Might the Kinetic Behavior of Hormone-Sensitive Lipase Reflect the Absence of the Lid Domain?

Yassine Ben Ali,[‡] Henri Chahinian,[‡] Stefan Petry,[§] Günter Muller,[§] Frédéric Carrière,[‡] Robert Verger,[‡] and Abdelkarim Abousalham*[,‡]

Enzymology at Interfaces and Physiology of Lipolysis, UPR 9025−CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France, and Aventis Germany, 65926 Frankfurt am Main, Germany

Received March 16, 2004; Revised Manuscript Received April 27, 2004

ABSTRACT: Hormone-sensitive lipase (HSL) is thought to contribute importantly to the mobilization of fatty acids from the triacylglycerols (TAGs) stored in adipocytes, providing the main source of energy in mammals. To investigate the HSL substrate specificity more closely, we systematically assessed the lipolytic activity of recombinant human HSL on solutions and emulsions of various vinyl esters and TAG substrates, using the pH-stat assay technique. Recombinant human HSL activity on solutions of partly soluble vinyl esters or TAG was found to range from 35 to 90% of the maximum activity measured with the same substrates in the emulsified state. The possible existence of a lipid−water interface due to the formation of small aggregates of vinyl esters or TAG in solution may account for the HSL activity observed below the solubility limit of the substrate. Recombinant human HSL also hydrolyzes insoluble medium- and long-chain acylglycerols such as trioctanoylglycerol, dioleoylglycerol, and olive oil, and can therefore be classified as a true lipase. Preincubation of the recombinant HSL with a serine esterase inhibitor such as diethyl *p*-nitrophenyl phosphate in 1:100 molar excess leads to complete HSL inhibition within 15 min. This result indicates that the catalytic serine of HSL is highly reactive and that it is readily accessible. Similar behavior was also observed with lipases with no lid domain covering their active site, or with a deletion in the lid domain. The 3-D structure of HSL, which still remains to be determined, may therefore lack the lid domain known to exist in various other lipases.

Free fatty acids (FFAs)[1] are the main source of energy in mammals. It has been recognized for a long time that hormone-sensitive lipase (HSL) plays a crucial role in the mobilization of FFA from the triacylglycerols (TAGs) stored in adipocytes (for a review see ref *1*). In vivo, HSL is activated by phosphorylation via cAMP-dependent kinase in response to various lipolytic hormones such as catecholamines. Phosphorylation of HSL leads to its translocation from the cytoplasm to the lipid droplet (*2*). Insulin acts as an antilipolytic hormone by phosphorylating and activating the phosphodiesterase 3B, which hydrolyzes cAMP and thus reduces the hydrolysis of TAG (*3*). In addition to adipocytes, HSL is expressed in other tissues (*4*), including skeletal muscle, heart, brain, pancreatic β cells, adrenal gland, ovaries, testes, and macrophages (*1*).

The primary sequence of HSL does not show any homology with any other mammalian lipases. This lipase shows some sequence homology, however, with various microbial carboxylester hydrolases such as brefeldin A esterase (BFAE) from *Bacillus subtilis* (*5*), acetylhydrolase from *Streptomyces viridochromogenes* (*6*), lipase 2 from *Moraxella* TA144 (*7*), heroin esterase from *Rhodococcus* sp. strain H1 (HerE) (*8*), and the thermophilic esterases from *Alicyclobacillus acidocaldarius* (EST2) (*9*, *10*) and from *Archaeoglobus fulgidus* (*11*). This group of proteins belongs to the large superfamily of carboxylester hydrolases and was identified as the HSL group (*12*), in which HerE (*8*), BFAE (*5*), EST2 (*10*), and thermophilic esterase from *A. fulgidus* (*11*) have been crystallized and structurally characterized. The 3-D structures of these proteins include a common topological α/β-hydrolase fold, which is also observed in lipases (*13*), consisting of a central parallel β-sheet core surrounded on both sides by α-helices containing the catalytic triad (Ser, Asp/Glu, His). These 3-D structures lack the lid domain covering the active site of many lipases, however.

In previous kinetic studies, it was established that HSL has a broad substrate specificity since it hydrolyzes long-chain TAG, diacylglycerols (DAGs), 1(3)-monoacylglycerols (MAGs), cholesteryl esters, retinyl esters, tributanoylglycerol (tributyrin), and *p*-nitrophenyl butyrate (PNPB) (*14*−*18*). HSL activity toward long-chain acyl esters is generally determined by measuring the release of radiolabeled oleic acid from phospholipid-stabilized emulsions of various substrates in a discontinuous assay system. Under standardized in vitro conditions, the HSL activity toward DAG was found to be about 10-fold and 5-fold higher than that toward

* To whom correspondence should be addressed. Phone: (33) 4 91 16 44 93. Fax: (33) 4 91 71 58 57. E-mail: abousal@ibsm.cnrs-mrs.fr.
[‡] UPR 9025−CNRS.
[§] Aventis Germany.

[1] Abbreviations: BSA, bovine serum albumin; cmc, critical micellar concentration; DAG, diacylglycerol; DGL, dog gastric lipase; E600, diethyl *p*-nitrophenyl phosphate; FFA, free fatty acid; FSC, *Fusarium solani pisi* cutinase; GPL-RP2, guinea pig lipase-related protein 2; HPL, human pancreatic lipase; HSL, hormone-sensitive lipase; PNPB, *p*-nitrophenyl butyrate; TAG, triacylglycerol; TLL, *Termomycess lanuginosus* lipase.

10.1021/bi049479o CCC: $27.50
Published on Web 06/26/2004

TAG and MAG, respectively (*19*). The enzyme activity toward cholesteryl esters is twice as high as its activity toward TAG (*19*, *20*).

Although HSL has been widely studied as a hormone target in adipocytes for over 40 years (*21*−*23*), its contribution to lipid mobilization in adipocytes was recently requestioned. Targeted disruption of the HSL gene in mice showed that the lipase activity is unaffected in brown adipose tissue, and a significant residual lipase activity was still observed in white adipocytes (*24*). By contrast, the HSL activity toward cholesteryl esters was completely abolished in knockout mice (*24*). Haemmerle et al. (*25*) recently reported that HSL deficiency in mice results in the accumulation of DAG in white adipose tissue, brown adipose tissue, skeletal muscle, cardiac muscle, and testes. These authors concluded that HSL is the rate-limiting enzyme for the cellular catabolism of DAG in adipose tissue and muscle (*25*). The hydrolysis of TAG in adipocytes may therefore be attributable to some other nonidentified lipase.

In 1958, Sarda and Desnuelle (*26*) defined lipases in kinetic terms, on the basis of the interfacial activation process. This amounts to the fact that the activity of lipases is enhanced on insoluble substrates (such as emulsions) in comparison with the same substrates in truly monomeric solutions. This property has been used for a long time to distinguish between lipolytic hydrolases and nonlipolytic hydrolases.[2] Moreover, the first 3-D structures of lipases to be published (*27*, *28*) suggested that the interfacial activation process might be due to the presence of the lid domain covering the active site of the enzyme in solution. When contact occurs with a lipid−water interface, a conformational rearrangement might result in the opening of this lid, making the active site accessible. These results were subsequently challenged in view of the kinetic and structural data carried out with true TAG lipases, namely, *Fusarium solani pisi* cutinase (FSC) as well as guinea pig pancreatic lipase-related protein 2 (GPL-RP2). It was observed that no sharp jump (interfacial activation) occurs in the lipase activity at substrate concentrations beyond the saturation point of the solution. The lack of interfacial activation of these true TAG lipases was interpreted in terms of the absence (in the case of FSC) or the partial deletion (in the case of GPL-RP2) of the lid domain. Interfacial activation and the presence of a lid domain are therefore not suitable criteria for defining a TAG lipase (*29*).

Since the presence of HSL in adipocytes was first established and its hormonal mode of regulation investigated, only a few in vitro studies have been carried out with the pure enzyme to determine its kinetic properties. In the present study, it was proposed to further document the kinetic behavior of pure recombinant HSL, using solutions and emulsions of short-chain vinyl esters and TAG, which are partially soluble in water, as well as emulsions of water-insoluble medium- and long-chain vinyl esters and TAG as substrates. In all the cases investigated, the hydrolytic activity of HSL was monitored using the pH-stat method, while the FFAs released were measured potentiometrically. It was previously established that vinyl esters are suitable substrates for pancreatic lipases (*30*, *31*) as well as for microbial lipases (*32*, *33*). As the lipid−water interfacial quality (*29*, *34*, *35*), in terms of the surface tension, is one of the most decisive parameters when working with lipolytic enzymes, valid comparisons can be made only between kinetic data obtained under identical experimental conditions. The results of this study should help to define this important lipase kinetically and gain further information about its substrate specificity.

Another aim of the present study was to investigate the inhibitory effects of the well-known serine esterase inhibitor diethyl *p*-nitrophenyl phosphate (E600) on HSL activity as well as on the activity of various digestive and microbial lipases with well-known structural characteristics. When incorporated into a micellar interface, E600 was reported to efficiently inhibit lipases containing a lid domain such as human gastric and pancreatic lipase (*36*−*38*). The explanation for this inhibitory effect may be that the presence of an interface may induce the opening of the lid covering the lipase active site. Alternatively, in the absence of an interface, the activity of these lipases was not affected by the presence of E600. In the present study, lipases lacking the lid domain, such as FSC and GPL-RP2, were found to be inhibited by E600, even in the absence of an interface. The inhibitory effect of E600 observed in the absence of an interface might be a convenient experimental criterion for predicting the absence of a lid domain in lipases such as HSL.

EXPERIMENTAL PROCEDURES

Reagents. Vinyl acetate, vinyl propionate, vinyl butyrate, vinyl laurate, dioleoylglycerol (diolein), Nonidet-P, E600, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). Triacetylglycerol (triacetin), tripropanoylglycerol (tripropionin), tributyroylglycerol (tributyrin), and trioctanoylglycerol (trioctanoin) were from Acros Organics (Noisy-Le-Grand, France). Crude olive oil was purchased from Lesieur (Aix-en-provence, France). Ni−NTA His.Bind resin was from Novagen. EX-CELL 400 insect cell culture medium was from Valbiotech. Antibiotic−antimycotic solution was from Gibco BRL-Life Science. Protease inhibitors (leupeptin, antipain, and pepstatin) were purchased from Roche Diagnostics. All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Proteins. Recombinant human pancreatic lipase (HPL) was expressed and purified from insect cells as described by Thirstrup et al. (*39*). Recombinant GPL-RP2 was expressed and purified from insect cells as described by Hjorth et al. (*40*). Recombinant dog gastric lipase (DGL) was provided by Meristem Therapeutics (Clermont-Ferrand, France). Recombinant FSC was kindly provided by Dr. M. R. Egmond (Utrecht University). Recombinant *Termomycess lanuginosus* lipase (TLL) was kindly provided by Dr. S. Patkar (Novo Nordisk, Copenhagen). Porcine colipase devoid of phospholipase contamination was purified by J. De Caro (EIPL-Marseille). The protein concentrations were determined using Bradford's procedure (*41*), with Bio-Rad dye reagent and BSA as the standard.

Expression and Purification of Recombinant Human HSL. The full-length human HSL gene was cloned into pFAST-Bac with a C-terminal hexa-His tag. The recombinant virus

[2] For the sake of clarity, among the members of the carboxylester hydrolases family, we now distinguish between the subclasses of lipolytic hydrolases (lipases acting on lipids) and nonlipolytic hydrolases (those not acting on lipids).

was generated using the Bac-to-Bac expression system from Life Technologies in *E. coli* DH10bac, the recombinant "bacmid" virus was transfected into Sf9 cells, and the cells were incubated for 72 h at 27 °C. The virus amplification was performed with 1 mL of the transfection supernatant along with Sf9 cells in a Spinner vessel subjected to bubble-free aeration filled with 1 L of Sf900 II culture medium from Life Technologies supplemented with 5% fetal calf serum. The virus titer was determined by plaque assay: 8×10^7 pfu/mL.

Recombinant human HSL was then expressed in High Five insect cells grown in suspension culture, in Ex-cell 400 medium supplemented with 1% antibiotic−antimycotic solution containing 10000 units/mL penicillin G, 10000 μg/mL streptomycin, and 25 μg/mL amphotericin B (Fungizone) in an orbital shaking incubator set at 27 °C and 140 rpm. When the cells were in the exponential growth phase, at a cell density of approximately 2×10^6 cells/mL, an inoculum of the recombinant baculovirus encoding HSL was added to the cell culture at a multiplicity of infection of around 5, and the culture was further incubated for 48 h. The baculovirus-infected incect cell cultures were harvested by centrifugation at 10000 rpm for 10 min at 4 °C and resuspended in 3 pellet-equivalent volumes of homogenization buffer (25 mM sucrose, 10% glycerol, 1% Nonidet-P, 1 mM β-mercaptoethanol, and protease inhibitors (leupeptin, pepstatin, and antipain at final concentrations of 2, 1, and 2 μg/mL, respectively). The homogenate was mildly sonicated using a Vibra Cell sonicator (2 min at setting 2 followed by 2 min at setting 4 with 1 min intervals on ice between each treatment) and centrifuged at 18000 rpm for 20 min. The supernatant (crude extract) containing HSL activity was used for the purification of the enzyme.

Since the expressed recombinant protein contained hexahistidine tag at its C-terminal end, it was conveniently purified in one step using metal (nickel) affinity chromatography (Ni−NTA resin) in line with the manufacturer's instructions. The crude extract was applied onto a Ni−NTA column (20 mL of resin) equilibrated with buffer A (50 mM sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl, 20% glycerol, 1% Nonidet-P, 1 mM β-mercaptoethanol, and protease inhibitors). The column was washed with 3 column volumes of buffer A and 3 column volumes of buffer A containing 50 mM imidazole. Recombinant human HSL was then eluted by an imidazole concentration gradient ranging from 50 to 250 mM in buffer A, over a total volume of 60 mL, and 2 mL fractions were collected at a flow rate of 0.8 mL/min. The elution of the recombinant HSL was monitored by measuring its activity on vinyl butyrate as a substrate using the pH-stat assay technique. The active fractions were pooled, concentrated, and stored at −80 °C.

HSL Activity Measurements. Lipase activity was assayed by measuring the FFA released from mechanically stirred acylglycerol or vinyl ester solutions or emulsions, using 0.1 N NaOH with a pH-stat (TTT80 radiometer, Copenhagen) adjusted to a constant end point value. Each assay was performed in a thermostated (37 °C) vessel containing 0.25 mM Tris−HCl buffer and 150 mM NaCl. When olive oil or diolein was used as the substrate, these glycerides were pre-emulsified with gum arabic (*42*), and 2 mM $CaCl_2$ (final concentration) was present in the assay performed at pH 8. The rate of spontaneous hydrolysis of the substrate was recorded for 2 min prior to HSL injection, and this background value was subtracted from the activity measurement to obtain the effects of substrate concentration on the specific activity of HSL. HSL activity is expressed as a percentage of the maximum activity ($100v/V_m$) measured on substrate emulsions. The specific lipolytic activities are expressed in international units (IUs) per milligram of enzyme. One unit corresponds to 1 μmol of fatty acid released per minute. To calculate the specific activity of pure HSL accurately, the protein mass was determined on the basis of the amino acid analysis.

Protein Identification by Matrix-Assisted Laser Desorption Ionization/Time-of-Flight (MALDI-TOF) Mass Spectrometry. After HSL was separated electrophoretically by performing SDS−PAGE (10%), the protein band was excised from the gel and subjected to in-gel tryptic cleavage. The resulting peptide fragments were extracted, desalted, and concentrated with pipet tips containing C-18 reversed-phase medium (ZipTip, Millipore). MALDI-TOF was performed using a voyager DE-RP mass spectrometer (Perspective). The peptide mass maps obtained were matched to the theoretical mass values obtained from the *Homo sapiens* genome protein database bank.

Effects of Serine Esterase Inhibitors on the HSL Activity. Pure HSL (0.2 mg/mL, final concentration, in buffer A at pH 7.5) was preincubated at 25 °C with E600 (0.4 mM, final concentration, in an ethanolic solution) at an enzyme:inhibitor molar ratio of 1:100. For the sake of comparison, the inhibitory effects of E600 were also tested with HPL, GPL-RP2, DGL, TLL, and FSC under the same experimental conditions. Phenylmethylsulfonyl fluoride (PMSF; 6 mM, final concentration) was preincubated with pure HSL (0.2 mg/mL, final concentration, in buffer A at pH 7.5) for 15 min at 25 °C. Control experiments were performed without any inhibitor. The residual lipase activity was measured at various preincubation times, using tributyrin as the substrate (see above).

Model Building. The HSL model was generated from the protein structure homology-modeling server with the SWISS-MODEL software program (*43*) using the coordinates of BFAE (PDB code 1JKM) as a template (*5*). The 3-D structures of TLL (open conformation, PDB code 1EIN) (*44*), FSC (PDB code 1CEX) (*45*), and *Torpedo californica* acetylcholinesterase (AchE; PDB code 1AMN) (*46*) were drawn up using the SPOCK program (*47*).

RESULTS

Purification and Characterization of Recombinant HSL Produced in Insect Cells. The purification of HSL was performed in a single step, using a Ni−NTA column. The bound lipolytic activity was eluted by increasing the imidazole concentration from 50 to 250 mM and measured using the pH-stat method with vinyl butyrate as the substrate (see the Materials and Methods). Highly purified HSL, devoid of any detectable contaminants, yielded a single protein band with an apparent molecular mass of 84 kDa in the SDS−PAGE analysis (Figure 1, lane 3). A 7-fold purification factor was achieved, starting with the crude insect cell extract, and the overall rate of HSL recovery was 30%. From 1 L of insect cell culture medium, about 5 mg of pure HSL was obtained, and the specific activity measured on a vinyl butyrate substrate was 143 U/mg.

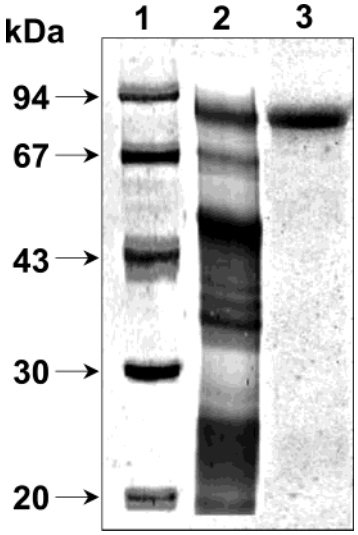

FIGURE 1: SDS−PAGE (12% acrylamide) of HSL at various purification stages. The gel was stained with Coomassie blue to reveal the proteins. Key: lane 1, molecular mass markers; lane 2, HSL crude extracts (see the Materials and Methods); lane 3, pooled fractions containing the active HSL eluted from the Ni−NTA chromatography step.

It was then attempted to determine the N-terminal amino acid sequence of the recombinant human HSL. As previously reported to occur with HSL of other origins (*48*), the N-terminal amino acid of the purified recombinant human HSL was found to be blocked. However, the identity of HSL protein was then determined by performing MALDI-TOF mass spectrometry after in-gel tryptic cleavage of the HSL electrophoretic band (see Materials and Methods). MALDI-TOF mass spectrometry analysis of the resulting peptides showed that they originated from human HSL (data not shown).

Kinetic Behavior of Recombinant HSL Studied with Vinyl Esters as Substrates. During the purification of HSL, using the Ni−NTA chromatographic procedure, we observed that the kinetics of the hydrolysis of vinyl butyrate by crude extracts of HSL-producing insect cells were linear for at least 20 min (data not shown), whereas the kinetics of the hydrolysis of vinyl butyrate by pure HSL fractions were nonlinear (Figure 2). This behavior probably results from the irreversible surface denaturation of pure HSL at the lipid−water interface. As established previously with gastric lipase (*49*, *50*), surface denaturation can be prevented by adding proteins such as BSA. Figure 2 shows the effect of BSA on the rate of hydrolysis of vinyl butyrate (Figure 2A) and diolein (Figure 2B) emulsions by pure HSL. It turned out that adding BSA (0.6 μM, final concentration) resulted in linear kinetic recordings and prevented HSL from undergoing interfacial denaturation. Similar effects of BSA were also observed using an olive oil substrate (data not shown). The final BSA concentration in the assay (0.6 μM) was optimized by measuring the rate of hydrolysis of vinyl butyrate and olive oil emulsions by HSL (data not shown).

Figure 3A shows the effects of increasing concentrations of vinyl butyrate on the rate of hydrolysis by HSL. It can be seen that the kinetic behavior of the enzyme showed no inflection point. The rate of hydrolysis of vinyl butyrate at a substrate concentration (22 mM) corresponding to saturation of the solution amounts to approximately 75% of the maximum rate measured when the emulsion was above the saturation point. The substrate concentration corresponding to half the maximum activity ($k_{0.5}$) was about 10 mM. The maximum specific activity of HSL measured on emulsified vinyl butyrate was 143 U/mg.

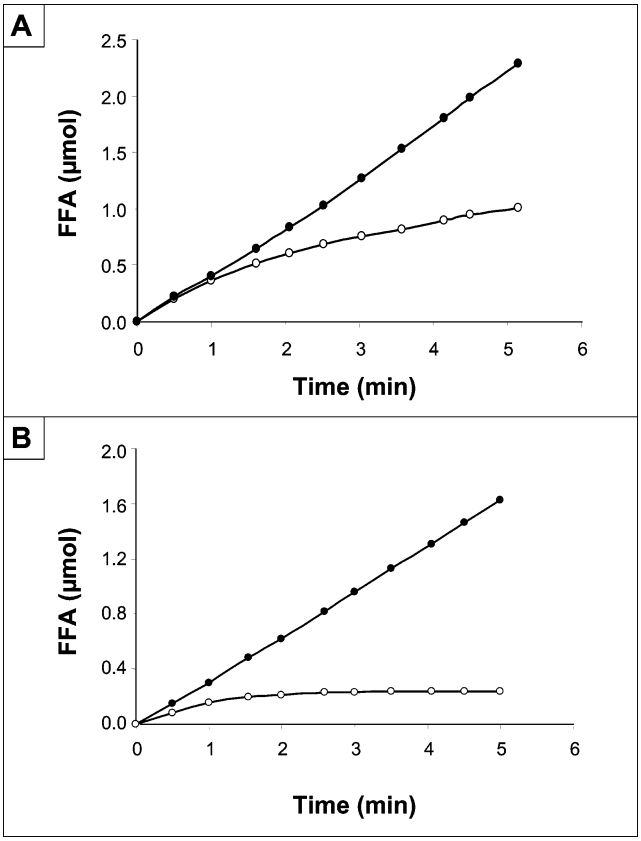

FIGURE 2: Effects of BSA on the kinetics of the hydrolysis of vinyl butyrate (A) and diolein (B) emulsions by pure HSL. Kinetic recordings were performed using the pH-stat technique in the absence (○) or presence (●) of 0.6 μM (final concentration) BSA. The experiments were carried out at 37 °C in 2.5 mM Tris−HCl buffer (15 mL, final volume), containing 150 mM NaCl, 125 μL of vinyl butyrate or diolein pre-emulsified with gum arabic, and 2 mM $CaCl_2$ at pH 7.5. The kinetic recordings shown here are typical of those obtained in three independent experiments.

The kinetic behavior of HSL on vinyl propionate is shown in Figure 3B. It can be seen from this figure that the shape of the curve differs markedly from that obtained with vinyl butyrate. The enzymatic activity reached a value corresponding to about 25% of its maximum activity at a vinyl propionate concentration of approximately 25 mM. The latter value corresponds to the critical micellar concentration (cmc), above which aggregates of vinyl propionate are detected (*51*, *52*). Upon increasing the substrate concentration, the HSL activity increased further, reaching a plateau at the vinyl propionate solubility limit (86 mM). The $K_{0.5}$ value recorded was approximately 70 mM, and the maximum specific activity of HSL toward vinyl propionate observed was 71 U/mg.

The substrate concentration dependence of the initial rate of hydrolysis of vinyl acetate by HSL is shown in Figure 3C. No detectable enzyme activity was measured below the cmc value (100 mM) of the substrate. Above the cmc, the HSL activity increased continuously, reaching a plateau at the solubility limit (300 mM). The $K_{0.5}$ value obtained was

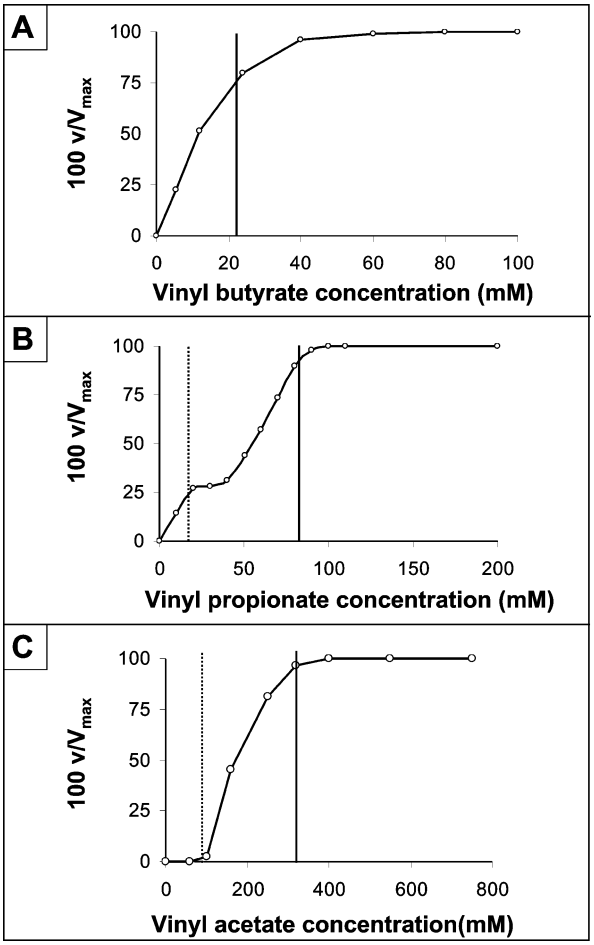

FIGURE 3: Effects of vinyl ester concentration on the rate of hydrolysis of vinyl butyrate (A), vinyl propionate (B) or vinyl acetate (C) by HSL. Activity is expressed as a percentage of the maximum activity ($100v/V_m$). The continuous vertical lines indicate the solubility limit of the esters. The dotted vertical lines indicate the cmc of the esters. Assay conditions were the same as in the experiments presented in Figure 2. The kinetic recordings shown here are typical of those obtained in three independent experiments.

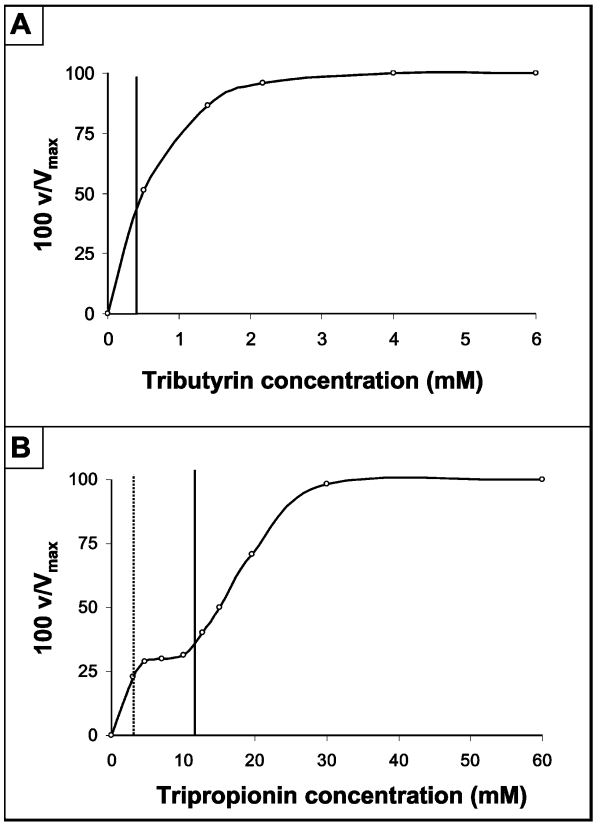

FIGURE 4: Effects of TAG concentration on the rate of hydrolysis of tributyrin (A) and tripropionin (B) by HSL. Symbols are the same as in Figure 3. Assay conditions were the same as in the experiments presented in Figure 2. The kinetic recordings shown here are typical of those obtained in three independent experiments.

approximately 170 mM, and the specific activity recorded was 96 U/mg.

Kinetic Behavior of Recombinant Human HSL Studied with TAG Substrates. The kinetic behavior of HSL toward tributyrin is shown in Figure 4. The enzyme was catalytically active toward a tributyrin solution and reached about 50% of its maximum activity below the solubility limit (0.41 mM (*35*)), with a $K_{0.5}$ value of approximately 0.4 mM. The specific activity of HSL measured on emulsified tributyrin was 12 U/mg.

The substrate concentration dependence of the rate of hydrolysis of tripropionin by HSL is shown in Figure 4B. The kinetic behavior observed here was similar to that of HSL on vinyl propionate (see Figure 3B). HSL activity on a soluble TAG substrate reached about 30% of the maximum value at a tripropionin concentration of approximately 2 mM. The latter value corresponds to the cmc of the substrate. Upon increasing the substrate concentration, the HSL activity increased further, reaching a plateau at the tripropionin solubility limit (12 mM (*35*)). The $K_{0.5}$ value was found to be around 15 mM, and the maximum HSL specific activity measured on emulsified tripropionin was 5 U/mg. Under all the experimental conditions tested (Figures 3 and 4), the rates of hydrolysis were found to be linearly proportional to the amount of recombinant human HSL used (data not shown).

The maximum specific activities of recombinant human HSL toward vinyl ester and TAG emulsions are summarized in Table 1. It can be observed that the rates of vinyl ester hydrolysis recorded here generally exceeded those of TAG hydrolysis, and the highest hydrolysis rates were observed with the C4 acyl chain. It is worth noting that the rate of hydrolysis of diolein by HSL is about 8 times higher than that measured with olive oil (Table 1).

Effects of Serine Esterase Inhibitors on the HSL Activity. Preincubating E600 with pure HSL for 15 min at a molar excess of 100 leads to 100% inactivation of the lipolytic activity observed with tributyrin (Figure 5). Under the same experimental conditions, FSC (which lacks the lid domain) and GPL-RP2 (a lipase with a large deletion within the lid domain) were also inhibited by E600. By contrast, preincubating E600 with HPL, DGL, and TLL, which are known to have a lid domain, does not significantly affect the catalytic activities of these lipases measured under the same experimental conditions (Figure 5). Preincubating PMSF with pure HSL for 15 min at a molar excess of 100 leads to 100% inactivation of the lipolytic activity measured with tributyrin (data not shown).

Surface Distribution of Hydrophobic Side Chain Residues of HSL, TLL, FSC, and T. californica AchE. A model for HSL was developed with the SWISS-MODEL server using the coordinates of BFAE (*5*) as a template. The N-terminal domain (amino acids 1−320) and the regulatory domain were

Table 1: Maximum Rates of TAG and Vinyl Ester Hydrolysis by HSL for Various Substrates[a]

	vinyl acetate	vinyl propionate	vinyl butyrate	vinyl laurate	triacetin	tripropionin	tributyrin	trioctanoin	diolein	olive oil
max specific activity (U/mg)	96	71	143	21	0	5	12	4	30	4

[a] Results are expressed as means of at least two independent measurements.

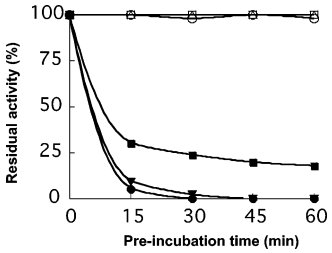

FIGURE 5: Time course of the residual activity of HSL (●), FSC (▼), GPL-RP2 (■), HPL (○), DGL (□), and TLL (△) during preincubation with E600 at a molar excess of 100. The residual lipase activity was measured using a tributyrin substrate, as described in the Materials and Methods.

not included in this model. Figure 6 shows the surface distribution of hydrophobic side chain residues (V, A, L, W, Y, and F) of TLL, FSC, HSL, and AchE viewed in a similar orientation, looking toward the active site serine. Upon comparing these figures, one can see that lipolytic hydrolases (TLL and FSC) are characterized by a tight cluster of hydrophobic side chain residues, whereas in the nonlipolytic hydrolase (AchE), these hydrophobic residues are more widely distributed. From the surface hydrophobic distribution in the HSL model (Figure 6C), this enzyme seems to resemble the lipolytic hydrolases (TLL, FSL) more than the nonlipolytic hydrolase (AchE).

DISCUSSION

HSL was first discovered in the early 1960s and described as an enzyme involved in the mobilization of FFA from the TAG stored in adipocytes. For over 40 years, this enzyme was thought to be regulated hormonally and to catalyze the rate-limiting step in the hydrolysis of intracellular TAG in the adipocytes, from which up to 150 g of FFA is released daily, corresponding to approximately half of the daily human calorie requirements (*1*, *53*−*55*). High levels of FFA have been found to be associated with increased insulin resistance and an increased risk of type 2 diabetes (*56*, *57*). HSL could therefore be a useful pharmacological target for lowering the plasma FFA levels. It is necessary to purify large amounts of this enzyme to be able to determine the 3-D structure, and detailed knowledge of the structure−activity relationship of HSL should be of great help for developing specific inhibitors. In this study, the recombinant human HSL was expressed and purified in a single chromatographic step in the form of a catalytically active enzyme from baculovirus-infected insect cells. Analysis of the primary amino acid sequence of human HSL showed the existence of three putative N-glycosylation sites (Asn 198, Asn 243 and Asn 572). The recombinant human HSL was therefore analyzed to determine whether it is glycosylated. The recombinant HSL expressed in insect cells was found to be nonglycosylated, on the basis of the data obtained using a DIG glycan detection kit supplied by Roche Biochemicals (data not shown). The N-terminal amino acid of the purified recombinant human HSL was found to be blocked and could therefore not be identified by N-terminal amino acid sequencing. A peptide fragment obtained after in-gel tryptic cleavage of the HSL protein band and identified by MALDI-TOF mass spectrometry analysis was found to occur at the N-terminal end of the protein (Thr 5−Arg 32), where it is preceded by an Arg 4 residue.

HSL has been described as an esterase, on the basis of the fact that this enzyme hydrolyzes solutions of PNPB at relatively high rates in comparison with the maximum activity observed on emulsions of the same substrate (*18*). One of the aims of the present study was to reassess the activity of this lipolytic enzyme and to determine kinetically whether it is a lipolytic hydrolase or a nonlipolytic hydrolase (see footnote 2) by measuring the catalytic activity on various vinyl esters and TAG solutions and emulsions. The results obtained show that HSL hydrolyzes water-insoluble long- and medium-chain acylglycerols such as olive oil, diolein, and trioctanoin (Table 1), whereas nonlipolytic hydrolases such as pig liver esterase and *Electrophorus electricus* acetylcholinesterase (*52*) were found to be completely inactive toward these water-insoluble substrates. Consequently, HSL fits the definition of TAG lipases as carboxylesterases that catalyze the hydrolysis of long-chain acylglycerols (*29*, *35*). In agreement with previous studies (*19*, *20*), the rate of hydrolysis of diolein by HSL was found to be about 8-fold higher than that measured with olive oil (see Table 1). This result is in line with recently obtained results on HSL-deficient mice showing that HSL is the rate-limiting enzyme for the cellular catabolism of DAG in adipose tissue (*25*). The specificity of HSL for DAG and the low specific activity of HSL toward TAG may indicate that the mobilization of TAG stores in adipocytes is catalyzed by other hitherto nonidentified lipases.

In addition to long- and medium-chain acylglycerols, the kinetics of the enzymatic hydrolysis of partly soluble vinyl esters (vinyl acetate, vinyl propionate, and vinyl butyrate) and TAG (tripropionin and tributyrin) were investigated. In aqueous media, these substrates sometimes form either isotropic solutions of monomers or micelles or turbid emulsions. They also form adsorbed monomolecular films at air−water interfaces. Substrates of these kinds usually coexist, as the result of a complex equilibrium between potential lipase "monomeric substrates" and supersubstrates (*29*, *35*). The substrate concentration corresponding to half the maximum activity ($K_{0.5}$) of HSL was found to be near the solubility limit of vinyl acetate, vinyl propionate, vinyl butyrate, tripropionin, and tributyrin, whereas in the case of "non-TAG lipases" such as pig liver esterase and acetylcholinesterase from *E. electricus*, the $K_{0.5}$ value was found to be far below the solubility limit of the substrate (*52*). This kinetic parameter has also been taken into account in characterizing carboxylester hydrolases and distinguishing between TAG lipases and non-TAG lipases (*52*). It is worth noting that HSL showed no detectable activity with triacetin

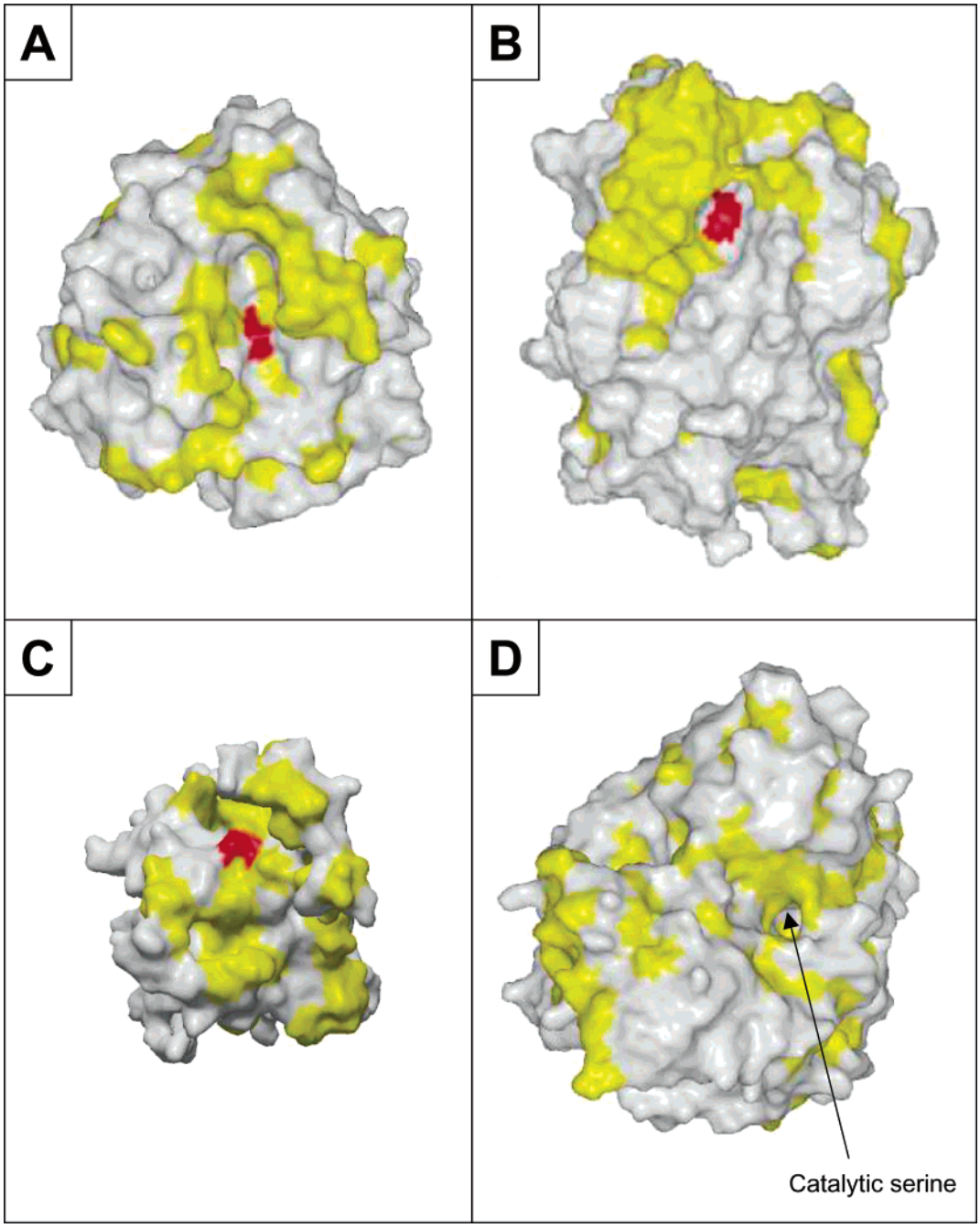

FIGURE 6: Surface distribution of hydrophobic side chain residues (V, A, L, I, W, and F), indicated in yellow, of TLL (open conformation, A), FSC (B), the catalytic domain of HSL (C), and *T. californica* AchE (D) viewed in a similar orientation, looking toward the active site serine (in red). In the case of AchE (D), the arrow indicates the active site gorge in which the catalytic serine is deeply buried (*46*). The HSL model and the 3-D structures of TLL (open conformation), FSC, and *T. californica* AchE were drawn using the SPOCK program.

(Table 1) under our assay conditions. As reported previously, HPL and TLL were found to have little or no activity, respectively, using this short-chain ester (*52*).

During the past decade, the crystal structures of various lipases have been elucidated. It has been established in some cases that the catalytic triad Ser-His-Asp/Glu is covered by a peptide loop (lid) which has to open to give the substrate access to the active site. This movement of the lid, which leads to an open active conformation of the enzyme, was thought to possibly result from specific hydrophobic lipase−substrate interactions at a lipid−water interface. It has been clearly demonstrated that neither interfacial activation nor the presence of a lid domain is a suitable criterion for defining lipases (*29*). In the present study, the kinetics of the interfacial activation of HSL were investigated using vinyl acetate, vinyl propionate, and tripropionin as substrates. As we expected, it is not possible to conclude from the results of these kinetics studies whether a lid domain is present in the structure of HSL. On the other hand, it was established here that HSL is inhibited by serine esterase inhibitors such as PMSF and E600 in the absence of an interface, which suggests that the catalytic serine of the enzyme is directly reactive and may therefore be readily accessible. In this study, similar effects of E600 were observed (Figure 5) on FSC, a lipase without a lid (*58*), and GPL-RP2, a lipase with a large deletion in the lid domain (*59*), whereas the HPL, DGL, and TLL activities were not affected when these lipases were preincubated with E600 in a molar excess of 100. It was

previously reported that HPL (*38*), porcine pancreatic lipase (*37*), and human gastric lipase (*38*) were inhibited by E600 only in the presence of bile salts at concentrations above their cmc. At values above their cmc, bile salts form mixed micelles with the E600 inhibitor, thus inducing the opening of the lid. All in all, it can be concluded from these data that the inhibitory effects exerted by E600 in the absence of an interface could be used as an experimental criterion for predicting the absence of a lid domain in carboxylester hydrolases. It therefore emerges from these results that HSL lacks the lid domain and that the catalytic serine of the hydrolytic enzyme is accessible to the solvent. The absence of a lid domain in HSL was also predicted by modeling the catalytic domain of HSL on the basis of the *Candida rugosa* lipase (*60*) and BFAE 3-D (*5*) structures. However, this model was developed without taking into account either the N-terminal domain (320 amino acid), the function of which is unknown, or the regulatory module of HSL (*5*, *60*). From the surface hydrophobic distribution in the HSL model (Figure 6C), this enzyme seems to resemble lipolytic hydrolases (TLL and FSL) more than the nonlipolytic hydrolase (AchE). The fact that a similar surface distribution of the hydrophobic side chain residues was observed in the active site serines of TLL (with the lid in its open conformation) and FSC (without a lid) argues against the idea that the lid may play a protective role by masking the hydrophobic surface residues of lipolytic hydrolases. It seems to be too early to be able to establish definite structural distinctions between lipolytic hydrolase and nonlipolytic hydrolase on the basis of the qualitative analysis of two TAG lipases (TLL and FSC) and one nonlipolytic hydrolase (AchE) (see Figure 6). Work is now in progress at our laboratory on these lines.

In conclusion, we have described here in detail for the first time, to our knowledge, the kinetics of the activity of HSL toward solutions and emulsions of various vinyl esters and TAG recorded using the pH-stat assay technique. Together with the current availability of large amounts of recombinant human HSL, these findings should lead to a better understanding of the substrate specificity and the structure−function relationships of this lipolytic enzyme. All these recent findings should contribute to developing specific HSL inhibitors which help to reduce the plasma FFA levels and thus to prevent insulin resistance and type 2 diabetes.

ACKNOWLEDGMENT

We are grateful to Dr. L. Sarda (University of Provence, Marseille) and Dr. Stephane Canaan (AFMB-CNRS, Marseille) for helpful discussions and R. Lebrun and D. Moinier (IBSM, Marseille) for N-terminal sequence and mass spectrometric analysis. English revision by Dr. Jessica Blanc is acknowledged.

REFERENCES

1. Holm, C., Osterlund, T., Laurell, H., and Contreras, J. A. (2000) Molecular mechanisms regulating hormone-sensitive lipase and lipolysis, *Annu. Rev. Nutr. 20*, 365−393.
2. Clifford, G. M., Kraemer, F. B., Yeaman, S. J., and Vernon, R. G. (2001) Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation during the lactation cycle of the rat, *Metabolism 50*, 1264−1269.
3. Degerman, E., Landström, T. R., Wijkander, J., Holst, L. S., Ahmad, F., Belfrage, P., and Manganiello, V. (1998) Phosphorylation and activation of hormone-sensitive adipocyte phosphodiesterase type 3B, *Methods 14*, 43−53.
4. Kraemer, F. B., Patel, S., Saedi, M. S., and Sztalryd, C. (1993) Detection of hormone-sensitive lipase in various tissues. I. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies, *J. Lipid Res. 34*, 663−671.
5. Wei, Y., Contreras, J. A., Sheffield, P., Osterlund, T., Derewenda, U., Kneusel, R. E., Matern, U., Holm, C., and Derewenda, Z. S. (1999) Crystal structure of brefeldin A esterase, a bacterial homolog of the mammalian hormone-sensitive lipase, *Nat. Struct. Biol. 6*, 340−345.
6. Wohlleben, W., Arnold, W., Broer, I., Hillemann, D., Strauch, E., and Puhler, A. (1988) Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tu494 and its expression in *Nicotiana tabacum*, *Gene 70*, 25−37.
7. Feller, G., Thiry, M., Arpigny, J. L., and Gerday, C. (1991) Cloning and expression in *Escherichia coli* of three lipase-encoding genes from the psychrotrophic antarctic strain *Moraxella* TA144, *Gene 102*, 111−115.
8. Zhu, X., Larsen, N. A., Basran, A., Bruce, N. C., and Wilson, I. A. (2003) Observation of an arsenic adduct in an acetyl esterase crystal structure, *J. Biol. Chem. 278*, 2008−2014.
9. Manco, G., Adinolfi, E., Pisani, F. M., Ottolina, G., Carrea, G., and Rossi, M. (1998) Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily, *Biochem. J. 332* (Part 1), 203−212.
10. De Simone, G., Galdiero, S., Manco, G., Lang, D., Rossi, M., and Pedone, C. (2000) A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase, *J. Mol. Biol. 303*, 761−671.
11. De Simone, G., Menchise, V., Manco, G., Mandrich, L., Sorrentino, N., Lang, D., Rossi, M., and Pedone, C. (2001) The crystal structure of a hyper-thermophilic carboxylesterase from the archaeon *Archaeoglobus fulgidus*, *J. Mol. Biol. 314*, 507−518.
12. Hemila, H., Koivula, T. T., and Palva, I. (1994) Hormone-sensitive lipase is closely related to several bacterial proteins, and distantly related to acetylcholinesterase and lipoprotein lipase: identification of a superfamily of esterases and lipases, *Biochim. Biophys. Acta 1210*, 249−253.
13. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., and et al. (1992) The alpha/beta hydrolase fold, *Protein Eng. 5*, 197−211.
14. Fredrikson, G., and Belfrage, P. (1983) Positional specificity of hormone-sensitive lipase from rat adipose tissue, *J. Biol. Chem. 258*, 14253−14256.
15. Cook, K. G., Yeaman, S. J., Stralfors, P., Fredrikson, G., and Belfrage, P. (1982) Direct evidence that cholesterol ester hydrolase from adrenal cortex is the same enzyme as hormone-sensitive lipase from adipose tissue, *Eur. J. Biochem. 125*, 245−249.
16. Lee, F. T., Adams, J. B., Garton, A. J., and Yeaman, S. J. (1988) Hormone-sensitive lipase is involved in the hydrolysis of lipoidal derivatives of estrogens and other steroid hormones, *Biochim. Biophys. Acta 963*, 258−264.
17. Wei, S., Lai, K., Patel, S., Piantedosi, R., Shen, H., Colantuoni, V., Kraemer, F. B., and Blaner, W. S. (1997) Retinyl ester hydrolysis and retinol efflux from BFC-1beta adipocytes, *J. Biol. Chem. 272*, 14159−14165.
18. Holm, C., and Osterlund, T. (1999) Hormone-sensitive lipase and neutral cholesteryl ester lipase, *Methods Mol. Biol. 109*, 109−121.
19. Fredrikson, G., Stralfors, P., Nilsson, N. O., and Belfrage, P. (1981) Hormone-sensitive lipase of rat adipose tissue. Purification and some properties, *J. Biol. Chem. 256*, 6311−6320.
20. Osterlund, T., Danielsson, B., Degerman, E., Contreras, J. A., Edgren, G., Davis, R. C., Schotz, M. C., and Holm, C. (1996) Domain-structure analysis of recombinant rat hormone-sensitive lipase, *Biochem. J. 319*, 411−420.
21. Rizack, M. A. (1961) Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3′,5′-phosphate, *J. Biol. Chem. 239*, 392−5.
22. Hollenberg, C. H., Raben, M. S., and Astwood, E. B. (1961) The lipolytic response to corticotropin, *Endocrinology 68*, 589−598.
23. Vaughan, M., Berger, J. E., and Steinberg, D. (1964) Hormone-Sensitive Lipase and Monoglyceride Lipase Activities in Adipose Tissue, *J. Biol. Chem. 239*, 401−409.

24. Osuga, J., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., Shionoiri, F., Yahagi, N., Kraemer, F. B., Tsutsumi, O., and Yamada, N. (2000) Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity, *Proc. Natl. Acad. Sci. U.S.A. 97*, 787−792.
25. Haemmerle, G., Zimmermann, R., Hayn, M., Theussl, C., Waeg, G., Wagner, E., Sattler, W., Magin, T. M., Wagner, E. F., and Zechner, R. (2002) Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis, *J. Biol. Chem. 277*, 4806−4815.
26. Sarda, L., and Desnuelle, P. (1958) Action de la lipase pancréatique sur les esters en émulsion, *Biochim. Biophys. Acta 30*, 513−521.
27. Winkler, F. K., D′Arcy, A., and Hunziker, W. (1990) Structure of human pancreatic lipase, *Nature 343*, 771−774.
28. Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christinasen, L., Huge-Jensen, B., Norskov, L., Thim, L., and Menge, U. (1990) A serine protease triad forms the catalytic centre of a triacylglycerol lipase, *Nature 343*, 767−770.
29. Verger, R. (1997) 'Interfacial activation' of lipases: facts and artifacts, *Trends Biotechnol. 15*, 32−38.
30. Brockerhoff, H. (1968) Substrate specificity of pancreatic lipase, *Biochim. Biophys. Acta 159*, 296−303.
31. Brockerhoff, H. (1970) Substrate specificity of pancreatic lipase. Influence of the structure of fatty acids on the reactivity of esters, *Biochim. Biophys. Acta 212*, 92−101.
32. Martinelle, M., Holmquist, M., Clausen, I. G., Patkar, S., Svendsen, A., and Hult, K. (1996) The role of Glu87 and Trp89 in the lid of *Humicola lanuginosa* lipase, *Protein Eng. 9*, 519−524.
33. Chahinian, H., Nini, L., Boitard, E., Dubes, J. P., Sarda, L., and Comeau, L. C. (2000) Kinetic properties of *Penicillium cyclopium* lipases studied with vinyl esters, *Lipids 35*, 919−925.
34. Verger, R., and de Haas, G. H. (1976) Interfacial enzyme kinetics of lipolysis, *Annu. Rev. Biophys. Bioeng. 5*, 77−117.
35. Ferrato, F., Carriere, F., Sarda, L., and Verger, R. (1997) A critical reevaluation of the phenomenon of interfacial activation, *Methods Enzymol. 286*, 327−347.
36. Desnuelle, P., Sarda, L., and Ailhaud, G. (1960) Inhibition of pancreatic lipase by diethyl-*p*-nitrophenyl phosphate in emulation, *Biochim. Biophys. Acta 37*, 570−571.
37. Maylie, M. F., Charles, M., Sarda, L., and Desnuelle, P. (1969) Action of organophosphates on pancreatic lipase, *Biochim. Biophys. Acta 178*, 196−198.
38. Moreau, H., Moulin, A., Gargouri, Y., Noel, J. P., and Verger, R. (1991) Inactivation of gastric and pancreatic lipases by diethyl *p*-nitrophenyl phosphate, *Biochemistry 30*, 1037−1041.
39. Thirstrup, K., Carrière, F., Hjorth, S., Rasmussen, P. B., Wöldike, H., Nielsen, P. F., and Thim, L. (1993) One-step purification and characterization of human pancreatic lipase expressed in insect cells, *FEBS Lett. 327*, 79−84.
40. Hjorth, A., Carrière, F., Cudrey, C., Wöldike, H., Boel, E., Lawson, D. M., Ferrato, F., Cambillau, C., Dodson, G. G., Thim, L., and Verger, R. (1993) A structural domain (the lid) found in pancreatic lipases is absent in the guinea pig (phospho)lipase, *Biochemistry 32*, 4702−4707.
41. Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-deye binding, *Anal. Biochem. 72*, 248−254.
42. Tiss, A., Carriere, F., and Verger, R. (2001) Effects of gum arabic on lipase interfacial binding and activity, *Anal. Biochem. 294*, 36−43.
43. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) SWISS-MODEL: An automated protein homology-modeling server, *Nucleic Acids Res. 31*, 3381−3385.
44. Brzozowski, A. M., Savage, H., Verma, C. S., Turkenburg, J. P., Lawson, D. M., Svendsen, A., and Patkar, S. (2000) Structural origins of the interfacial activation in *Thermomyces (Humicola) lanuginosa* lipase, *Biochemistry 39*, 15071−15082.
45. Longhi, S., Czjzek, M., Lamzin, V., Nicolas, A., and Cambillau, C. (1997) Atomic resolution (1.0 A) crystal structure of *Fusarium solani* cutinase: stereochemical analysis, *J. Mol. Biol. 268*, 779−799.
46. Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science 253*, 872−879.
47. Christopher, J. A. (1998) *SPOCK: The Structural Properties Observation and Calculation Kit*, The Center for Macromolecular Design, Texas A&M University, College Station, TX.
48. Holm, C., Belfrage, P., and Fredrikson, G. (1989) Human adipose tissue hormone-sensitive lipase: identification and comparison with other species, *Biochim. Biophys. Acta 1006*, 193−197.
49. Gargouri, Y., Pieroni, G., Lowe, P. A., Sarda, L., and Verger, R. (1986) Human gastric lipase. The effect of amphiphiles, *Eur. J. Biochem. 156*, 305−310.
50. Gargouri, Y., Moreau, H., and Verger, R. (1989) Gastric lipases: biochemical and physiological studies, *Biochim. Biophys. Acta 1006*, 255−271.
51. Nini, L., Sarda, L., Comeau, L. C., Boitard, E., Dubes, J. P., and Chahinian, H. (2001) Lipase-catalysed hydrolysis of short-chain substrates in solution and in emulsion: a kinetic study, *Biochim. Biophys. Acta 1534*, 34−44.
52. Chahinian, H., Nini, L., Boitard, E., Dubes, J. P., Comeau, L. C., and Sarda, L. (2002) Distinction between esterases and lipases: a kinetic study with vinyl esters and TAG, *Lipids 37*, 653−662.
53. Langin, D., Holm, C., and Lafontan, M. (1996) Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism, *Proc. Nutr. Soc. 55*, 93−109.
54. Yeaman, S. J., Smith, G. M., Jepson, C. A., Wood, S. L., and Emmison, N. (1994) The multifunctional role of hormone-sensitive lipase in lipid metabolism, *Adv. Enzyme Regul. 34*, 355−370.
55. Kraemer, F. B., and Shen, W. J. (2002) Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis, *J. Lipid Res. 43*, 1585−1594.
56. Roden, M., Stingl, H., Chandramouli, V., Schumann, W. C., Hofer, A., Landau, B. R., Nowotny, P., Waldhausl, W., and Shulman, G. I. (2000) Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans, *Diabetes 49*, 701−707.
57. Bergman, R. N., and Ader, M. (2000) Free fatty acids and pathogenesis of type 2 diabetes mellitus, *Trends Endocrinol. Metab. 11*, 351−356.
58. Longhi, S., Mannesse, M., Verheij, H. M., De Haas, G. H., Egmond, M., Knoops-Mouthuy, E., and Cambillau, C. (1997) Crystal structure of cutinase covalently inhibited by a triglyceride analogue, *Protein Sci. 6*, 275−286.
59. Carriere, F., Thirstrup, K., Hjorth, S., Ferrato, F., Nielsen, P. F., Withers-Martinez, C., Cambillau, C., Boel, E., Thim, L., and Verger, R. (1997) Pancreatic lipase structure−function relationships by domain exchange, *Biochemistry 36*, 239−248.
60. Contreras, J. A., Karlsson, M., Osterlund, T., Laurell, H., Svensson, A., and Holm, C. (1996) Hormone-sensitive lipase is structurally related to acetylcholinesterase, bile salt-stimulated lipase, and several fungal lipases. Building of a three-dimensional model for the catalytic domain of hormone-sensitive lipase, *J. Biol. Chem. 271*, 31426−31430.

BI049479O