, using a thin platinum plate attached to the beam of an electromicrobalance. Enzymatic activities were determined at constant surface pressure using the barostat technique (Verger & de Haas, 1973) with a "zero order" trough composed of a reaction compartment (volume 120 cm^3; surface 100 cm^2) and a reservoir compartment (length 24.8 cm; width 14.8 cm). In all the experiments the subphase was composed of a 10 mM Tris-HCl buffer (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA.

pH-Stat Technique. The lipase activity (HPL at 1.5 μg mL^{-1} final concentration) was measured titrimetrically on purified soybean oil as the substrate at pH 8 and 37 °C using the pH-stat (TTT 80 Radiometer) under the following nonstandard assay conditions: 500 μL of soybean oil mechanically emulsified in 10 mM Tris-HCl buffer (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA in the presence or absence of 5 mM NaTDC and in the presence or absence of colipase (lipase−colipase, ratio molar 1/5). The total volume assayed was 15 mL.

Kinetic Model of Enzymatic Hydrolysis of Long-Chain Lipids at the Oil−Water Interface. The adapted version of the Michaelis−Menten kinetic scheme for use at the air−water interface has been described and used by previous authors (Verger & de Haas, 1976; Verger, 1980; Jain & Berg, 1989; Ransac et al., 1991). In the case of the hydrolysis of monolayers of medium-chain lipids, it was experimentally checked that the lipolytic products of the reaction desorbed rapidly into the water subphase (Verger et al., 1973). Raneva et al. (1995) recently adapted this original kinetic model to study the phospholipase A_2 action on liposomes of long-chain phosphatidylcholine spread at the air−water interface. This kinetic scheme may also be used to describe the enzymatic hydrolysis at the oil−water interface. The model shown in Figure 1 consists of several successive steps. In

the first step, a water-soluble enzyme (E) reversibly adsorbs to the oil drop surface. This adsorption step, which leads to a more favorable catalytic state of the enzyme (E*), results in a two-dimensional Michaelis−Menten kinetic equilibrium. The enzyme at the interface (E*) binds to a substrate molecule (S) and forms the complex (E*S) prior to its decomposition. In the physiologically relevant case of the hydrolysis of long-chain triacylglycerols at the oil−water interface, the lipolytic products P* remain transiently at the oil drop surface where either a reorganization process (P* → P**) takes place and/or the lipolytic products desorb slowly into the aqueous or the oil phase.

According to this model (see Figure 1), the enzyme-catalyzed reaction occurs at the interface and the concentrations of E*, S, E*S, and P* must be expressed as surface concentration values (molecules cm^{-2}). According to the nomenclature proposed by Verger et al. (1973), the following expression for the enzymatic lipolysis velocity measured with the oil drop system (V_d) can be used:

$$V_d = \frac{d\Gamma_{p^*}}{dt} = \frac{k_2}{(k_d/k_p)K_m^*}\Gamma_s C_{Eo}$$

where $K_m^* = (k_2 + k_{-1})/k_1$ is the interfacial Michaelis−Menten constant.

We define, as follows, a global kinetic constant (Q), previously denoted as "interfacial quality" (Verger et al., 1973) and expressed in cubic centimeters per second per molecule

$$Q = \frac{k_2}{(k_d/k_p)K_m^*} = \frac{d\Gamma_{p^*}}{dt}\frac{1}{\Gamma_s C_{Eo}}$$

where Γ_s and Γ_{p^*} are the interfacial concentrations of substrate molecules (triglycerides) and lipolytic products (fatty acids and diglycerides), respectively, expressed in molecules per squared centimeter; $d\Gamma_{p^*}/dt$ is the hydrolysis velocity, expressed in molecules per squared centimeters per second; C_{Eo} is the enzyme concentration in the aqueous phase, expressed in molecules per cubic centimeters.

Biotin-Labeling of HPL. In order to obtain approximately 5% of bionylated HPL lysine residues, we used a procedure previously described by Guesdon et al. (1979), which was adapted at our laboratory by Aoubala et al. (1995b) for studying the interfacial binding of human gastric lipase to lipid monolayers.

The lipolytic activity of HPL was determined before and after biotin labeling with purified soybean oil as the substrate, using the pH-stat method. It was observed that HPL could be biotin-labeled with a slight loss of catalytic activity. An HPL activity of 100% was observed, except in the presence of NaTDC and in the absence of colipase, when the residual activity was 75%.

The native HPL and HPL* concentrations were checked spectrophotometrically at 280 nm using an adsorption coefficient of $A_{1cm}^{1\%} = 13, 3$.

Identical linear relationships were found to exist between the initial decrease in the interfacial tension with the concentration using native HPL and HPL* at concentrations of up to 2 μg mL^{-1} (data not shown).

Oil Drop Recovery and ELISA Experiments. At the end of a kinetic experiment, the oil drop and part of the aqueous phase associated with it were recovered using a Pasteur pipette adapted to the tip of a pipetting device (pipettman P200) and transferred into a glass tube containing 300 μL of a gum arabic mixture used to ensure an efficient emulsification (30 s with an Ultra Turax T25, Janke and Klunkle). The emulsification of the recovered oil drop containing adsorbed HPL* was performed in a solution containing gum arabic (10% w/v dissolved in TBS under stirring at 50 °C and filtered with a cheese cloth having a pore size of 50 μm) and Tween 20 (0.05% v/v) and BSA (0.05%).

In parallel with the oil drop recovery procedure, an equal volume of the aqueous phase from the cuvette was sampled and emulsified with a gum arabic mixture after adding of 50 μL of pure soybean oil.

The ELISA test procedure was adapted from the method developed at our laboratory by Aoubala et al. (1995b) for studying the interfacial binding of human gastric lipase to lipid monolayers. All the ELISA tests were performed in 96 wells of polyvinylchloride microplates (Maxisorb, Nunc). Each well was coated with 250 ng of a specific polyclonal anti-HPL antibody solubilized in 50 μL of 150 mM Tris-HCl buffer containing 140 mM NaCl and 3 mM KCl (TBS) and incubated overnight at 4 °C. The subsequent steps in the procedure were as described previously (Aoubala et al., 1995b), except for the preparation of the HPL* samples. The wells, previously coated with polyclonal anti-HPL antibody, were incubated for 2 h at 37°C with 50 μL of various dilutions of the emulsion, as described above from the recovered biotinylated lipase (HPL*).

A reference curve was drawn using pure HPL* samples, at known concentrations, emulsified as described above in the gum arabic mixture after adding of 50 μL of pure soybean oil. Each assay was carried out in duplicate.

The reliability of the sandwich ELISA was tested with HPL*. The recovery levels of HPL* injected into the glass cuvette were determined at each oil drop kinetic experiment and found to be excellent (90% ± 10). This means that negligeable amounts of HPL* were lost by adsorption to the walls of the glass cuvette or during the emulsification procedure.

In order to calculate the amount of HPL* adsorbed to the oil drop surface, the following equation was used:

$$\Gamma_E = \frac{[O,B]V_a - [B]V_b}{S_d}$$

where Γ_E is the surface excess of HPL* adsorbed to the oil surface, expressed in nanograms per squared centimeters; V_b is the volume of the aspirated bulk phase and S_d the oil drop surface; [B] is the HPL* concentration in the aspirated bulk phase; V_a is the total volume of the oil drop and the simultaneously aspirated bulk phase and $[O,B]V_a$ is the total amount of HPL* detected in the aspirated oil drop and in the simultaneously aspirated bulk phase.

Specific Activity of HPL.* The initial accumulation of insoluble lipolytic products (an equimolar mixture of oleic acid and diolein) at the drop surface results in a decrease in the interfacial tension. Our aim was to transform this decrease in the oil−water interfacial tension ($\gamma_{o/w}$) into the number of molecules of lipolytic products developing per unit time ($d\Gamma_{p^*}/dt$), which reflects the action of the lipase more realistically. For this purpose, we recently determined the relationship between the surface tension (γ) and the

surface density of lipolytic products (Γ_{p*}) at the argon−water interface in the presence or absence of excess triglycerides (Labourdenne et al., 1996). The variations in the surface density ($d\Gamma_{p*}$) of the lipolytic products were therefore correlated with the variations in the surface tension ($d\gamma$), using the monolayer technique. This correlation, which is the derivative of the surface compression isotherm, reflects the compressibility of the monolayer and can be used to interpret the variations in the interfacial tension at the oil−water interface, assuming the interfacial behavior of the lipolytic products at the oil−water and air−water interfaces to be comparable. The lipase SA can thus be estimated from the oil drop tensiometer measurements, given the interfacial lipase excess determined in the ELISA test. The following equation was used here:

$$SA = \frac{(d\Gamma_{p*}/dt)}{\Gamma_E}$$

By carrying out a simple transformation, we obtained:

$$SA = \frac{\left(\frac{d\gamma}{dt}\right)\left(\frac{d\Gamma_{p*}}{d\gamma}\right)}{\Gamma_E}$$

where SA is the specific activity expressed in micromoles of fatty acid released per minute per milligram of lipase; $d\gamma/dt$ is the decrease in the interfacial tension, determined directly from the recorded kinetics of the oil drop tensiometer and expressed in millinewtons per meter per minute; $d\Gamma_{p*}/d\gamma$ was defined above and was expressed here in molecules meters/per squared centimeters per millinewtons; $d\gamma$ is the difference between the initial and final values of the interfacial tension; Γ_E is the amount of HPL* adsorbed at the drop surface, as determined by the ELISA test and expressed in milligrams per squared centimeters.

RESULTS

Kinetics of HPL Hydrolysis in the Presence and Absence of Colipase.* The lipolysis at the oil−water interface was monitored by recording the decrease with time in the interfacial tension, after injecting lipase into the aqueous phase. Figure 2A shows the effects on the decrease in the interfacial tension of injecting colipase or HPL* in two separate kinetic experiments. Colipase, at a final concentration of 0.2 μg mL^{-1}, displayed no detectable tensioactive effect at the surface of the soybean oil drop. At concentrations greater than 0.4 μg mL^{-1}, the colipase adsorption at the oil−water interface slightly decreased the interfacial tension, which may have affected the accuracy of the lipase activity assessments (data not shown). These findings are in agreement with the results of previous studies on the effects of colipase on the hydrolysis of monomolecular films by lipase (Verger et al., 1977). Furthermore, pure HPL* displayed very low enzymatic activity levels on soybean oil substrates, and the rate of the decrease in the corresponding interfacial tension was 0.4 ± 0.1 mN m^{-1} min^{-1} (see Figure 2A).

Figure 2B shows the rapid decrease in the interfacial tension (6 ± 2 mN m^{-1} min^{-1}) recorded as a function of time, after HPL* injection followed by colipase injection. Figure 2C shows the effects of simultaneously injecting

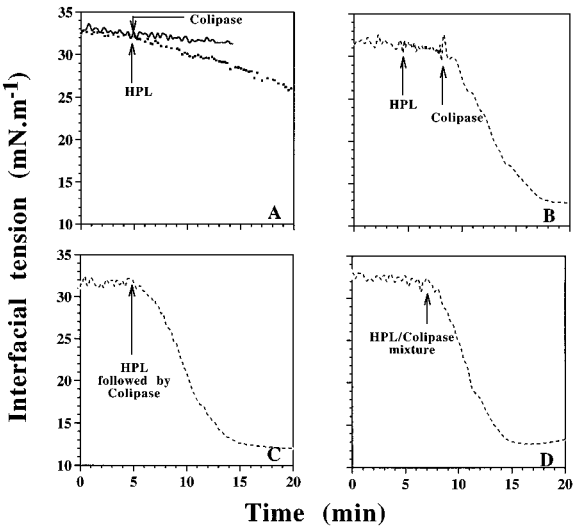

FIGURE 2: Variation as a function of time in the interfacial tension at a soybean oil−water interface: A, Separate colipase (—) and pure HPL (- - -) injections; B, HPL was injected first and colipase second; ***C***, HPL and colipase were injected simultaneously; D, the preformed HPL/colipase mixture (1/5 molar ratio) was injected. The bulk phase (volume 5 mL) was composed of 10 mM Tris-HCl (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA and was stirred continuously. The final concentrations of colipase and HPL were 0.2 μg mL^{-1}.

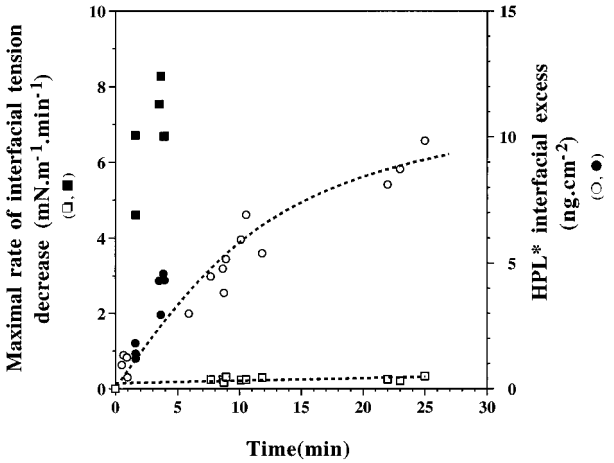

FIGURE 3: Maximal rate of interfacial tension decrease (□, ■) and the HPL* interfacial excess (○, ●) as a function of time. At each point, we recovered the oil drop and determined the interfacial HPL* excess. The measurements were performed without (○, □) and with (●, ■) colipase (0.2 μg mL^{-1}, final concentration). The bulk phase (volume 5 mL) was composed of 10 mM Tris-HCl (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA and was stirred continuously.

HPL* and colipase. Figure 2D shows the decrease in the interfacial tension which occurred after the injection of a HPL/colipase mixture (1/5 molar ratio). It can be noted that in all the cases where an efficient lipolysis occurred, the same final interfacial tension value (12 ± 1 mN m^{-1}) was reached. The presence of colipase considerably increased the activity of HPL (approximately 10−15-fold) and the stimulatory effect of colipase did not depend upon the mode of cofactor injection. However the time lag, required to obtain the maximal interfacial tension decrease when a preformed lipase/colipase mixture was injected, was shorter (50 s) than when the HPL and colipase were injected separately (90 s).

Figure 3 gives the plots of the maximal rates of decrease in the interfacial tension and the corresponding interfacial

HPL* excess resulting from a series of kinetic experiments which were interrupted after a predetermined period of time. The amount of adsorbed HPL* at the drop surface was time dependent and the maximum amount of adsorbed HPL* was 1% of the total HPL* injected.

The specific activity of HPL* was calculated as a function of time (and consequently, of the interfacial tension). After 1, 6, 10, and 23 min of enzymatic reaction, corresponding to 31, 28, 25, and 17 mN m^{-1}, the specific activity of HPL* was found to be 41 ± 11, 7 ± 3, 3 ± 0.7, 2 ± 0.3 μmol min^{-1} mg^{-1}, respectively, in the absence of colipase. In the presence of colipase, after 2 and 4 min of enzymatic reaction, corresponding to 26 and 14 mN m^{-1}, the specific activity was found to be 148 ± 13 and 34 ± 4 μmol min^{-1} mg^{-1}, respectively.

Kinetics of HPL Hydrolysis in the Presence of Bile Salts.* The surface properties of NaTDC have been measured by Brass et al. (unpublished results) at the oil−water interface, using the oil drop tensiometer. Using the Gibbs expression, the surface density of NaTDC at the oil−water interface was determined as a function of the interfacial tension. A value of 110 $Å^2$/molecule was obtained at the CMC (1.5 mM at pH 8), which is in agreement with data from the literature (Small, 1968).

To evaluate the effects of bile salts on HPL* hydrolysis, we injected them after the formation of the drop, before injecting the HPL/colipase mixture. After the bile salts injection, a rapid decrease in the interfacial tension was observed and a new equilibrium value was reached. After the HPL/colipase injection and after a given period of lipolysis, the oil drop was recovered with a view to determining the HPL* interfacial excess. Figure 4, panels A and B give several curves showing the kinetics of an oil drop hydrolysis by HPL*, in the presence of colipase, as a function of the NaTDC concentration. Throughout the NaTDC concentration range investigated, the rate of hydrolysis was found to be practically proportional to the lipase concentration (see Figure 4A). Furthermore, the presence of NaTDC, up to 100 μM, was not found to significantly affect the hydrolysis rates. However, it can noted that at various initial $\gamma_{o/w}$ values, various final $\gamma_{o/w}$ values were reached. For instance, in the absence of NaTDC, the $\gamma_{o/w}$ dropped from 32 to 12 mN m^{-1}, whereas in the presence of 400 μM NaTDC, the $\gamma_{o/w}$ decreased from 14 to 5 mN m^{-1}. In the absence of colipase, the presence of bile salts enhanced the hydrolysis rate from 0.4 ± 0.1 mN m^{-1} min^{-1} without NaTDC to 2 mN m^{-1} min^{-1} at 10 and 100 μM NaTDC (data not shown).

Figure 4B illustrates the fact that the presence of NaTDC (up to 100 μM) had no significant effects on the interfacial HPL* excess. The calculated SA of HPL* averaged 140 ± 15 μmol min^{-1} mg^{-1}.

Table 1 shows that the presence of bile salts, below their CMC, drastically decreased the lag time. Assuming that the lag time corresponded to a rate limiting step associated with lipase adsorption (E ↔ E* in Figure 1), as previously suggested (de la Fournière et al., 1994), it can be concluded from our data that the presence of negative charges at the drop surface, facilitated the binding of the HPL/colipase to the oil surface. This interpretation is in agreement with the shortening of the lag phase previously observed in the presence of ionized fatty acids (Wieloch et al., 1982).

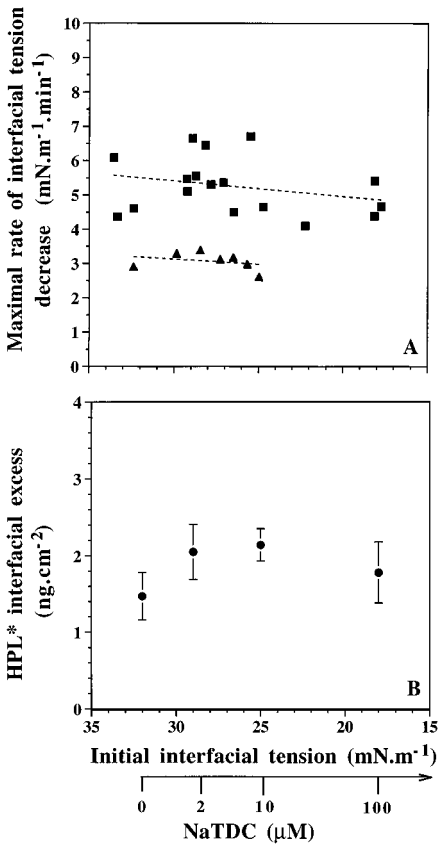

FIGURE 4: A, The maximal rates of interfacial tension decrease at a soybean oil−water interface induced by HPL*/colipase mixture (1/5 molar ratio) as a function of the NaTDC concentration. (▲) 0.1 μg mL^{-1}, (■) 0.2 μg mL^{-1} HPL*; B, (●) Interfacial excess of HPL* (0.2 μg mL^{-1}) in the presence of colipase (1/5 molar ratio). The interfacial excess of HPL* was measured 100 s after its injection into the aqueous phase, as described in Materials and Methods. The aqueous phase was composed of 10 mM Tris-HCl (pH 8), 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

Table 1: Lag Times Measured during the Hydrolysis of a Soybean Oil Drop, as a Function of the Bile Salts Concentration

NaTDC (μM)	lag time (s)	NaTDC (μM)	lag time (s)
0	50	60	11
2	18	100	8
4	16	200	0
10	17	400	0
30	12		

DISCUSSION

Lipid−water interfaces are obviously not all equivalent, and they do not act as "inert mirrors" for lipolytic enzymes, whereas the "interfacial quality" plays an important regulatory role in enzymatic processes. Gargouri et al. (personal communication) have measured HPL activity on triolein emulsified in gum arabic substrate and obtained values of 800 μmol min^{-1} mg^{-1} and 1100 μmol min^{-1} mg^{-1}, without and with colipase, respectively. We performed independent HPL activity measurements on pure soybean oil, emulsified by mechanical stirring, in the absence or presence of colipase and obtained specific activities of around 80 and 800 μmol min^{-1} mg^{-1}, respectively. It is clear from the above examples that the substrate emulsification method used (gum arabic or mechanical stirring) influences the catalytic activity, the effect of colipase, and the surface denaturation. Several authors have reported that a rapid surface denaturation of

Table 2: Comparison between the Global Kinetics Constants (Q) Expressed in ($cm^3\ s^{-1}$ molecule^{-1}) $\times\ 10^{14}$, as Measured with the Oil Drop Method and with the Monolayer Technique, Using Various Di- and Triglycerides as Substrates[a]

	oil drop method (soybean oil)		monolayer technique ($\pi = 12$ mN m^{-1})			
	−NaTDC	+NaTDC	dicaprin	dilaurin	diolein with β-CD	soybean oil with β-CD
HPL	0, 31	0, 95	4, 7	3, 2	3, 2	3, 1
HPL + CO	1, 4	1, 7	5, 4	4, 1	3, 5	2, 9

[a]NaTDC was used at a final concentration of 10 μM, with the oil drop method and β-cyclodextrin at a final concentration of 0.77 mM using long-chain acylglyceride monomolecular films. We checked that β-cyclodextrin did not affect the rate of hydrolysis when dilaurin monolayers were used as the lipase substrate.

pancreatic lipase occurs and is particularly pronounced in the absence of colipase and bile salts (Gargouri et al., 1995), at high temperatures (Granon & Sémériva, 1980; Borgström, 1982) and at high interfacial energies (Rietsch et al., 1977). In our experiments, we did not note any significant surface denaturation of pure HPL, since identical hydrolysis rates were measured whether colipase was injected simultaneously or a few minutes after injecting HPL (see Figure 2). In order to look more closely at the "interfacial quality", we studied lipid hydrolysis by HPL on various monomolecular films of dicaprin, dilaurin, diolein, and purified soybean oil. In the case of the long-chain lipids (diolein and soybean oil) the β-cyclodextrin was used to solubilize the lipolytic products as described previously (Laurent et al., 1994; Ivanova et al., 1996). On the basis of these kinetic data, we calculated the global catalytic constant (Q) which takes into account the substrate surface concentration and the HPL concentration in the bulk phase. From Table 2, it can be seen first that HPL does not require the presence of the cofactor to hydrolyze lipid monolayers. This is in agreement with previous data on the hydrolysis of dicaprin by HPL (de la Fournière et al., 1994). Secondly, Table 2 shows that HPL hydrolyses monomolecular films of di-and triglycerides with various chain lengths at about the same rate. For the sake of comparison, the global kinetic constants (Q) of HPL at the oil drop surface are also presented in Table 2. With the oil drop system, in the absence of colipase, the global kinetic constant was found to be 10-fold lower than the value obtained using monomolecular films of soybean oil in the presence of β-cyclodextrin. In the presence of colipase, the global kinetic constant was found using the oil drop method to be only two times lower. Once again, it can be concluded that the type of interface used, *i.e.*, oil−water versus air−water, strongly influences the catalytic activity of HPL as well as the colipase effect. Lairon et al. (1980) have previously investigated the effects of bile salts and colipase on the pancreatic lipase hyrolysis of monomolecular films of dilaurin. It is not possible however to make any quantitative comparaisons between the present data and the kinetic constants obtained by these authors, because the composition of their mixed monolayer substrate is not known in the presence of bile salts in the subphase.

A few years ago, Verger et al. (1977), using radioactively labeled lipase, showed that the amount of pancreatic lipase adsorbed to a didecanoylglycerol film at low surface pressures (2 mN m^{-1}) was not affected by the presence of colipase although the catalytic rate increased significantly. A clear-cut effect (3−4-fold) of colipase on the pancreatic lipase turnover number was also observed, using mixed monomolecular films of triglyceride−phosphatidylcholine (Piéroni & Verger, 1979). When the interface was a pure anionic phosphatidylglycerol monomolecular film, mimicking the presence of bile salts, it was observed on the contrary that the colipase was adsorbed first and served as an anchor for lipase in a narrow range of surface pressures (23−30 mN m^{-1}). In the present study, we clearly established that colipase induces a highly significant increase in the turnover of HPL, whereas its adsorption at the triacylglycerol surface did not require the assistance of the cofactor (Figure 3). Colipase probably contributes to stabilizing the open HPL conformation preexisting at the interface, resulting in an HPL active site with an increased accessibility and efficacy (van Tilbeurgh et al., 1993). An alternative, nonexclusive, hypothesis might be that colipase has "substrate-channeling" effects on the interfacial activity of HPL, as recently suggested by Momsen et al. (1995).

With the oil drop technique, it is unfortunately impossible to study the lipase hydrolysis at NaTDC concentrations above their CMC, because the surface of the drop is saturated with bile salts molecules and the interfacial tension therefore does not vary when other tensioactive agents (lipolytic products) are generated. In emulsified systems, such as those usually used with the pH-stat method, the specific surface area of the emulsion is generally unknown but plays an important role. At a given bile salts concentration, the amount of adsorbed bile salt is therefore unknown, but not negligible and much higher concentrations of bile salts will be required to saturate the surface of an emulsion than at the surface of a single drop. Consequently, one cannot quantitatively compare the effects of varying the bile salts concentration on lipase activity when using either emulsified bulk methods or the oil drop technique.

In the present investigation, we established (Figure 4A and B) that the bile salts below their CMC affected neither the HPL binding nor the hydrolysis rates at the oil−water interface. This means that the HPL/colipase complex has the same binding and catalytic efficacy at electrically neutral or negatively charged oil−water interfaces. Our data are in agreement with findings made some years ago by Momsen and Brockman (1976), using the silicon-coated glass beads system. These authors showed that pure pancreatic lipase readily adsorbs to a bile salt-substrate-covered surface below the CMC. At the oil−water interface, in the presence or absence of bile salts below their CMC, colipase behaves like a true lipase cofactor, *i.e.*, it increases the enzyme turnover and does not affect interfacial lipase adsorption.

ACKNOWLEDGMENTS

This research was carried out with financial and technical support from the ITC company as well as the Bridge T-lipase Programme of the European Communities under contract BIOT-CT91-0274 (DTEE) and the BIOTECH G-Programme BIO 2-CT94-3041 and BIO 2-CT94-3041. We are grateful

to I. Douchet and Dr. M. Aoubala (UPR9025, Laboratoire de Lipolyse enzymatique du CNRS, Marseille) for their technical assistance in performing the ELISA test as well as their gift of polyclonal antibodies. We are grateful to Dr. Y. Gargouri and J. de Caro (UPR9025, Laboratoire de Lipolyse enzymatique du CNRS, Marseille) for their generous gift of HPL and colipase, respectively. Our thanks are also due to Dr. J. Blanc for correcting the English.

REFERENCES

Aoubala, M., de la Fournière, L., Douchet, I., Abousalham, A., Daniel, C., Hirn, M., Gargouri, Y., Verger, R., & de Caro, A. (1995a) *J. Biol. Chem. 270*, 3932−3937.

Aoubala, M., Ivanova, M., Douchet, I., de Caro, A., & Verger, R. (1995b) *Biochemistry 34*, 10786−10793.

Borgström, B. (1975) *J. Lipids Res. 16*, 411−417.

Borgström, B. (1976) *FEBS Lett. 71*, 201−204.

Borgström, B. (1982) *Biochim. Biophys. Acta 712*, 490−497.

Borgström, B., & Erlanson-Albertson, C. (1971) *Biochim. Biophys. Acta 242*, 509−513.

Borgström, B., & Erlanson-Albertsson, C. (1973) *Eur. J. Biochem. 37*, 60−68.

Canioni, P., Julien, R., Rathelot, J., & Sarda, L. (1976) *Lipids 12*, 393−397.

Chaillan, C., Kerfelec, B., Foglizzo, E., & Chapus, C. (1992) *Biochem. Biophys. Res. Commun. 184*, 206−211.

Chapus, C., Sari, H., Sémériva, M., & Desnuelle, P. (1975) *FEBS Lett. 58*, 155−158.

Chapus, C., Desnuelle, P., & Fogglizzo, E. (1981) *Eur. J. Biochem. 115*, 99−105.

Chattoraj, D. K., & Birdi, K. S. (1984) *Adsorption and the Gibbs surface excess*, Plenum Press, New York.

de Caro, A., Figarella, C., Amic, J., Michel, R., & Guy, O. (1977) *Biochim. Biophys. Acta 490*, 411−419.

de la Fournière, L., Ivanova, M. G., Blond, J.-P., Carrière, F., & Verger, R. (1994) *Colloids Surf. B2*, 585−593.

Desnuelle, P. (1986) in *Molecular and cellular basis of digestion* (Desnuelle, P., Sjöström, H., & Norén, O., Eds.) pp 275−296, Elsevier, Amsterdam, New York, Oxford.

Egloff, M.-P., Marguet, F., Buono, G., Verger, R., Cambillau, C., & van Tilbeurgh, H. (1995a) *Biochemistry 34*, 2751−2762.

Egloff, M.-P., Sarda, L., Verger, R., Cambillau, C., & van Tilbeurgh, H. (1995b) *Protein Sci. 4*, 44−57.

Erlanson-Albertsson, C. (1992) *Biochim. Biophys. Acta 1125*, 1−7.

Gargouri, Y., Bensalah, A., Douchet, I., & Verger, R. (1995) *Bba-Lipid Lipid Metab. 1257*, 223−229.

Granon, S., & Sémériva, M. (1980) *Eur. J. Biochem. 929*, 1−8.

Grimaldi, M., Bois, A., Nury, S., Rivière, C., Verger, R., & Richou, J. (1991) *Opto. 91*, 104−110.

Guesdon, J. L., Térnynck, T., & Avrameas, S. (1979) *J. Histochem. Cytochem. 8*, 1131−1139.

Ivanova, M. G., Ivanova, T., Verger, R., & Panaiotov, I. (1996) *Colloids Surf. B6*, 9−17.

Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127−156.

Labourdenne, S., Gaudry-Rolland, N., Letellier, S., Lin, M., Cagna, A., Esposito, G., Verger, R., & Rivière, C. (1994) *Chem. Phys. Lipids 71*, 163−173.

Labourdenne, S., Ivanova, M. G., Brass, O., Cagna, A., & Verger, R. (1996) *Colloids Surf. 6*, 173−180.

Lairon, D., Charbonnier-Augeire, M., Nalbone, G., Léonardi, J., Hauton, J. C., Piéroni, G., Ferrato, F., & Verger, R. (1980) *Biochim. Biophys. Acta 618*, 106−118.

Laurent, S., Ivanova, M. G., Pioch, D., Graille, J., & Verger, R. (1994) *Chem. Phys. Lipids 70*, 35−42.

Mahe-Gouhier, N., & Leger, C. (1988) *Biochim. Biophys. Acta 962*, 91−97.

Maylié, M. F., Charles, M., Gache, C., & Desnuelle, P. (1971) *Biochim. Biophys. Acta 229*, 286−289.

Maylié, M. F., Charles, M., Astier, M., & Desnuelle, P. (1973) *Biochem. Biophys. Res. Commun. 52*, 291−297.

Momsen, W. E., & Brockmann, H. L. (1976) *J. Biol. Chem. 251*, 384−388.

Momsen, W. E., Momsen, M. M., & Brockman, H. L. (1995) *Biochemistry 34*, 7271−7281.

Morgan, R. G. H., & Hoffman, N. E. (1971) *Biochim. Biophys. Acta 280*, 143−148.

Nury, S., Piéroni, G., Rivière, C., Gargouri, Y., Bois, A., & Verger, R. (1987) *Chem. Phys. Lipids 45*, 27−37.

Piéroni, G., & Verger, R. (1979) *J. Biol. Chem. 254*, 10090−10094.

Raneva, V., Ivanova, T., Verger, R., & Panaiotov, I. (1995) *Colloids Surf. B3*, 357−369.

Ransac, S., Rivière, C., Gancet, C., Verger, R., & de Haas, G. H. (1990) *Biochim. Biophys. Acta 1043*, 57−66.

Ransac, S., Moreau, H., Rivière, C., & Verger, R. (1991) *Methods Enzymol. 197*, 49−65.

Rietsch, J., Pattus, F., Desnuelle, P., & Verger, R. (1977) *J. Biol. Chem. 252*, 4313−4318.

Small, D. M. (1968) *Adv. Chem. Ser. 84*, 31−52.

van Tilbeurgh, H., Sarda, L., Verger, R., & Cambillau, C. (1992) *Nature 359*, 159−162.

van Tilbeurgh, H., Egloff, M.-P., Martinez, C., Rugani, N., Verger, R., & Cambillau, C. (1993) *Nature 362*, 814−820.

Vandermeers, A., Vandermeers-Piret, M. C., Rathé, J., & Christophe, J. (1976) *Biochem. Biophys. Res. Commun. 69*, 790−797.

Verger, R. (1980) *Methods Enzymol. 64*, 340−392.

Verger, R. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 83−149, Elsevier, Amsterdam.

Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127−136.

Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77−117.

Verger, R., Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023−4034.

Verger, R., Rietsch, J., & Desnuelle, P. (1977) *J. Biol. Chem. 252*, 4319−4325.

Wieloch, T., Borgström, B., Piéroni, G., Pattus, F., & Verger, R. (1982) *J. Biol. Chem. 257*, 11523−11528.

Wilhelmy, L. (1863) *Ann. Physik. 119*, 177.

BI961331K