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Inhibition of Lipases by Proteins: A Binding Study Using Dicaprin Monolayers†

Youssef Gargouri, Gérard Piéroni, Claude Rivière, Louis Sarda,‡ and Robert Verger*
Centre de Biochimie et de Biologie Moléculaire du Centre National de la Recherche Scientifique, 13402 Marseille Cedex 9, France

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ABSTRACT: We previously reported that the inhibition of pancreatic and *Rhizopus delemar* lipases by proteins is due to the protein associated with lipid and is not caused by direct protein–enzyme interaction in the aqueous phase [Gargouri, Y., Piéroni, G., Rivière, C., Sugihara, A., Sarda, L., & Verger, R. (1985) *J. Biol. Chem. 260*, 2268–2273]. In this study, using radiolabeled lipases, serum albumin, and β-lactoglobulin A, we investigated their respective binding with respect to lipolysis of dicaprin monolayers. Lipase inhibition was found to be correlated with a lack of lipase binding to mixed protein–dicaprin films or to a desorption of lipase from the interface when inhibitory protein was added later. Since a large proportion of the lipid film remained potentially accessible to the enzyme in the presence of inhibitory protein, it was concluded that the observed decrease in lipase binding to the interface was due to a variation of the physicochemical properties of the lipid–water interface following binding of inhibitory protein. On the basis of the results presented here, it is proposed that mixed protein–glyceride films could be used to characterize the interaction of various lipases with lipid substrates and to classify these enzymes according to their penetration power.

Hydrolysis of triacylglycerol by lipase occurs at the oil–water interface where the enzyme binds. As shown by several authors, pancreatic and microbial lipase activities are inhibited by bile salts at concentrations in the millimolar range (Canioni et al., 1977; Borgström et al., 1979; Sémériva & Desnuelle, 1979). Several research groups (Borgström, 1975; Chapus et al., 1975; Vandermeers et al., 1975, 1976; Momsen & Brockman, 1976a) have presented evidence that inactivation of pancreatic lipase by bile salts results from enzyme desorption from the interface. A hypothesis was initially put forward according to which the lipase might be prevented from reaching its substrate by a layer of bile salt molecules formed at the surface of the emulsified substrate (Morgan et al., 1969;

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* Address correspondence to this author.
‡ Present address: Laboratoire de Biochimie, Faculté des Sciences, St-Charles, 13003 Marseille, France.

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Borgström & Erlanson, 1973; Borgström, 1975; Chapus et al., 1975; Vandermeers et al., 1976). As an alternative hypothesis, Momsen and Brockman (1976b) have suggested that lipase desorption might result from the cooperative formation of a lipase–bile salt complex between molecules dispersed in the aqueous phase. Later, it was recognized that a wide variety of ionic and nonionic synthetic detergents had an inhibitory effect on the activity of lipases of various origins (Borgström & Erlanson, 1973; Canioni et al., 1976; Gargouri et al., 1983).

As shown in earlier reports (Fraser & Nicol, 1966; Brockerhoff, 1971; Borgström & Erlanson, 1978; Blackberg et al., 1979), proteins inactivate lipase as do other amphiphilic compounds. By studying lipase activity remaining in the supernatant after centrifugation of tributyrin preemulsified with bovine serum albumin or by using siliconized glass beads as model substrate, Borgström and Erlanson (1978) showed that "lipases and serum albumin compete for the interface of the substrate and that the inhibition is an effect of the physical separation of lipase from the interface". In these bulk systems, however, no quantification of the respective surface concentrations of lipase and inhibitory proteins could be achieved. We recently reinvestigated the inactivation of pancreatic and microbial lipases by several proteins including serum albumin, β-lactoglobulin A, ovalbumin, myoglobin, and melittin and by a protein-inhibiting lipase (PIL)[1] isolated from soybean (Gargouri et al., 1984a,b). We were able to show that inhibition of lipase activity neither resulted from the interaction between enzyme and protein in the bulk phase nor was related to the lowering of interfacial tension. We demonstrated, using the monolayer technique, that lipase inactivation is due to the protein associated with lipid substrate (Gargouri et al., 1984b). In the present investigation, we determined the amounts of radiolabeled enzyme and inhibitory protein bound to the lipid monolayer during lipolysis. These experiments were aimed at reaching a better understanding of the inhibitory mechanisms of proteins on lipase activity.

Materials and Methods

Lipids. A mixture of 1,2- and 1,3-didecanoylglycerol (dicaprin) was prepared at the laboratory as described previously (Rietsch et al., 1977). Phosphatidylcholine (PC) was purified from egg yolk L-α-lecithin (Sigma) by chromatography on a silicic acid column.

Enzymes and Proteins. Hog pancreatic lipase and lipase from the mold *Rhizopus delemar* were purified at the laboratory by previously described procedures (Verger et al., 1969; Iwai & Tsujisaka, 1974). Horse pancreatic lipase was a gift from Dr. R. Julien (Faculty of Science, Marseille).

Bovine serum albumin (BSA), β-lactoglobulin A, ovalbumin, and myoglobin were from Sigma. Melittin was a gift from Dr. Gould (New York State Institute for Basic Research in Mental Retardation, Staten Island). The protein-inhibiting lipase (PIL) was prepared from soybean meal as recently reported (Gargouri et al., 1984b).

Protein Concentration. Protein concentration was determined as described by Lowry et al. (1951) or by spectrophotometry at 280 nm using the following absorption coefficients ($E^{1\%}_{1cm}$): BSA, 6.7; β-lactoglobulin A, 9.6; ovalbumin, 7.5; myoglobin, 20.5; melittin, 16.6.

Enzyme and Protein Labeling. (*A*) *Pancreatic Lipases.* Pure hog and horse pancreatic lipases were labeled with 5,5′-dithiobis(2-nitro[^{14}C]benzoic acid) (CEA, Saclay, France) by a published method (Verger et al., 1977). Specific radioactivities of horse and hog pancreatic [^{14}C]TNB lipases calculated after scintillation counting on samples, using a liquid scintillation spectrophotometer (Packard, Model 2450), were 10.5×10^{12} and 15.5×10^{12} dpm/mol, respectively, corresponding to 0.24 and 0.60 modified sulfhydryl residue/mol of enzyme.

(*B*) *Rhizopus delemar Lipase.* Lipase from *R. delemar* was labeled at 25 °C with Na^{125}I by the lactoperoxidase method (Thorell & Johansson, 1971). The enzyme (0.5 mg, 11 nmol) was dissolved in 0.5 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 33 nmol of NaI and 1 mCi of Na^{125}I (Amersham, Great Britain). Lactoperoxidase (0.11 nmol) obtained from Sigma was then added to the solution followed by four additions (110 nmol each) of hydrogen peroxide at 4-min intervals. The final solution was dialyzed exhaustively at 4 °C against 10 mM acetate buffer, pH 5.6. Radioactivity was counted in a Packard Autogamma scintillation spectrometer (Model 3002). The specific radioactivity of the ^{125}I-labeled enzyme was 81.6×10^{15} dpm/mol, corresponding to the incorporation of 1.8 iodine atoms/mol of enzyme. The enzymatic activity of *R. delemar* and pancreatic lipases was not affected by labeling.

(*C*) *Serum Albumin and β-Lactoglobulin A.* The technique described above was used for iodination of serum albumin and β-lactoglobulin A. In both cases, 285 nmol of protein was dissolved in 0.5 mL of phosphate buffer containing 0.029 mCi of Na^{125}I and 840 nmol of NaI. Lactoperoxidase (2.85 nmol) was then added to the solution followed by four additions of 2.85 nmol of hydrogen peroxide at 4-min intervals. The solution was finally dialyzed against 10 mM Tris-HCl buffer, pH 8. The specific radioactivities of serum albumin and β-lactoglobulin A were 21.2×10^{13} and 8.8×10^{13} dpm/mol, respectively, corresponding to the incorporation of 2.8 and 1.2 iodine atoms/mol of protein. No change was observed in the inhibitory properties of these proteins after iodination.

Monolayer Techniques. Surface pressure (π) was measured by the Wilhelmy plate method using a thin platinum plate (perimeter, 3.94 cm) attached to a Beckman electromicrobalance (Model LM 600). A Teflon trough (volume, 50 mL; surface, 31 cm^2) was used to measure surface pressure increase ($\Delta\pi_{max}$) during adsorption of radiolabeled proteins at lipid–water interfaces. The aqueous subphase was continuously stirred at 250 rpm with a magnetic rod.

Lipase activity was determined by the barostat technique (Verger & De Hass, 1973) with a "zero-order" trough composed of a reservoir and one compartment. In all assays, the hydrolysis reaction was stopped by placing a barrier across the canal connecting the two compartments. When necessary, mixed films containing radiolabeled proteins were recovered in a scintillation vial, as described previously (Rietsch et al., 1977). In these cases, an equal volume of the subphase was placed in another vial. The difference in radioactivity between these two vials was attributed to the protein molecules bound to the lipid film. The protein surface concentration was expressed as ng/cm^2.

Mixed lipid–protein films were transferred as described previously (Gargouri et al., 1985). After transfer, no significant change in the surface protein concentration of the mixed lipoprotein films, as determined by surface radioactivity, could be detected after 1 h, showing that β-lactoglobulin A and serum albumin were quasi-irreversibly bound to the dicarprin monolayer.

Results

Adsorption of Radiolabeled Proteins and Pancreatic Lipase

[1] Abbreviations: PC, phosphatidylcholine; BSA, bovine serum albumin; PIL, protein-inhibiting lipase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TNB, 5-thio-2-nitrobenzoic acid.

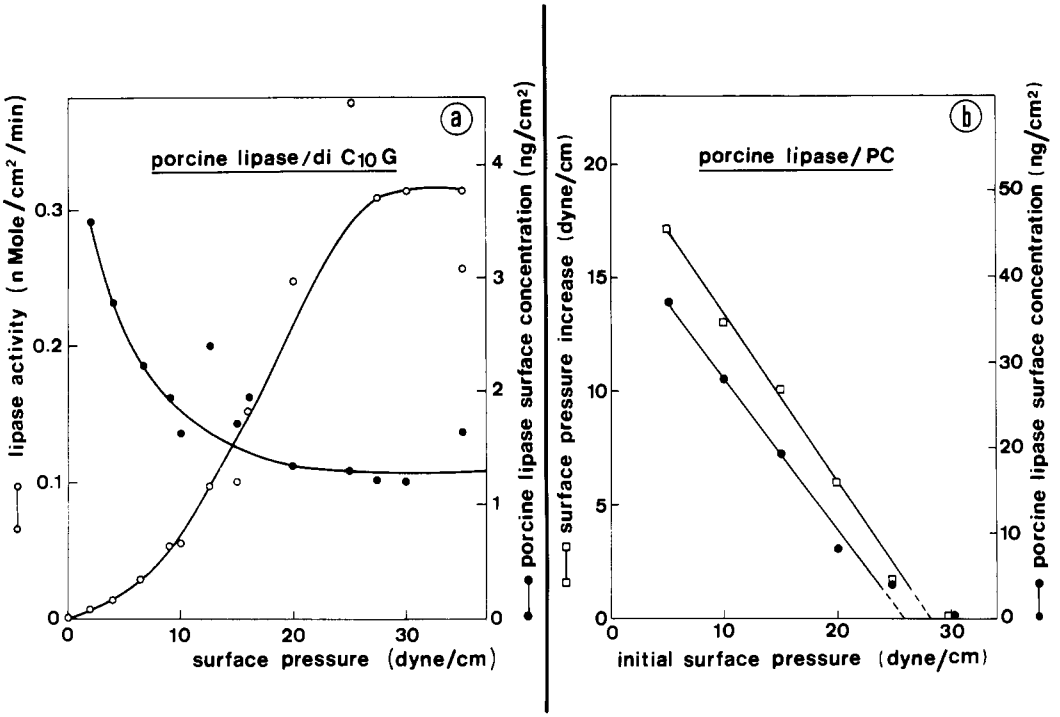

FIGURE 1: Interaction of hog pancreatic [^{14}C]TNB lipase with dicaprin and phosphatidylcholine monolayers. Panel a: Variations with surface pressure in lipase activity on a dicaprin monolayer (○) and hog pancreatic [^{14}C]TNB lipase surface concentration (●). Final lipase concentration 2.4 nM injected into the reaction compartment (volume, 100 mL; surface, 127.7 cm^2) of a "zero-order" trough. Panel b: Variations with initial surface pressure in protein surface concentration (●) and maximal surface pressure increase (□) at equilibrium (20 min) after hog [^{14}C]TNB lipase injection under monomolecular films of phosphatidylcholine (PC) spread in a cylindrical Teflon trough (volume, 50 mL; surface, 31 cm^2). Final enzyme concentration, 100 nM. Buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

to Monomolecular Films of Dicaprin and Phosphatidylcholine. ^{125}I-Labeled serum albumin or β-lactoglobulin A was injected under monomolecular films of didecanoylglycerol (dicaprin) or phosphatidylcholine (PC) at initial surface pressures (π_i) varying in the range 5–35 dyn/cm, and the change in surface pressure at equilibrium ($\Delta\pi_{max}$) was determined as previously reported (Gargouri et al., 1985). The protein surface concentration was also measured as a function of π_i as described under Materials and Methods. For both proteins, with phosphatidylcholine films, the $\Delta\pi_{max}$ value decreased linearly from 18 dyn/cm to 0, and the protein surface concentration decreased, in parallel, from 75 ng/cm^2 to 0 when π_i was increased from 5 to 25 dyn/cm; with dicaprin films, $\Delta\pi_{max}$ decreased linearly from 18 dyn/cm at π_i = 10 dyn/cm to 0 at π_i = 36 dyn/cm, and in sharp contrast to what had been observed on phosphatidylcholine films, the protein surface concentration remained constant (60 ng/cm^2) for π_i, varying from 10 to 35 dyn/cm (data not shown).

In a second series of experiments, hog pancreatic [^{14}C]TNB lipase (final concentration, 100 nM) was injected under phosphatidylcholine (PC) films at initial surface pressure (π_i) varying in the range 5–30 dyn/cm. The change in surface pressure at equilibrium ($\Delta\pi_{max}$) and the hog lipase surface concentration were determined. It can be seen from the curves in Figure 1b that the surface protein concentration and $\Delta\pi_{max}$ decreased linearly with increasing π_i. Thus, pancreatic lipase behaves similarly as BSA or β-lactoglobulin in the monolayer system.

A "zero-order" trough was used to study hog pancreatic [^{14}C]TNB lipase interaction with dicaprin monolayers. After injection of the lipase, when steady-state kinetics of hydrolysis were obtained, the film over the reaction compartment was recovered and the lipase surface concentration was determined. The variations in lipase activity and interfacial concentration with surface pressure are shown in Figure 1a. Lipase activity increased continuously from 0 to 35 dyn/cm while lipase surface concentration in the dicaprin film remained constant from 10 to 35 dyn/cm.

A dicaprin film maintained at a surface pressure of 35 dyn/cm was chosen for the inhibition studies reported below. This value allows minimal change in surface pressure due to protein adsorption and maximal lipase activity.

Effect of Increase in Protein Surface Concentration on Rate of Dicaprin Monomolecular Film Hydrolysis Induced by Pancreatic and *R. delemar Lipases.* Increasing amounts of ^{125}I-labeled serum albumin or β-lactoglobulin A were injected under films of dicaprin maintained at a surface pressure of 35 dyn/cm. The requisite time (20 min) was allowed to elapse for equilibrium to be reached. Then hog pancreatic lipase (final enzyme concentration, 160 pM) was added to the aqueous phase. Hydrolysis of dicaprin was observed over a 10-min period so that steady-state kinetics were reached. Films were then collected, and the surface concentration of the radioactive protein bound to dicaprin was determined. The curves in Figure 2 indicate that the rate of hydrolysis of dicaprin by hog pancreatic lipase decreased as the amount of protein injected under the films increased. Ninety percent inhibition of pancreatic lipase was obtained at concentrations of about 50 nM and 60 nM for β-lactoglobulin A (Figure 2a) and serum albumin (Figure 2b), respectively. No significant difference in lipase inhibition was observed when experiments were performed with transfer of the mixed dicaprin–protein film (data not shown).

As shown in Figure 2, the surface concentration of β-lactoglobulin A and serum albumin bound to dicaprin increased as more protein was injected under dicaprin monolayers. Fifty percent inhibition of hog lipase activity occurred at a protein surface concentration of 15 ng/cm^2 and 14 ng/cm^2 for β-lactoglobulin A and serum albumin, respectively.

Comparable results were obtained with horse pancreatic

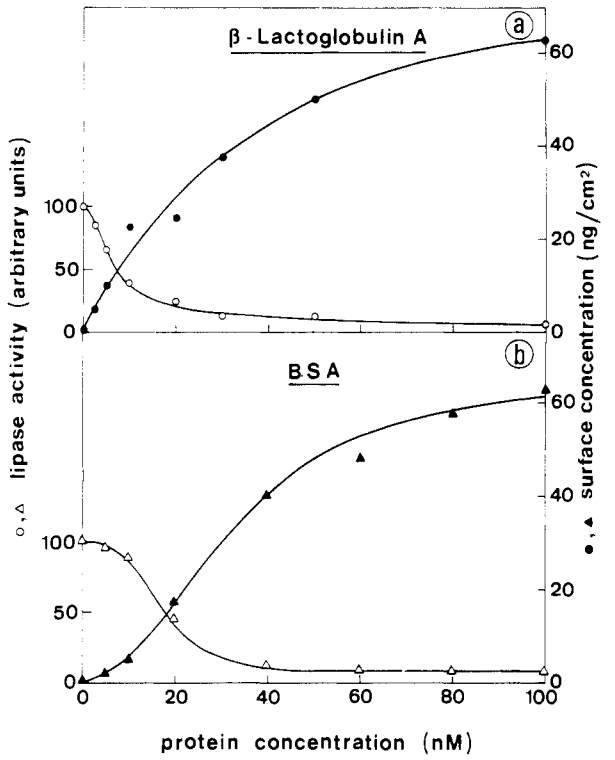

FIGURE 2: Variations with bulk concentrations of β-lactoglobulin A (panel a) and serum albumin (panel b) in surface concentration (solid symbols) and lipase activity (open symbols). ^{125}I-Labeled β-lactoglobulin A and ^{125}I-labeled serum albumin were allowed to equilibrate for 20 min under a dicaprin film (35 dyn/cm). Then, hog lipase was injected (final concentration, 160 pM), and activity was measured by using a "zero-order" trough composed of a reservoir and four identical compartments (volume, 50 mL; surface, 31 cm^2) (Gargouri et al., 1985). Buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA. One hundred arbitrary units represent the hydrolysis of 65.4 pmol/(cm^2·min) dicaprin.

Table I: Inhibition of Pancreatic Lipases by β-Lactoglobulin A and BSA

	I_{50} (ng/cm^2)[a]			
	without film transfer		after film transfer	
enzymes	β-lactoglobulin A	BSA	β-lactoglobulin A	BSA
hog lipase	15	14	nd[b]	nd
horse lipase	20	17.5	9	5

[a] I_{50} represents the surface protein concentration that produces half-inactivation of the enzyme. [b] nd, not determined.

lipase (Table I). As shown in Table I, the surface protein concentration that produces half-inactivation of horse lipase is reduced after transfer of the mixed film from 20 to 9 ng/cm^2 and from 17.5 to 5 ng/cm^2 when β-lactoglobulin A or serum albumin is used, respectively.

Experiments were performed under the same conditions with hog or horse pancreatic [^{14}C]TNB lipases and unlabeled inhibitory proteins (1 μM). The enzyme (3.75 nM) was injected either before or after protein addition. In both cases, the rate of hydrolysis was reduced to less than 5% of the values measured in the absence of inhibitory protein. Lipase surface concentration was concomitantly reduced to 8% of the control values (data not shown). It thus appears that, in the presence of serum albumin or β-lactoglobulin A, pancreatic lipases do not adsorb to or are desorbed from dicaprin films.

Parallel experiments were performed with *R. delemar* lipase. Taking advantage of the fact that activity of the microbial enzyme, in contrast to pancreatic lipases, is not inactivated by iodination, radiolabeled lipase could be prepared with a high

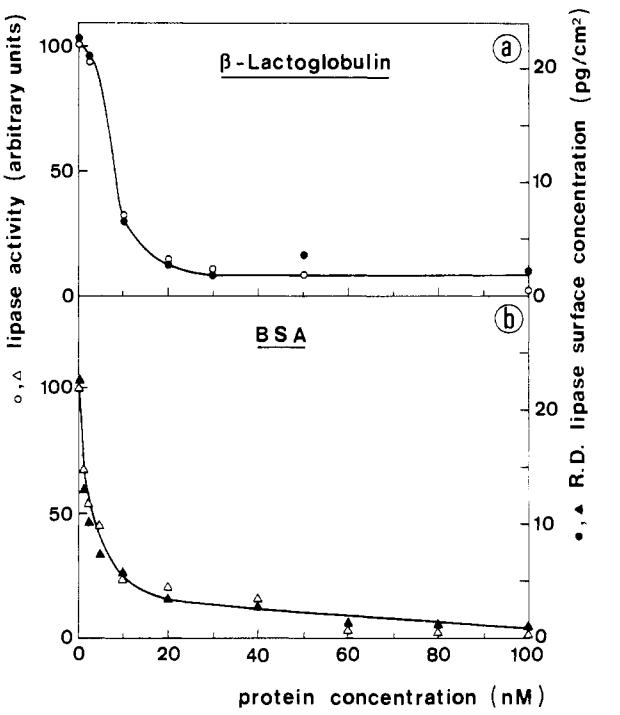

FIGURE 3: Variations with bulk concentrations of β-lactoglobulin A (panel a) in the rate of hydrolysis by ^{125}I-radiolabeled *R. delemar* lipase (open symbols) of dicaprin films at constant surface pressure and lipase surface concentration (solid symbols). Proteins were injected into the reaction compartment of a "zero-order" trough (volume, 200 mL; surface, 127.7 cm^2) (Verger & De Haas, 1973) under a dicaprin film (35 dyn/cm). Lipase (final concentration, 12 pM) was injected 20 min later. Buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA. One hundred arbitrary units represent the hydrolysis of 78 pmol/(cm^2·min) dicaprin.

Table II: Effect of Proteins on Rate of Hydrolysis and on Adsorption of ^{125}I-Labeled *R. delemar* Lipase (Final Concentration 12 pM) to Dicaprin Film (35 dyn/cm)[a]

	lipase activity		lipase adsorbed to dicaprin	
proteins	units[b]	%	pg/cm^2	%
none	78	100	23	100
serum albumin	5.1	7	2.3	10
β-lactoglobulin A	6.5	8	1.4	6
melittin	9.2	12	4.6	20
PIL	10.8	14	1.9	8.3
myoglobin	74	94	17	74
ovalbumin	72	92	22	96

[a] All proteins (0.5 μM) were added 10 min after lipase injection. [b] Units were expressed as pmol/(cm^2·min).

specific radioactivity with a view to relating lipase binding to inactivation by proteins.

Serum albumin or β-lactoglobulin A was injected at increasing concentrations up to 0.1 μM under films of dicaprin maintained at 35 dyn/cm. Twenty minutes later, ^{125}I-labeled *R. delemar* lipase was added to the aqueous subphase (12 pM final concentration) and the rate of dicaprin hydrolysis was determined. Finally, the mixed film was collected and the surface concentration of the labeled enzyme was measured. The enzyme surface concentration and the rate of dicaprin hydrolysis were plotted against inhibitory protein concentration. Results are presented in Figure 3. Curves in Figure 3 show that the hydrolysis rate decreased as inhibitory protein concentration increased. A direct correlation was observed between the decrease in lipase activity and the decrease in lipase surface concentration.

When serum albumin and β-lactoglobulin A (final con-

centration 0.5 μM) were injected after *R. delemar* lipase, activity was markedly reduced, as was enzyme surface concentration (Table II). The effects of other proteins—melittin, myoglobin, and ovalbumin—and the protein-inhibiting lipase (PIL) from soya were also investigated. Data in Table II fully confirm that inhibition of lipase is directly related to enzyme desorption from the dicaprin film in the case of serum albumin, β-lactoglobulin, melittin, and PIL. As observed previously (Gargouri et al., 1985), neither myoglobin nor ovalbumin caused inhibition.

Discussion

The rate of hydrolysis of emulsified triacylglycerol by pancreatic and microbial lipases decreases greatly in the presence of bile salts or synthetic detergents irrespective of charge and structure. Amphiphilic compounds act as lipase inhibitors by preventing the enzyme from binding to its substrate at the oil–water interface. Proteins also have the capacity to inactivate lipase (Fraser & Nicol, 1966; Brockerhoff, 1971; Borgström & Erlanson, 1978; Blackberg et al., 1979; Gargouri et al., 1984a). The molecular mechanism underlying lipase inhibition has still to be elucidated.

We recently reinvestigated the inhibition of pancreatic and *R. delemar* lipases by various proteins including serum albumin and β-lactoglobulin A with the monolayer technique using films of didecanoylglycerol (dicaprin) as substrate (Gargouri et al., 1984b, 1985).

Experiments using mixed lipid–protein film transfer clearly show that the inhibition of pancreatic lipase is due solely to the protein associated with lipid and is not caused by direct protein–enzyme interaction in the aqueous phase. A positive correlation was observed between values of the initial rates of surface pressure changes occurring after protein injection and their respective capacity to inhibit pancreatic lipase (Gargouri et al., 1985).

With a view to obtaining further information on how protein adsorbed to dicaprin inhibits interfacial lipolysis, we prepared radiolabeled lipases, serum albumin, and β-lactoglobulin A in order to quantify the amount of protein bound to dicaprin that causes enzyme inactivation. At the same time, we determined the amount of radiolabeled lipase bound to the film in the presence or absence of inhibitory protein.

Serum albumin and β-lactoglobulin A bind to films of dicaprin or egg lecithin. Binding of the proteins resulted, in both cases, in an increase in surface pressure ($\Delta\pi_{max}$). As observed in both cases, the value of $\Delta\pi_{max}$ decreased as the initial surface pressure of the lipid film increased. With films of egg lecithin, it was observed that the total amount of bound protein decreases with increasing initial surface pressure. If one takes the increase in surface pressure of the film to be indicative of its penetration by protein, it appeared, in the case of PC films, that protein penetration and binding varied simultaneously and linearly with initial surface pressure as previously observed (Quinn & Dawson, 1960; Phillips et al., 1975; Teissié, 1981; Mayer et al., 1983). By contrast, with dicaprin films at an initial surface pressure varying in the range 10–35 dyn/cm, the amount of surface-bound proteins remained constant whereas film penetration decreased at high lipid packing. This difference in behavior reveals the existence of at least two main steps in protein–monolayer interaction, i.e., adsorption occurring with or without film penetration, as already shown by Schulman (1957) and Quinn and Dawson (1970).

Similar behavior was also observed after pancreatic lipase injection under a phospholipid or dicaprin film. As shown in Figure 1b, it was observed that the surface concentration of hog pancreatic lipase decreases linearly with increasing initial surface pressure of phosphatidylcholine monolayers. Above the critical lipid packing (corresponding to 28 dyn/cm) of phosphatidylcholine films, pure pancreatic lipase was unable to bind to the latter. Figure 1a further shows that in the 10–35 dyn/cm range a constant amount of radiolabled lipase is associated with a dicaprin film even though enzymatic activity rises continuously. Thus, protein binding to phosphatidylcholine presents a penetration threshold, whereas binding to dicaprin seems to be fairly lipid packing independent. This type of behavior appears to be a general feature of enzymatic or nonenzymatic proteins and to our knowledge has not been reported before.

In further experiments we determined the rate of hydrolysis of dicaprin monolayer by *R. delemar* lipase and the amount of enzyme adsorbed to the film as a function of the concentration of inhibitory protein (serum albumin and β-lactoglobulin A) in the aqueous phase (Figure 3). A parallel decrease was found between the lipase surface concentration and lipase activity as the inhibitory protein concentration increased. Furthermore, it is worth noting, from data shown Table II, that several proteins could desorb lipase from a dicaprin monolayer. This confirms that it is unlikely that lipase inhibition could result from direct enzyme–protein interaction in the dicaprin monolayer. Mixed lipid–protein films were prepared under conditions where surface concentration of inhibitory proteins was maximal. Under these conditions, they contained radiolabled serum albumin and β-lactoglobulin A at surface concentrations of 100 and 89 ng/cm^2, respectively (calculated from Hanes–Woolf plots of the data in Figure 2).

If one assumes that proteins adsorbed to dicaprin were in a fully expanded state (Phillips et al., 1975; MacRitchie, 1978; Graham & Phillips, 1979a–c) at the lipid–water interface, corresponding to an average 17 Å^2/amino acid residue (1 mg/m^2), only a maximal percentage of 7.5% of the total surface area of the dicaprin monolayer after film transfer (or about 17% without film transfer; see Table I) will have been covered by inhibiting protein at 50% lipase inhibition.

At this inhibition level, one can calculate that the *R. delemar* lipase surface concentration is equal to 10 pg/cm^2 (see Figure 3); this value has to be compared with the 14 and 2.5 ng/cm^2 obtained for the surface concentration of β-lactoglobulin A and BSA, respectively, responsible for 50% *R. delemar* lipase inhibition. Even though surface concentration of inhibitory protein is in very large excess (a factor of about 1000) with respect to lipase surface concentration, we are still far from saturating conditions for the protein binding capacity to the dicaprin film (see Figure 2). Consequently, it cannot be argued that protein inhibition proceeds via direct competition for the same adsorption sites. Furthermore, since a large proportion of the lipid film remained potentially accessible to the enzyme in the presence of inhibitory protein, the possibility that steric hindrance on lipase binding to the interface may exist can be ruled out. Proteins, which are polyelectrolytes, may induce long-range electrostatic forces after their binding to the lipid interface as well as variations in several physicochemical parameters (surface potential, surface viscosity, etc.) that might control the interaction of lipase with the interface evidenced by a penetration threshold.

Among the lipases, only some were found to be able to hydrolyze some mixed protein–dicaprin films (Gargouri et al., 1984b, 1985). As we reported in a previous study, *Geotrichum candidum* and *Rhizopus arrhizus* lipases hydrolyze a mixed protein–dicaprin film that is resistant to pancreatic and *R. delemar* lipase action (Gargouri et al., 1985). Other authors have suggested that the critical surface pressure of phospholipid

monolayer penetration by snake venom or pancreatic phospholipase A could be used to classify enzymes with respect to their hemolytic (Demel et al., 1975) or anticoagulative (Verheij et al., 1980) properties. Tentatively, we propose that various mixed protein–glyceride films could be used as a reference scale to classify lipases according to their penetration power.

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