Interfacial Binding of Human Gastric Lipase to Lipid Monolayers, Measured with an ELISA†

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ABSTRACT: Two sandwich enzyme linked immunosorbent assays (ELISA) were developed for evaluating the surface excess at the lipid/water interface of the human gastric lipase (HGL) and two anti-HGL monoclonal antibodies (mAbs). These assays were adapted to the monomolecular film technique used previously for measuring lipase kinetics. HGL and the two anti-HGL mAbs (4-3 and 218-13) were biotinylated without any significant loss of their biological activities occurring. They were further detected by ELISA using either anti-HGL or anti-mouse IgG polyclonal antibodies as specific captors before being revealed using a streptavidin−peroxidase conjugate as tracer. The detection limit was 25 and 85 pg in the case of HGL and mAb, respectively. By combining the above sandwich ELISA technique with the monomolecular film technique, it was possible for the first time to measure the enzymatic activity of HGL on 1,2-didecanoyl-*sn*-glycerol (dicaprin) monolayers as well as to determine the corresponding interfacial excess of the enzyme. The HGL turnover number increased steadily with the lipid packing. The specific activities determined on dicaprin films spread at 35 $mN \cdot m^{-1}$ were found to be in the range of the values measured under optimal bulk assay conditions, using tributyrin emulsion as a substrate [*i.e.,* 1000 μmol/(min·mg of enzyme)]. At a given lipase concentration in the water subphase, the interfacial binding of HGL to the nonhydrolyzable egg yolk phosphatidylcholine (egg PC) monolayers was found to be 10 times lower than that in the case of dicaprin monolayers. Given the low tensioactivity of the mAbs of the IgG isotype [Ivanova et al. (1993) *Colloids Surf. B1,* 17−22], we also investigated the effects of five anti-HGL mAbs (mAbs 4-3, 25-4, 35-2, 83-15, and 218-13) on the catalytic activity as well as on the interfacial binding of HGL to lipid/water interfaces. Four out of these five mAbs (mAbs 4-3, 25-4, 35-2, and 83-15) were found to significantly reduce the lipolytic activity of HGL. Moreover, three of the four inhibitory mAbs (mAbs 4-3, 25-4, and 35-2) were found to reduce the specific activity of HGL, while mAb 83-15 had no effect on the specific activity. These results clearly indicate that the latter mAb (83-15) complexed with HGL mainly affects the binding of the enzyme to the lipid/water interface, while the other three inhibitory mAbs (mAbs 4-3, 25-4, and 35-2) affect both the binding and the catalytic steps of HGL.

Since lipolytic reactions take place at the lipid/water interface, the monomolecular film technique seems to be optimally suited for studying the interfacial enzyme kinetics of lipolysis (Ransac et al., 1991; Verger & de Haas, 1976). Several investigators using this methodology have reported that an optimum occurs in the velocity−surface pressure profile. The exact value of the optimum varies considerably with the particular enzyme/substrate combination used.

Qualitative interpretations have been given to explain this phenomenon. The first hypothesis, proposed by Hughes (1935) and supported by later workers (Momsen et al., 1979; Muderhwa & Brockman, 1991), was that a packing-dependent orientation of the substrate may be one of the factors on which the regulation of lipolysis depends. Another interpretation was put forward by Esposito et al. (1973), who explained the surface pressure optimum in terms of changes in the lipase conformation upon adsorption at the interface, resulting in an optimal conformation at intermediate values of the interfacial free energy (film pressure). It was suggested that lower and higher values of surface pressure would lead to inactive forms either because of denaturation or because the conformational changes in the enzyme structure are not sufficiently marked. This view was challenged by Verger et al. (1976) and Pattus et al. (1979). Using radiolabeled enzymes, these authors showed that the observed maxima in the velocity−surface pressure profile disappear when they are correlated with the interfacial excess of enzyme.

At our laboratory, one of the favorite lipolytic enzymes for studying the surface pressure dependency has been the human gastric lipase (HGL)[1] present in gastric juice (Carrière

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[1] Abbreviations: HGL, human gastric lipase; HGL*, biotinylated human gastric lipase; IgG, immunoglobulin G; mAb, monoclonal antibody; mAb*, biotinylated monoclonal antibody; pAb, polyclonal antibody; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; ACNHS−biotin, ϵ-aminocaproic *N*-hydroxysuccinimide ester D-biotin; OPD, *o*-phenylenediamine dihydrochloride; PBS, 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl; BSA, bovine serum albumin; PVC, polyvinyl chloride; egg PC, egg phosphatidylcholine; dicaprin, 1,2-didecanoyl-*sn*-glycerol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB-HGL, 2-nitro-5-thiobenzoic acid−human gastric lipase conjugate.

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et al., 1994). This enzyme has been described as a member of the acid lipase family (Anderson & Sando, 1991) which shares no sequence homology with other lipase families (Petersen & Drabløs, 1994).

Chemical modifications of HGL with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Gargouri et al., 1988) or diethyl *p*-nitrophenyl phosphate (Moreau et al., 1991) showed that both a free sulfhydryl and a serine residue are essential for the enzyme to show lipolytic activity. Iodination of HGL as well as dog and rabbit gastric lipases also leads to complete inactivation of these enzymes (Gargouri et al., personal communication). Using radiolabeled DTNB, Gargouri et al. (1989) investigated the interactions between covalently labeled [^{14}C]TNB-HGL and monomolecular lipid films. It is worth noting that [^{14}C]TNB-HGL is an inactive enzyme and, moreover, to facilitate detection, that the [^{14}C]-TNB-HGL concentrations used by Gargouri et al. (1989) to study its binding to monomolecular films were about 40 times higher than the usual catalytic concentrations of HGL. Under these conditions, the existence of a correlation between the increase in surface pressure and the amount of protein bound to the interface was the only possible conclusion which could be drawn by the authors. They showed that, in the presence of egg PC films, the total amounts of surface-bound [^{14}C]TNB-HGL decreased linearly as the initial surface pressure increased. On the contrary, using lipase substrates such as dicaprin films, the amounts of surface-bound inactive [^{14}C]TNB-HGL remained constant at variable surface pressures (Gargouri et al., 1989).

The ELISA/biotin−streptavidin system has been found to be as sensitive as the use of radiolabeled proteins (Guesdon et al., 1979). In addition, biotinylation preserves the biological activities of many proteins. Using this labeling procedure, we recently developed a specific double sandwich ELISA to measure the HGL levels of duodenal contents (Aoubala et al., 1993b). The aim of the present study was to develop a sensitive sandwich ELISA, using the biotin−streptavidin system, and to measure the amount of surface-bound HGL* and anti-HGL mAbs* adsorbed to monomolecular lipid films. Using an enzymatically active HGL*, it was possible for the first time to correlate the lipase activity with the surface excess of the enzyme present at the interface.

MATERIALS AND METHODS

Chemicals. Monomolecular film experiments were carried out with dicaprin purchased from Serdary Research Laboratory (London, Ontario, Canada), and egg PC was obtained from Sigma. Horseradish peroxidase-labeled streptavidin (HRP-streptavidin) was purchased from Immunotech (Marseilles); bovine serum albumin (BSA), *o*-phenylenediamine (OPD), and ϵ-aminocaproic *N*-hydroxysuccinimide ester D-biotin (ACNHS-biotin) were from Sigma.

Purification of HGL from Gastric Juice. Human gastric juice was collected with the patient's consent from the gastroenterology department at St Marguerite's Hospital (Marseille) as previously described by Moreau et al. (1988). Freshly collected gastric juice was centrifuged for 30 min at 10000*g*, and the supernatant was neutralized and kept frozen at −20 °C. HGL was purified using the methods developed at our laboratory by Moreau et al. (1992) and Aoubala et al. (1993b), involving ion exchange chromatography followed by immunoaffinity chromatography on an Affi-gel 10-immobilized specific mAb (35-2) column.

HGL Activity Measurement. The lipase activity was measured titrimetrically at pH 6.0 and 37 °C using a pH-stat (TTT 80 radiometer) and tributyrin as substrate, under the standard assay conditions previously described by Gargouri et al. (1986b).

Anti-HGL Polyclonal and Monoclonal Antibodies. Monoclonal and polyclonal antibodies (mAbs and pAbs) against native HGL were prepared and characterized as previously described (Aoubala et al., 1994, 1993a; Ivanova et al., 1993). To prepare the pAb, rabbits were injected every 4 weeks with pure HGL, and the sera were tested for anti-HGL reactivity by immunoblotting procedure. Lastly, the rabbit anti-HGL pAbs were purified by immunoaffinity, using immobilized HGL (Aoubala et al., 1993b). Anti-HGL mAbs were generated by fusion of spleen cells from a mouse immunized with pure HGL with mouse myeloma (P 3 × 63Ag 8.653) (Aoubala et al., 1993a). The mAbs were purified from mouse ascitic fluids by precipitation with 50% saturated ammonium sulfate followed by affinity chromatography on protein A−Sepharose CL-4B (Pharmacia).

Protein Measurement. The protein concentrations of solutions of purified HGL and immunoglobulin G (mAbs and pAbs) were determined spectrophotometrically at 280 nm using absorption coefficients of $A_{1cm}^{1\%} = 15.8$ and 14, respectively.

Biotin-Labeling of HGL and mAbs (4-3 and 218-13). ACNHS-Biotin was used to couple biotin moieties to ϵ-amines of HGL lysine residues using a modified procedure previously described by Guesdon et al. (1979). In order to obtain the desired stoichiometric ratio of ACNHS-Biotin to total lysine residues of the lipase, a solution of HGL (1 mg·mL^{-1}) in 20 mM borate buffer (pH 8.0) containing 150 mM NaCl was mixed with various volumes of a solution of ACNHS-Biotin (10 mg·mL^{-1}) in dimethylformamide. The reaction mixture was incubated at room temperature for 20 min under stirring and stopped by adding NH_4Cl (final concentration 0.1 M). The mixture was then immediately dialyzed at 4 °C against 10 mM MES buffer (pH 7).

Two anti-HGL mAbs (4-3 and 218-13) were also biotinylated as previously described (Aoubala et al., 1993b).

ELISA Experiments. All the ELISA tests were performed in 96-wells poly(vinyl chloride) (PVC) microplates (Maxisorb, Nunc). The wells were coated with 250 ng of a specific pAb (anti-HGL or anti-IgG for the titration of HGL or mAb, respectively) solubilized in 50 μL of 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (buffer A) and incubated overnight at 4 °C. The wells were then washed three times, for 5 min each, with 300 μL/well of buffer A using an automatic multireagent washer (MRW, Dynatech), and the excess protein binding sites were saturated by incubation with 200 μl/well of blocking agent (3% w/v Régilait skimmed milk powder in buffer A) for 2 h at 37 °C. The plates were then washed three times with buffer B (buffer A containing 50 μM BSA and 0.05% v/v Tween 20), and the pAb-coated wells were incubated 2 h at 37 °C with 50 μL of various solutions of biotinylated proteins (HGL* or mAbs*) prepared in buffer B. In some experiments, the dilutions of biotinylated proteins were carried out in buffer A without any BSA. The wells were then washed three times with buffer B, and the biotin-labeled protein was revealed using 2 ng/well of HRP−streptavidin conjugate in 50 μL of buffer B. The HRP−streptavidin was incubated

for 30 min at room temperature. After washing the plates three times with buffer B, 50 μl of peroxidase substrate solution (*o*-phenylenediamine (5 g/L) in 50 mM sodium phosphate/citrate (pH 5) containing 0.4% fresh hydrogen peroxide) was added to each well and incubated for 20 min at room temperature in dark. The enzyme reaction was stopped by adding 50 μL of 2 M H_2SO_4 to each well, and the optical density (OD) was measured at 492 nm using a micro ELISA reader (MR 5000, Dynatech).

Monomolecular Film Experiments. (i) Kinetics of Hydrolysis and Binding to Dicaprin Films. Measurements were performed with the KSV Barostat equipment (KSV-Helsinki). The principle of the method has been described by Verger and de Haas (1973). It involved the use of a special "zero order trough" (Lairon et al., 1980) drilled into a Teflon block with two compartments: a reaction compartment containing 30 mL of solution with a total surface of 39.3 cm^2 and a reservoir compartment (length, 19.7 cm; width, 2 cm). The two compartments were connected to each other by a small surface channel. Enzyme solution was injected into the subphase of the reaction compartment only, whereas the lipid film covered both of them. A mobile Teflon barrier, automatically driven and controlled by the barostat, moved back and forth over the reservoir to keep the surface pressure at a given constant end point value, thus compensating for the substrate molecules removed from the film by enzyme hydrolysis. The surface pressure was measured continously with a Wilhelmy plate (perimeter 3.94 cm) attached to an electromicrobalance connected in turn to a microprocessor programmed to regulate the movement of the mobile barrier. The reaction compartment was continuously stirred at 250 rpm by one 2.5 cm magnetic bar and the temperature maintained at 37 °C by means of a circulating water bath. The lipid films were spread from a chloroform solution (aroun 1 $mg \cdot mL^{-1}$). The aqueous subphase was composed of 10 mM acetate (pH 5) containing 20 mM $CaCl_2$ and 100 mM NaCl. The kinetics of hydrolysis were recorded for 10 min, and the remaining film was aspirated as previously described (Rietsch et al., 1977). The film was recovered into a glass tube (0.5−1.5 mL) by placing a barrier across the surface channel and by sweeping it over the reaction compartment and aspirating the film thus collected. After completely recovering the film, an equal volume of the subphase was sampled and placed in another tube. The difference between the total amounts of proteins between these two samples was attributed to the surface excess of protein molecules bound to the lipid film.

(ii) Binding to Egg PC Monomolecular Films. Since phospholipids are not hydrolyzed by gastric lipases, it was thus possible to study, at high protein concentrations, the binding of HGL* or mAbs* and their respective complexes to the lipid/water interface, using an egg PC monomolecular film. In these experiments, only the reaction compartment of the "zero order trough" described above was used. The amounts of protein bound to the lipid/water interface were correlated with the surface pressure increases. During the protein adsorption to egg PC monolayers, the subphase composed of a 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl was stirred continuously as described above. At the maximal surface pressure increase, films containing proteins in excess and the corresponding subphases were recovered as described above, and their protein content was quantified by performing ELISA tests.

Before being used, the trough was first cleaned each time with tap water and gently brushed in the presence of distilled ethanol to remove any protein which may be bound to the trough. It was washed again with tap water and finally rinsed with distilled and filtered water.

Determination of the Amount of Protein Adsorbed to the Lipid Monolayer. A reference curve was drawn up for each test and was used to determine the amount of protein adsorbed to the lipid monolayer. For this purpose, biotinylated proteins (HGL* or mAb*) at known concentrations in 50 μL of buffer B were incubated in the wells of a microtitration plate previously coated with a specific pAb (anti-HGL or anti-IgG1). An ELISA was performed as described above. The optical density values at 492 nm were plotted as a function of the concentration of biotinylated protein. Each assay was carried out in duplicate. The obtained reference curve was used to calculate the concentration of biotinylated protein in the aspirated samples recovered from the monomolecular experiments as described above. In order to calculate the amount of protein bound to the monomolecular lipid film, the volume occupied by the lipid film was not taken into account since it was negligible with respect to the aspirated subphase. We used the following equation:

$$\Gamma = \frac{([F + B] - [B])}{S} \times V_a$$

where Γ is the surface excess of protein bound to the lipid monolayer, expressed in $ng \cdot cm^{-2}$. [F + B] is the concentration of protein present in the aspirated film with the aspirated bulk subphase, as determined by ELISA test. [B] is the concentration of protein in the bulk sample, also determined by ELISA. V_a is the aspirated volume (ranging from 0.5 to 1.5 mL) and S the area of the reactional compartment of the trough (39.3 cm^2).

Reliability of the Sandwich ELISA for HGL Adsorbed to Lipid Monolayers.* During the monolayer experiments, the validity of the sandwich ELISA for HGL* was tested in the presence of monomolecular films of either dicaprin or egg PC. The recovery levels of HGL* injected under lipid monomolecular films were determined after each experiment as:

$$\text{total recovery } (\%) = \frac{[B]V_t + \Gamma S}{T} \times 100$$

where [B] is the concentration of HGL* in the subphase (Bulk); V_t is the total volume of the reactional compartment measured after each monolayer experiment (30 ± 2 mL); ΓS is the total amount of HGL* adsorbed to the monomolecular film; and T is the total amount of HGL* (200 ng) injected under the monomolecular film.

RESULTS

Biotin Labeling of HGL and mAbs. Various stoichiometric ratios of ACNHS-biotin to the ε amines of HGL lysine residues were used to introduce biotin moieties into some of the 21 lysine residues of HGL (Bodmer et al., 1987). The lipolytic activity of HGL was determined both before and after biotin labeling using the pH-stat method with tributyrin as the enzyme substrate. It was observed at all three stoichiometric ratios used that HGL could be biotin-labeled

without any appreciable loss of catalytic activity. Residual activities of 86%, 96% and 100% were observed at stoichiometric ratios of ACNHS-biotin to HGL lysine residues of 0.6, 0.3 and 0.15, respectively. This is a considerable advantage over previous methods for chemically labeling HGL with radioactive DTNB, which led to a catalytically inactive lipase (Gargouri et al., 1989).

mAbs (IgG1 isotype) were biotin-labeled as previously described (Aoubala et al., 1993b), and their capacity to immunoreact with HGL was tested using an ELISA test with native HGL directly coated to the wells of the PVC microplates. No decrease in the antigen binding capacity of mAbs 4-3* and 218-13* was observed (data not shown).

Sandwich ELISA for HGL and mAbs**. Since we have produced a specific pAb directed against HGL and since anti-mAbs (of IgG isotype) pAb are commercially available, two specific sandwich ELISAs were developed for titrating HGL* and mAbs*. Specific anti-HGL or anti-mouse Ig G pAbs were coated on the PVC microplate and used as the antigen captor. The saturating concentration of pAb adsorbed on the PVC plate corresponds to 125 ng/well, as previously determined (Aoubala et al., 1993b). Various concentrations of mAb* or HGL* were then applied to the above pAb-coated plates and the immunoreactive mass of each protein of interest was detected with streptavidin–peroxidase conjugate as tracer and revealed by the peroxidase substrate as described in Materials and Methods.

The sensitivity of the sandwich ELISA test corresponds to the minimum amount of mAb* or HGL* which gave a signal 4 times higher than the mean background signal (0.05 ± 0.005). Figure 1A shows the titration curves of three HGL* preparations. ELISA sensitivity values of 15, 25, and 135 pg/well were obtained at stoichiometric ACNHS-Biotin to HGL lysine residue ratios of 0.6, 0.3, and 0.15, respectively. As expected, this result shows that the sensitivity of the sandwich ELISA test increases with increasing amounts of biotin groups introduced into the HGL molecule (Figure 1A). Figure 1B gives the titration curve of mAb 4-3* and shows the immunoreactive mass toward anti-IgG pAb adsorbed to the PVC plate. The sensitivity of the mAb* detection corresponds to 50 pg/well. The titration curves and the sensitivity for the detection of mAbs 4-3* and 218-13* were similar (data not shown).

During the monolayer experiments, the validity of the above sandwich ELISA for HGL* was tested. The recovery levels of HGL* injected under lipid monomolecular films were determined after each experiment as described in Materials and Methods. The results are shown in Table 1, where one can observe that low recovery rates of HGL* occurred in the presence of dicaprin (total recovery = 21 ± 2.3%) while good yields were obtained in the presence of egg PC (total recovery = 74 ± 5.5%). The effects of BSA, present in buffers used for ELISA, on the recovery of HGL* after monolayer experiments were studied. The BSA is absent in buffer used for the monomolecular experiments. As shown in Table 1, a considerable increase in the total recovery values was observed in the presence of BSA with dicaprin (from 21 ± 2.3% to 78 ± 6.2%) while no significant changes in the recovery values were observed with egg PC.

Adsorption of HGL to Dicaprin and Egg PC Monomolecular Films.* Using monomolecular films of dicaprin at variable surface pressures (ranging from 5 to 35 $mN \cdot m^{-1}$),

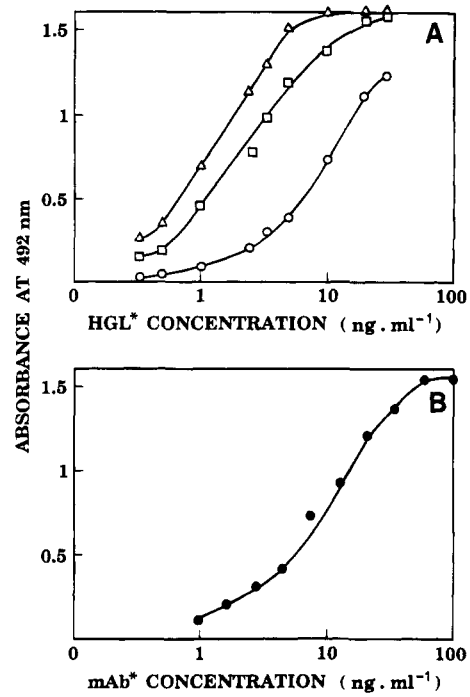

FIGURE 1: Reference ELISA curves of HGL* (A) and mAb 4–3* (B). (A) (△), (□), and (○) indicate the titration curves of HGL biotinylated at molar ratios of ACNHS-Biotin to HGL lysine amino groups of 0.6, 0.3, and 0.15, respectively. (B) The reference curve of mAb 4-3 biotinylated at one constant molar ratio of ACNHS-Biotin to mAb lysine amino groups (Aoubala et al., 1993b). HGL* and mAb* were dissolved in washing buffer at the concentrations indicated on the abscissa, and the immunoreactivity mass of biotinylated proteins was measured with the specific sandwich ELISA as described in Materials and Methods.

the HGL activity against dicaprin monolayers was measured before and after biotinylation (ACNHS-biotin/HGL lysine residues: 0.3). Figure 2A shows that biotinylated HGL hydrolyzes dicaprin films similarly as native HGL, indicating that the labeling does not alter the interfacial HGL activity. Moreover, we continuously recorded the enzyme activity, and at the end of the kinetic experiment, we determined the amount of biotinylated enzyme in excess at the interface. The amounts of HGL* present at or close to the interface increased linearly with increasing surface pressures up to 25 $mN \cdot m^{-1}$ (Figure 2A). Furthermore, a decrease in the amount of adsorbed enzyme was observed above this optimal surface pressure value. The lipolytic activity of HGL* was also measured simultaneously at the corresponding surface pressures. As shown in Figure 2A, HGL* does not significantly hydrolyze dicaprin films at surface pressures of less than 15 $mN \cdot m^{-1}$. Above this value the enzymatic activity increases rapidly, reaching a maximum value at 30 $mN \cdot m^{-1}$. The ratio of observed enzyme activity to the amount of adsorbed protein, as determined by the specific sandwich ELISA, allows one to calculate the specific activity of the enzyme acting on a monomolecular film of dicaprin. As shown in Figure 2B, the specific activity increased continuously from 15 up to 35 $mN \cdot m^{-1}$. At the latter value, the maximal specific activity reached was 1100 ± 97 $units \cdot mg^{-1}$, whereas at 15 $mN \cdot m^{-1}$, its value was zero.

The amount of HGL* adsorbed to egg PC monomolecular films was also measured (Figure 2A), and a similar bell-shaped HGL* adsorption curve was observed. At high surface pressures (30 and 35 $mN \cdot m^{-1}$), no significant

Table 1: Sandwich ELISA for Measuring the HGL* Bound to Lipid Monomolecular Films[a]

		dicaprin			egg PC		
	surface pressure	HGL* concn in recovered film ($ng \cdot mL^{-1}$)	HGL* concn in bulk phase ($ng \cdot mL^{-1}$)	tot HGL* recovery (%)	HGL* concn in recovered film ($ng \cdot mL^{-1}$)	HGl* concn in bulk phase ($ng \cdot mL^{-1}$)	tot HGL* recovery (%)
−BSA	5	2.4/2.9/2.9	1.2/1.4/1.5	19/21/22	6.0/5.3/5.6	5.7/5.1/5.3	67/73/69
	10	2.7/2.6/2.7	1.7/1.2/1.2	25/19/19	5.6/5.3/6.1	5.2/4.7/5.5	79/67/81
	15	3.2/2.7/3.1	1.4/1.2/1.4	22/19/22	5.9/6.6/5.7	5.2/5.8/5.2	75/80/77
	20	3.5/2.9/3.1	1.1/1.3/1.5	17/21/23	5.8/6.2/5.6	4.9/5.3/4.5	72/77/69
	25	6.6/4.0/4.4	1.2/1.2/1.3	19/20/20	6.0/5.7/6.0	4.9/4.8/5.1	79/72/81
	30	2.7/4.1/3.2	1.3/1.4/1.1	20/22/19	4.6/5.5/5.4	4.8/5.5/5.8	73/76/83
	35	2.7/3.1/3.0	1.4/1.6/1.7	22/24/26	4.3/4.7/4.9	4.3/5.0/4.2	67/74/64
+BSA	5	8.0/8.2/7.4	5.2/5.4/4.6	80/82/71	5.1/5.5/5.2	4.9/5.3/4.9	67/73/69
	10	8.6/8.0/8.5	4.8/4.7/5.1	74/73/79	6.0/5.4/5.6	5.6/5.1/5.1	83/73/76
	15	6.7/8.0/8.4	4.3/4.2/4.0	67/66/65	6.0/6.1/6.4	5.4/5.6/4.8	83/79/69
	20	9.6/9.7/9.5	6.0/5.4/5.3	86/84/82	5.7/6.2/6.0	4.7/4.2/5.1	71/80/76
	25	10.9/11.1/10.7	5.4/5.4/5.7	76/77/80	5.6/6.4/6.0	4.9/5.5/5.2	71/77/80
	30	12.8/9.6/8.9	6.0/5.7/5.5	83/79/80	5.1/4.7/4.8	5.2/4.9/5.0	71/74/70
	35	9.8/8.1/7.9	6.0/5.8/5.4	84/83/80	4.4/5.0/4.6	4.8/5.1/4.8	68/75/74

[a] The data show the effect of the BSA present in the ELISA buffers on the total HGL* recovery rates expressed as %. Monolayer experiments and the total recovery determinations were carried out as described in Materials and Methods.

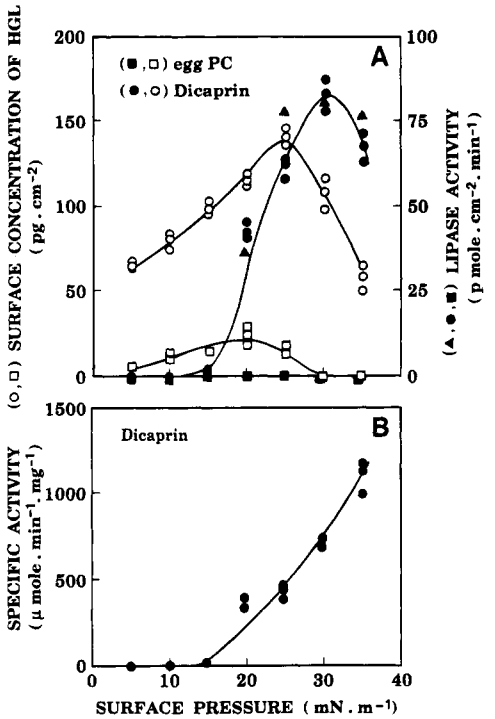

FIGURE 2: (A) Variations of the initial velocity of hydrolysis of a dicaprin monolayer with surface pressure (●) by HGL* (200 ng) and (▲) by native nonbiotinylated HGL (200 ng) injected into the subphase (30 ± 2 mL) of a zero order trough. No enzymatic activity was detected with egg PC films (■). The interfacial excess of HGL* was measured 10 min after its injection under a dicaprin (○) or egg PC (□) monomolecular film. (B) Variations with surface pressure of the minimal specific activity of HGL* acting on dicaprin monolayers.

amounts of biotinylated enzyme were adsorbed to the phospholipid film.

At fixed surface pressures (20 and 25 $mN \cdot m^{-1}$ for egg PC and dicaprin, respectively), the adsorption of HGL* to lipid films was further examined, in the presence of variable concentrations of biotinylated enzyme. The amounts of HGL* bound to the lipid films as well as lipase activity increased linearly with increasing enzyme concentrations (Table 2). Consequently, the percentage values of adsorbed enzyme did not vary significantly with the enzyme concentration. At a given HGL* concentration, the amounts of enzyme adsorbed to dicaprin films were about 10 times higher than the corresponding values obtained in the case of adsorption to egg PC films (see Figure 2A and Table 2).

Effects of mAbs on the Interfacial Binding and the Catalytic Activity of HGL. Five anti-HGL mAbs (4-3, 25-4, 35-2, 83-15, and 218-13) were previously characterized at our laboratory to determine their immunoreactivity to HGL (Aoubala et al., 1994, 1993a; Ivanova et al., 1993). Using two different substrates, four mAbs (4-3, 25-4, 35-2, and 83-15) were observed to inhibit lipolytic activity of HGL, yielding very similar inhibition patterns and reaching a maximum at an HGL/mAb molar ratio of 2 (Aoubala et al., 1993a; Ivanova et al., 1993). In the present work, we investigated the inhibitory effects of these antibodies on HGL* activity using monomolecular films of dicaprin at a constant surface pressure (25 $mN \cdot m^{-1}$). HGL* (2 μM final concentration) was preincubated with each mAb (HGL* to mAb molar ratio of 2 to 1) for 1 h at 37 °C. The residual catalytic activity, along with the amount of enzyme bound to the interface, was determined. The results are given in Table 3. Among the five anti-HGL mAbs tested, four (4-3, 25-4, 35-2, and 83-15) were found to reduce the residual activity of the enzyme, while mAb 218-13 had no detectable effects. By measuring the binding of HGL* it was established, in the presence of inhibitory mAbs, that the amounts of adsorbed enzyme decreased concomitantly with the decreasing residual activity (Table 3).

As shown in Figure 2A, no enzymatic activity of HGL was detected with egg PC films. This is a considerable advantage since it was also possible to simultaneously measure the surface concentration of HGL* and the corresponding increase in the surface pressure ($\Delta\Pi$) as a function of the initial surface pressure (Π_i), using quite high and noncatalytic enzyme concentrations (see Figure 3). The specific sandwich ELISA described above for mAbs of the IgG 1 isotype was used to quantify the amount of mAbs* adsorbed, alone or complexed to HGL, to egg PC monomolecular films as a function of the surface pressure (Figure 3A). The amounts of the two mAbs* (4-3* and 218-13*) adsorbed either alone or complexed to HGL (mAb* to HGL molar ratio of 0.5) at the lipid/water interface were much

Table 2: Amounts of Biotinylated HGL (HGL*), Expressed in pg/cm^2, Adsorbed on a Monomolecular Film of Dicaprin (25 mN·m^{-1}) and Egg PC (20 mN·m^{-1})[a]

	initial HGL* concn (pM)	HGL* concn in recovered film (ng·mg^{-1})	HGL* concn in bulk phase (ng·mL^{-1})	tot HGL* recovery (%)	HGL* act. (pmol·cm^{-2} min^{-1})	HGL* surface concn (pg·cm^{-1})	film adsorbed HGL* (%)	sp act. of HGL* (μmol·min^{-1}·mg^{-1})
dicaprin	20	1.1/1.1	0.7/0.8	68/82	5.3/5.6	10.0/8.5	1.4/1.2	530/659
	33	2.4/2.6	1.1/1.3	66/79	26.3/24.1	37.0/31.4	2.9/2.5	710/767
	66	6.0/5.8	2.7/2.7	76/82	58.0/61.4	70.0/81.0	2.8/3.2	828/758
	132	11.1/10.7	5.4/5.2	78/79	73.8/72.7	145.0/124.0	2.8/2.5	509/586
egg PC	132	5.2	4.2	60	0	14.7	0.29	0
	400	7.6	6.2	65	0	33.8	0.22	0
	1190	28.0	24.0	62	0	86.5	0.19	0
	3560	138.0	124.0	62	0	438.0	0.32	0
	13200	440.0	375.0	66	0	1600.0	0.33	0

[a] The lipase activity was measured as a function of enzyme concentration. The monolayer experiments and the total recovery determinations were carried out as described in Materials and Methods.

Table 3: Effects of Five Anti-HGL mAbs on the Catalytic Activity and Adsorption of Biotinylated HGL (HGL*) on a Dicaprin Monomolecular Film at a Constant Surface Pressure (25 mN·m^{-1})[a]

HGL*/mAb	HGL* concn in recovered film (ng·mL^{-1})	HGL* concn in bulk phase (ng·mL^{-1})	tot HGL* recovery (%)	residual HGL* act. (%)	HGL* surface concn (pg·cm^{-2})	film adsorbed HGL* (%)	sp act. of HGL* (μmol·min^{-1}·mg^{-1})
HGL*	9.8	5.5	77	100	136	2.6	520
HGL*/mAb 4-3	5.6	4.5	68	8	26	0.5	185
HGL*/mAb 25-4	6.1	5.1	73	7	26	0.5	186
HGL*/mAb 35-2	7.4	5.6	80	28	54	1.1	387
HGL*/mAb 83-15	8.3	5.4	71	50	63	1.3	560
HGL*/mAb 218-13	9.7	5.3	82	98	123	2.4	547

[a] HGL*/mAb complexes were formed by preincubating the enzyme (final concentration 2 μM) with each mAb (HGL* to mAb molar ratio of 2 to 1) for 1 h at 37 °C. The binding and the activity of the enzyme were determined after injecting the complexes (HGL* at 132 pM, final concentration) under the lipid monomolecular film.

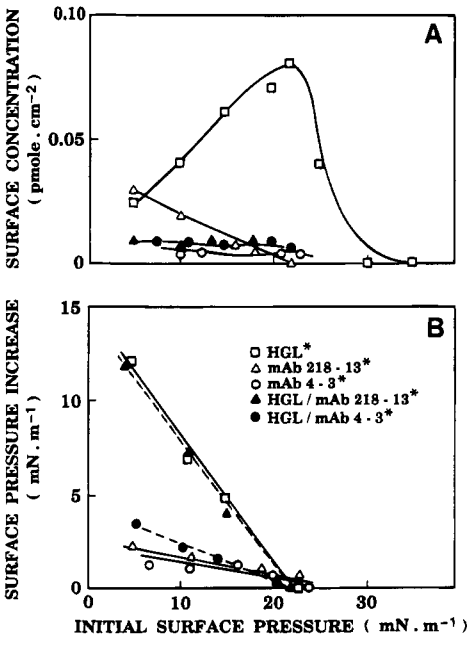

FIGURE 3: (A) Interfacial excesses of HGL*, mAbs*, and their complexes, 20 min after being injected under egg PC monolayers kept at various surface pressures. (B) Maximal increase in surface pressure as a function of the initial surface pressure of an egg PC film, after protein injection. The final protein concentrations were as follows: 13.2 nM with HGL*, 13.2 nM with mAb*, and 20 nM with mAb*/HGL complexes. (□) HGL*, (△) mAb 218-13*, (○) mAb 4-3*, (▲): HGL/mAb 218-13*, (●) HGL/mAb 4-3*.

lower than the amounts of HGL* adsorbed alone (see Figure 3A).

The surface pressure increase at equilibrium was determined and plotted as a function of the initial surface pressure of an egg PC film. The results given in Figure 3B show that, in general, the penetration capacity of mAbs into egg PC films was much lower than that of biotinylated HGL alone. Furthermore, the inhibitory mAb 4-3 was found to significantly decrease the penetration capacity of HGL, while the noninhibitory mAb 218-13 had no detectable effect on this process.

DISCUSSION

Biotin Labeling of HGL and Development of a Specific Sandwich ELISA. Two sandwich ELISA procedures were developed and used to measure the HGL* and mAbs* in excess at the lipid/water interface. The reliability of the above sandwich ELISA for measuring the amounts of HGL* adsorbed to the lipid/water interfaces was ascertained experimentally by estimating the overall recovery rates. Initially, we observed that the total recovery (expressed as a percentage) was significantly lower in the presence of dicaprin films (21 ± 2.3%), while good recovery rates (74 ± 5.5%) were observed in the presence of egg PC films (see Table 1). This result can be explained by the fact that dicaprin, unlike egg PC, is a good substrate for HGL and the negatively charged fatty acids liberated during the hydrolysis process may have interacted electrostatically with the biotinylated enzyme and lowered its immunoreactivity against the pAb used as antigen captor. This interpretation was confirmed by the fact that low recovery values were obtained with oleic acid (around 20%, data not shown). BSA, which is one of the physiological fatty acid carriers, was used throughout the ELISA procedure and induced in all cases an appreciable increase of up to 78 ± 6.2% in the measured total recovery rates (see Table 1). This observation

confirms that BSA, which is commonly used in *in vitro* lipolytic assays, plays an important role in stabilizing the lipolytic activity and desorbing free fatty acids from the lipid/water interface.

Interaction of HGL with Lipid Monolayers. Using catalytically active HGL*, it was possible for the first time to directly correlate the HGL activity with its surface concentration at any surface pressure. We can see from Figure 2A that both the lipase activity and the enzyme interfacial excess depended in a bell-shaped manner on the initial surface pressure, which directly reflects the influence of the "quality" of the lipid/water interface (Verger & de Haas, 1976). It is worth noting that, at surface pressures of less than 15 $mN \cdot m^{-1}$, no lipase activity was detectable, although significant amounts of HGL* were found to be associated with the film.

Since HGL activity is expressed in $molecules \cdot cm^{-2} \cdot min^{-1}$ and the interfacial excess of enzyme is expressed in $pg \cdot cm^{-2}$, a value of the minimal specific activity of HGL* (expressed as usual in $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was obtained at each surface pressure (see Figure 2B). The expression "minimal specific activity" previously coined by Verger et al. (1976) was used here because the experimentally measured enzyme in excess at the interface includes not only the enzyme molecules directly involved in the catalysis but also an unknown amount of lipase molecules present (adsorbed) close to the lipid monolayer. Until now, it is not possible to distinguish between these two enzyme pools, and therefore under our monolayer conditions, it is clear that the estimates of the specific activity are only a minimal value. Furthermore, it can be seen from the data presented in Figure 2B that a continuous increase in the minimal specific activity occurred between 15 and 35 $mN \cdot m^{-1}$ without reaching any optimal value in the range of surface pressures investigated. It is worth noting that the variations in the lipase specific activity with the surface pressure were directly related neither to the enzyme surface concentration nor to the enzyme activity. This result is in agreement with previous data obtained with pancreatic phospholipase A_2 (Verger et al., 1976) and pancreatic lipase (Gargouri et al., 1986a). A linear dependency of the minimal specific activity of these enzymes on the surface density of the lipid monolayer was observed. It can be concluded from these data that the turnover number of these lipolytic enzymes increases steadily with the lipid packing. Furthermore, it can be noted that minimal specific HGL activity obtained at a surface pressure of 35 $mN \cdot m^{-1}$ was in the range of the values obtained under optimal bulk conditions (1000 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) using a tributyrin emulsion in the presence of BSA and bile salts.

When increasing concentrations of HGL* (from 20 to 132 pM final concentration) were injected under a monomolecular film of dicaprin maintained at a constant surface pressure (25 $mN \cdot m^{-1}$), we observed the existence of a good correlation between the lipase activity and the amounts of HGL* in excess at the interface, resulting in a fairly constant minimal specific activity value (see Table 2). A similar type of relationship between HGL* (bulk concentrations ranging from 0.13 to 13 nM final concentrations) and the amount of enzyme present at the lipid/water interface was also observed using nonhydrolyzable egg PC monomolecular films maintained at a constant surface pressure (20 $mN \cdot m^{-1}$). Consequently, the fraction (expressed in percent) of HGL* adsorbed to the interface remained quite constant (see Table 2). This result shows that, under the above experimental conditions (nanomolar range), we are still far from Langmuir's saturating conditions for performing HGL* adsorption to dicaprin and egg PC films. We also studied the adsorption of two mAbs* and their complexes with HGL. The results show that much lower amounts of these antibodies were adsorbed to the egg PC films in comparison with HGL* (see Figure 3A). Moreover, the surface pressure increase experiments showed that these mAbs* had a lower penetration capacity than HGL (see Figure 3B). These results are in good agreement with the finding previously reported by Ivanova et al. (1993) that mAbs of the IgG isotype have low tensioactive properties in comparison with those of HGL or other tensioactive proteins such as BSA, ovalbumin, and β-lactoglobulin (Gargouri et al., 1985). The weak tensioactive properties of IgG molecules are probably due to their Fc fragment, since Fab fragments are even less tensioactive than the intact antibodies (de la Fournière, personal communication).

It is also worth noting that the fraction of adsorbed HGL* (expressed in percent) was 10 times lower using an egg PC film at 20 $mN \cdot m^{-1}$ than with a dicaprin monolayer at 25 $mN \cdot m^{-1}$ (see Table 2 and Figure 2A). Similar findings were previously obtained by Piéroni and Verger (1979). Using mixed trioctanoylglycerol/didodecanoylphosphatidylcholine monomolecular films, the above authors showed that the amount of bound TNB-labeled porcine pancreatic lipase, in the absence of colipase, decreased linearly with increasing proportions of phospholipid in the above mixed films, kept at a constant surface pressure of 12 $mN \cdot m^{-1}$. Under some circumstances, colipase was required for pancreatic lipase to bind to phosphatidylcholine monolayers (Piéroni & Verger, 1979). This behavior was used to advantage 15 years later for the cocrystallisation of the lipase/colipase complex with mixed phosphatidylcholine micelles (van Tilbeurgh et al., 1993). At high surface pressures (30 and 35 $mN \cdot m^{-1}$), above the critical lipid packing corresponding to a surface pressure of 23 $mN \cdot m^{-1}$, HGL* was unable to bind to egg PC films. This finding is in agreement with previous reports by Gargouri et al. (1989) showing that HGL was completely desorbed from the monomolecular film of egg PC at surface pressures of more than 30 $mN \cdot m^{-1}$. These observations suggest that the weak affinities of both digestive lipases for phosphatidylcholine are probably due to repulsive forces originating from the zwitterionic phospholipid head groups, whereas the strong interactions observed between HGL as well as pancreatic lipase and the electrically neutral dicaprin monomolecular films in fact reflect the occurrence of strong enzyme−substrate interactions.

Effects of Anti-HGL mAbs on the Activity and Interfacial Binding of HGL. The immunochemical properties of HGL have been previously studied at our laboratory using several mAbs raised against the native enzyme (Aoubala et al., 1994, 1993a; Ivanova et al., 1993). Four mAbs (4-3, 25-4, 35-2, and 83-15) have been previously found to inhibit the lipolytic activity of HGL, while mAb 218-13 had no effect. Using the monomolecular film technique combined with the sandwich ELISA, we reinvestigated the effects of the above five mAbs on HGL activity and binding to the lipid/water interface. We established clearly that the four inhibitory mAbs significantly decreased the amount of HGL* adsorbed to the dicaprin monomolecular film (see Table 3). Moreover, three of the four inhibitory mAbs (mAbs 4-3, 25-4, and 35-

2) were found to reduce the specific activity of HGL*, while mAb 83-15 had no effect on the specific activity (see Table 3). These results clearly indicate that the immunoreactivity of the latter mAb (83-15) mainly affects the binding of the enzyme to the lipid/water interface, while the other three inhibitory mAbs (mAbs 4-3, 25-4, and 35-2) interfere with both the binding and the catalytic steps of HGL*. Moreover, the four inhibitory mAbs have been found to recognize overlapping epitopes located in the same antigenic region (Aoubala et al., 1994, 1993a; Ivanova et al., 1993). All in all, these results clearly show that the latter epitopic region is probably involved in both the binding and the catalytic activity of HGL at a lipid/water interface and suggest that the lipid binding site and the catalytic site of HGL are close in space.

A further remote epitope which is recognized by the noninhibitory mAb 218-13 seems not to interfere with the interfacial binding of HGL (see Table 3). Under these circumstances, one might expect at first glance that HGL might possibly anchor mAb 218-13 at the interface. In fact, this possibility was ruled out since the mAb 218-13* was not found to be associated with the egg PC film in the presence or absence of HGL (see Figure 3A). This result might be attributable to the fact that the lipid and mAb 218-13* compete for the same region in HGL and that the mAb 218-13*/HGL complex is therefore dissociated in the presence of lipid because HGL has a lower affinity for this mAb (Aoubala et al., 1993a) than for lipid/water interfaces. This interpretation is in agreement with the fact that HGL and the HGL/mAb 218-13* complex display exactly the same tensioactivity when injected under an egg PC monolayer, which indicates that antibody−antigen dissociation occurs in the presence of a lipid film. From the latter example, it is clear that lipids can influence antigen−antibody recognition.

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