Inactivation of Pancreatic Lipases by Amphiphilic Reagents 5-(Dodecyldithio)-2-nitrobenzoic Acid and Tetrahydrolipstatin. Dependence upon Partitioning between Micellar and Oil Phases†

C. Cudrey,‡ H. van Tilbeurgh,§ Y. Gargouri,‡,∥ and R. Verger*,‡

ERS 26 du GDR 1000, Centre de Biochimie et de Biologie Moléculaire du CNRS, 31 chemin J. Aiguier, 13402 Marseille Cedex 20, France, LCCMB du CNRS, Faculté de Médecine Nord, Boulevard P. Dramard, 13916 Marseille Cedex 20, France, and Laboratoire de Biochimie, Ecole Nationnale d'Ingénieurs de Sfax, Route de Soukra, 3038 Sfax, Tunisia

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ABSTRACT: We have reported previously that Cys103 (SH_{II}) of human pancreatic lipase (HPL), unlike the nonessential Cys181 (SH_I), was buried and inaccessible to classical water-soluble sulfhydryl reagents. The lipolytic activity of HPL was lost after the labeling of the above two SH groups with the amphiphilic sulfhydryl reagent, 5-(dodecyldithio)-2-nitrobenzoic acid (C_{12}-TNB), suggesting that the SH_{II} residue may play an important role in the hydrolytic process [Gargouri, Y., Cudrey, C., Medjoub, H., & Verger, R. (1992) *Eur. J. Biochem. 204*, 1063–1067]. For the present experiments, we selected dog pancreatic lipase (DPL), purifying it for the first time, and recombinant guinea pig pancreatic lipase (r-GPL), which both contain a buried SH_{II} group but no accessible SH_I group. The single SH_{II} of DPL and r-GPL reacted only with the amphiphilic SH reagent (C_{12}-TNB), and its labeling was correlated with a rapid lipase inactivation. Although it is spatially remote from the catalytic triad, the SH_{II} group of pancreatic lipases, when chemically labeled, was found to be responsible for the loss of their lipolytic activity. The presence of a bulky dodecyl chain, linked by a disulfide bond to the SH_{II}, may have prevented the critical β-5 loop (residues 76–85) movement by steric hindrance and consequently disturbed the formation of the oxyanion hole. Thus, pancreatic lipase inactivation by the amphiphilic sulfhydryl reagent can be said to be due to the prevention of a productive induced fit. Tetrahydrolipstatin (THL) is an amphiphilic inactivator reacting with the essential serine of the lipase active site. Comparisons of the partitioning between the micellar and oil phases of THL and C_{12}-TNB were made in order to estimate the hydrophobic–lipophilic balance of each inactivator. Its preferential micellar partitioning makes C_{12}-TNB inefficient in the presence of NaTDC, whereas THL is mostly associated with the triglyceride phase even in the presence of bile salts. The latter physicochemical property is probably a requirement for prototypic lipase inactivators to be effective under physiological conditions, i.e., in the presence of bile and lipids.

It has been established that porcine pancreatic lipase (PPL)[1] possesses two cysteine residues, named SH_I and SH_{II} (Verger et al., 1971). SH_I can be selectively labeled with classical water-soluble sulfhydryl reagents, such as DTNB or 4-PDS, whereas SH_{II} does not react with these compounds except in the presence of denaturing agents (0.3% SDS or 8 M urea). Similar results have been obtained for human pancreatic lipase (HPL) (De Caro et al., 1981).

Benkouka et al. (1982) have shown, by performing peptide analysis, that the SH_I group belongs to the Cys181 residue of PPL. The other free cysteine was attributed to either C101 or C103 as the result of ambiguity in the assignment of a disulfide bridge. The SH_{II} group was definitely identified as the side chain of Cys103 by Winkler et al. (1991) after determining the three-dimensional structure of HPL on the basis of X-ray crystallographic data. In searching for the amino acid residues important for the expression of lipase activity, Gargouri et al. (1992) described the stoichiometric modification of SH_I and SH_{II} by 5-(dodecyldithio)-2-nitrobenzoic acid (C_{12}-TNB), a new amphiphilic reagent designed to react with SH_{II} under nondenaturing conditions. However, the loss of lipase activity could not be easily interpreted since Gargouri et al. (1992) were unable to selectively modify SH_{II}. In order to obtain a clear-cut answer concerning the role of SH_{II} in pancreatic lipase activity, we sought other mammalian sources of lipase having only one free cysteine residue at position 103. Dog pancreatic lipase (DPL), the cDNA sequence of which was published by Kerfelec et al. (1986), and the recombinant guinea pig pancreatic lipase (r-GPL) (Hjorth et al., 1993) satisfy this condition. In order to selectively modify the single cysteine residues of DPL and r-GPL, we used the amphiphilic sulfhydryl reagent (C_{12}-TNB), which was found to be preferentially localized and oriented at the oil/water interface (Gargouri et al., 1988, 1991). This SH reagent was found to be totally

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* Author to whom correspondence should be addressed.
‡ Centre de Biochimie et de Biologie Moléculaire du CNRS.
§ Faculté de Médecine Nord.
∥ Ecole Nationnale d'Ingénieurs de Sfax.

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[1] Abbreviations: THL, tetrahydrolipstatin; DPL, dog pancreatic lipase; r-GPL, recombinant guinea pig pancreatic lipase; THL/r-GPL, THL-inactivated recombinant guinea pig pancreatic lipase; HPL, human pancreatic lipase; PPL, porcine pancreatic lipase; C_{12}-TNB, 5-(dodecyldithio)-2-nitrobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, 4,4′-dithiopyridine; NaTDC, sodium taurodeoxycholate; SDS, sodium dodecyl sulfate; IU, international unit, expressed as micromoles of fatty acid released per minute.

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water-insoluble and to form stable monomolecular films at the air/water interface (Ransac et al., 1991).

In order to investigate the effects of the hydrophobic–lipophilic balance of inactivators upon lipase inactivation, we also used tetrahydrolipstatin (THL) as a prototypic serine esterase reagent, in addition to C_{12}-TNB. Tetrahydrolipstatin, derived from lipstatin, which is produced by *Streptomyces toxytricini*, acts in vitro as a potent inhibitor of pancreatic lipases (Hadvàry et al., 1988; Borgström, 1988). It was suggested by Borgström that a stoichiometric covalent acyl–enzyme complex with a long life span was formed. The enzymatically inactive complex of THL and carboxyester lipase was slowly hydrolyzed, regenerating an active enzyme and an open form of THL. More recently, Lüthi-Peng et al. (1992) have shown that THL reacted with the active-site serine (152) of HPL. Ransac et al. (1991) demonstrated that even very low surface concentrations of THL, in water-insoluble mixed monomolecular films of THL and substrate, suffice to obtain complete pancreatic lipase inactivation. The surface molar fraction of THL that reduces lipase activity to 50% is 0.013 mol %. Furthermore, the same authors showed that the THL inactivation process was independent of the surface pressure.

MATERIALS AND METHODS

Dog Pancreatic Juice Collection and Storage. Dog pancreatic juice was collected from healthy dogs, bearing a pancreatic cannula, under caerulein (Cerulex, Farmitalia Carlo Erba, 68 ng/kg/h) and secretin (Sekretolin, Diagnosticum Hoechst, 1.3 units/kg/h) stimulation. Pancreatic juice was collected on a solution of protease inhibitors: PMSF and benzamidine each at a final concentration of 2 mM. Dog pancreatic juice was lyophilized and kept at −20 °C.

Purification of DPL. Crude lyophilized pancreatic juice (500 mg) was dissolved in 13 mL of 50 mM acetate buffer (pH 5.5), 2 mM benzamidine, and 2 mM PMSF and passed through a Sephadex G25 coarse column (length: 53 cm; diameter: 3 cm) equilibrated in the same buffer. The protein fractions eluted with the breakthrough volume were pooled and incubated with a batch of 40 mL of Mono Q gel (trimethylammonium anion exchanger, Pharmacia) in 50 mM acetate buffer (pH 5.5). The nonadsorbed lipase activity (63 000 units) was added onto a column (2.5 cm internal diameter and 11 cm high) of Mono S gel (sulfopropyl cation exchanger, Pharmacia) equilibrated in 50 mM acetate buffer (pH 5.5) and 75 mM NaCl. Ninety-nine percent of the lipase activity was adsorbed. A linear concentration gradient (over 90 min) was applied from 75 to 200 mM NaCl in 50 mM acetate buffer (pH 5.5). The flow rate was adjusted to 1 mL/min. The protein elution was monitored by measuring the absorbance at 280 nm. We obtained three unresolved peaks, the third of which contained lipase activity. At this stage, DPL contained 47% colipase as determined after lipase reactivation. The active fractions were pooled and concentrated with an Amicon cell using a PM 30 membrane. S-200 gel filtration chromatography (column 2.5 cm in diameter and 76 cm high) was performed in 0.1 M Tris-HCl buffer (pH 8) and 150 mM NaCl. The lipase activity was detected in the first protein peak, eluted at a K_{av} of 0.29.

The pure lipase fractions expressed a specific activity of 6000 units/mg with no detectable colipase activity. Polyacrylamide gel electrophoresis in the presence of SDS showed one band with a molecular mass of around 50 kDa. The polyacrylamide gel electrophoresis analysis in the presence of SDS at various purification steps is given in Figure 1. The purification flow-sheet is given in Table I.

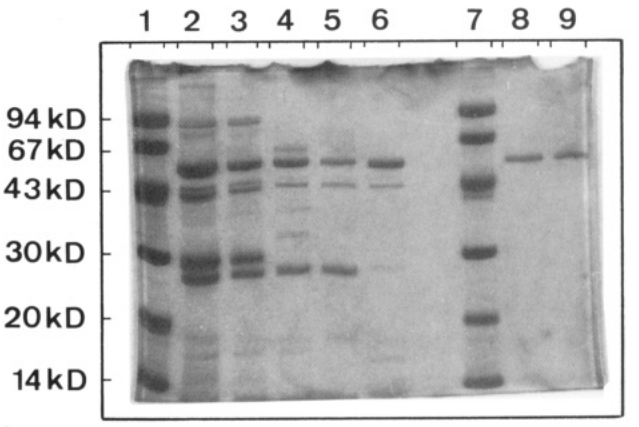

FIGURE 1: SDS–polyacrylamide (12.5%) gel electrophoresis patterns of DPL at various purification steps: lanes 1 and 7, molecular weight markers; lane 2, freeze-dried dog pancreatic juice; lane 3, pooled fractions eluted from G-25 filtration; lane 4, pooled fractions eluted from Mono Q column; lane 5, pooled fractions of proteins adsorbed on the Mono S column; lane 6, pooled fractions eluted from Mono S column; lanes 8 and 9, pooled fractions eluted from S-200 gel filtration.

Table I: DPL Purification Flow Sheet[a]

step	total activity (units)	protein (mg)	specific activity (units/mg)	activity recovery (%)	purification factor
pancreatic juice	65000	196	330	100	1
G-25	62400	148	420	96	1.3
Mono Q	38500	56	1964	59	5.9
Mono S	18400	3.2	5800	28	17.6
S-200	8820	1.5	6000	14	18.2

[a] The results are representative of three independent purifications. One unit is the amount of lipase catalyzing the release of 1 μmol of fatty acid per minute using tributyrin as substrate. Protein amounts were determined using Lowry's method.

Electrofocalization analysis in an ampholine pH gradient between pH 3 and 9.0 (Pharmacia) revealed at least four bands, ranging from pH 5.7 to 6.6. It is worth noting that the N-terminal sequence analysis performed on purified DPL (2 nmol) yielded a double pattern of two amino acid sequences amounting to 20% and 80% of the initial amount of protein. The first amino acid sequence, KEVCYEQIGC FSDAEPWAGT AIRPLKVLPW SPERIGTRFL LYTNKNPN NFQTL, is identical to the results of the cDNA sequence analysis performed by Kerfelec et al. (1986). The second amino acid sequence, KEVCFPRLGC FSDDSPWAGF VERPLKILPW APKDVNWRLL LWWNENPD, shows 70% similarity with the above sequence. It is thus likely that in dogs at least two genes encode pancreatic lipase-like sequences.

Lipids. Tributyrin (puriss) was from Fluka (Buchs, Switzerland). Taurodeoxycholate was from Sigma (St. Louis, MO).

Proteins. r-GPL was a generous gift from Dr. L. Thim and Dr. A. Hjorth from Novo Nordisk (Copenhagen). Porcine pancreatic colipase was from Boehringer. Protein concentration was determined as described by Lowry et al. (1951) using BSA as a reference protein.

Reagents. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Aldrich. 5-(Dodecyldithio)-2-nitrobenzoic acid (C_{12}-TNB) was synthesized at the laboratory as described by Gargouri et al. (1988). An ethanolic solution of C_{12}-TNB was added to 0.1 M Tris-HCl buffer (pH 8) and 1 mM EDTA

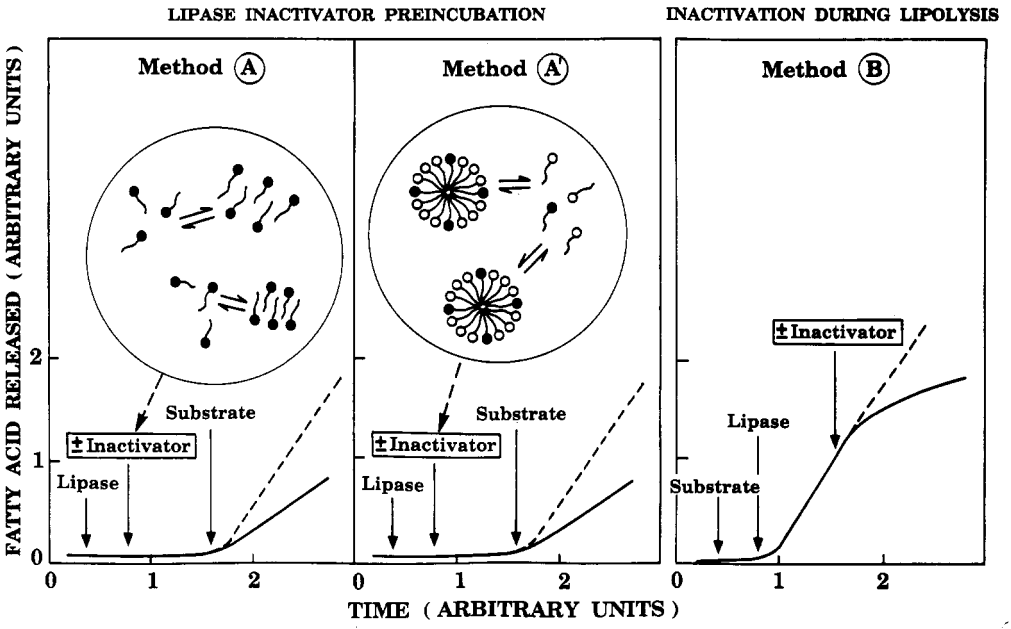

FIGURE 2: Three methods, A, A', and B, were used to study the effects of THL and C_{12}-TNB on lipase activity. Solid arrows indicate the order of the successive injections of inactivator (THL or C_{12}-TNB), lipases (DPL or r-GPL), and substrate (tributyrin). See Materials and Methods for details.

to reach a final concentration of 2 mM in C_{12}-TNB. Tetrahydrolipstatin (THL) samples were a generous gift from Professor A. E. Fischli (F. Hoffman-La Roche, Basel, Switzerland). Radiolabeled [^{14}C]THL (F. Hoffman-La Roche, Basel, Switzerland) was 23.8 mg/mL in toluene and had a specific radioactivity of 4.61×10^8 dpm/mmol.

N-Terminal Sequence Analysis. Automated Edman degradation of the protein was performed using an Applied (Model 470A) gas-phase microsequencer. The phenylthiohydantoin amino acid derivatives were identified by reversed-phase HPLC on an ODS column (4.6 mm/15 cm, Beckman) and were quantitated by an integration program on a Waters 840 data control station (Esch, 1984; Hewick et al., 1981).

Standard Lipase Activity Measurement. Lipase activity was measured titrimetrically at pH 8.0 and 37 °C with a pH–stat (TTT 80, Radiometer) using a tributyrin emulsion as substrate: 0.5 mL of tributyrin added to 14.5 mL of 0.3 mM Tris-HCl, 0.15 M NaCl, 1.4 mM $CaCl_2$, and 4 mM NaTDC. DPL activity was measured in the presence of a 5-fold molar excess of colipase to lipase.

Interfacial Binding of Pancreatic Lipases. Here we used a modified version of the original procedure developed by Borgström to measure the binding of PPL and colipase to a tributyrin emulsion (Borgström, 1975), which was subsequently adapted by Moreau et al. (1991) and Gargouri et al. (1992) to estimate the interfacial binding of native and chemically modified lipases. The lipases DPL (42 IU), HPL (45 IU), and r-GPL (70 IU) were incubated in the absence or presence (4 mM) of NaTDC, with 0.25 mL of tributyrin emulsified in a final volume of 7.5 mL containing 0.3 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 1.4 mM $CaCl_2$. This incubation was performed at 37 °C for 1 min under stirring, and the lipase activity was recorded using the pH–stat method. The reaction medium was then centrifuged at 10000*g* for 10 min. The lipase activity of 1 mL from the top layer of the supernatant was tested under optimal standard assay conditions (0.5 mL of tributyrin in a final volume of 15 mL, as described above). During the subsequent titration assay, in the same vial, we also added the complete contents of the centrifuge tube in order to estimate the total lipolytic activity recovered.

Reaction of DTNB, 4-PDS, or C_{12}-TNB with Lipases. A 0.6-mL cuvette was filled with 0.6 mL of 0.25 M Tris-HCl buffer (pH 8) containing 0.5 mg of DPL or r-GPL and a 100 molar excess of DTNB or 4-PDS. A 0.6-mL cuvette was filled with 0.6 mL of 0.1 M Tris-HCl buffer (pH 8) and 1 mM EDTA containing 0.5 mg of DPL or r-GPL and a 20-fold molar excess of C_{12}-TNB. The number of titrated sulfhydryl groups was calculated from the maximal absorbance using molar extinction coefficient values of 13 600 at 412 nm for TNB (Ellman, 1959) and 19 800 at 324 nm for thiopyridone (Grassetti & Murray, 1967). In some experiments, SDS (0.3% (w/v) final concentration) was added.

Methods To Test Lipase Inactivation. The three methods depicted in Figure 2 [adapted from Moulin et al. (1989)] were used to determine the inhibitory capacity of C_{12}-TNB and THL. These methods differ in their order of addition of lipase, substrate, and inactivator.

Method A. Lipase/inactivator preincubation in the absence of substrate (i.e., no interface). Inactivator aggregates, if existing, are nevertheless available to lipase under these conditions.

Method A'. Lipase/inactivator preincubation in the presence of bile salts in order to create a micellar interface, eventually containing the incorporated inactivator.

Method B. Inactivation during lipolysis.

Phase Partitioning between NaTDC Micelles and Tributyrin Emulsion. A fixed amount of C_{12}-TNB (3 mg) or THL (0.5 mg) was injected into a tributyrin emulsion under conditions identical to those described previously for the lipase activity measurement, and the pH was adjusted with a pH–stat (TTT 80 Radiometer, Copenhagen). After 6 min, the emulsions were centrifuged (6500*g* for 10 min). In the clear supernatant, the C_{12}-TNB concentration was estimated by reading the optical density at 412 nm after the addition of β-mercaptoethanol in order to release the colored TNB ions. Alternatively, the THL concentration in the supernatant (micellar phase) was measured using radiolabeled [^{14}C]THL and a liquid scintillation spectrometer (Beckman LS 3800).

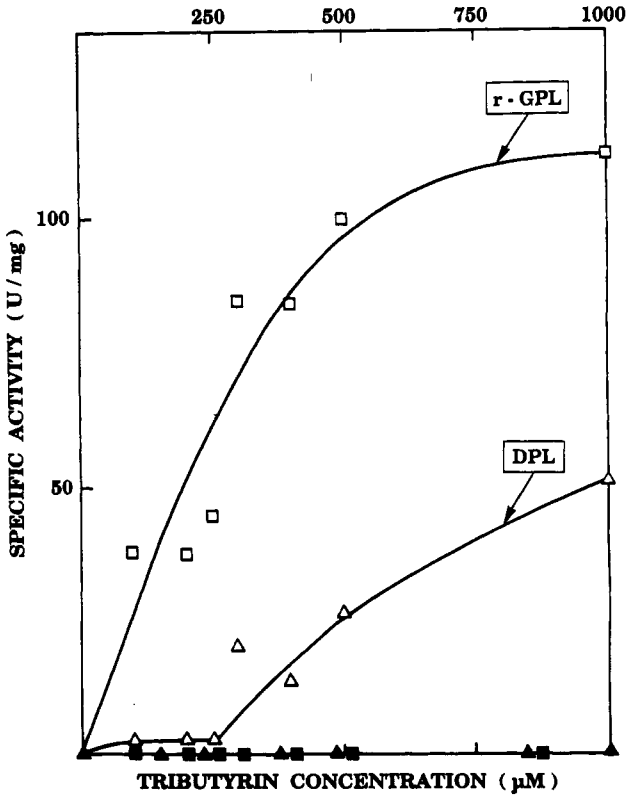

FIGURE 3: Hydrolysis of tributyrin at various concentrations below and above its saturation (400 μM) by DPL (△), C_{12}-TNB-treated DPL (▲), r-GPL (□), and C_{12}-TNB-treated r-GPL (■). Assays were performed either with 2 nmol of enzyme (DPL and C_{12}-TNB-treated DPL) or with 0.3 nmol of enzyme (r-GPL and C_{12}-TNB-treated r-GPL). No interference was observed with C_{12}-TNB in excess during the lipase activity measurement: 40 nmol of C_{12}-TNB in the case of DPL or 6 nmol of C_{12}-TNB in the case of r-GPL was added to the assays performed with untreated lipases (data not shown).

RESULTS

One peculiarity of the kinetic behavior of r-GPL is its insensitivity to the presence of a tributyrin/water interface, unlike DPL, which shows a jump in the enzymatic activity profile when the tributyrin solubility limit is reached (i.e., interfacial activation), as illustrated in Figure 3. Hjorth et al. (1993) have observed that the amino acid sequence of GPL is highly homologous to that of other pancreatic lipases, with the remarkable exception of a deletion in the so-called flap domain. These authors suggested that this deletion may be directly responsible for the anomalous behavior of this enzyme, i.e., the absence of interfacial activation. With a view to interpreting the results of the inactivation experiments using amphiphilic reagents such as C_{12}-TNB and THL, it was of importance to determine experimentally the phase partitioning of the various pancreatic lipases used in the present study in an emulsified system in the absence and presence of 4 mM NaTDC (see Table II). From these results it is clear that, unlike the "classical" pancreatic lipases (HPL and DPL), which preferentially adsorb to a tributyrin emulsion, the outcome of r-GPL partitioning is mainly in favor of the aqueous phase: in the presence of 4 mM NaTDC, the partitioning coefficients of HPL and r-GPL were 0.1 and 2.8, respectively.

Inactivation of DPL and r-GPL by C_{12}-TNB and THL. Method A. When DPL or r-GPL was incubated with classical sulfhydryl reagents such as DTNB or 4-PDS at a molar excess of 100, no SH group was titrated and no inactivation was observed (data not shown). The addition of 0.3% SDS (final concentration) to the reaction medium liberates 0.6 mol of TNB/mol of DPL or r-GPL. These experiments confirm the absence of SH_I in pancreatic lipases of both dog and guinea pig origin. As shown in Figure 4, when DPL or r-GPL was incubated with C_{12}-TNB (at a molar ratio of 20), 0.8 and 0.52 mol of TNB were liberated per mole of DPL and r-GPL, respectively. In both cases, SH group titration was accompanied by a loss of lipase activity on emulsified tributyrin. The above treatment by C_{12}-TNB of both enzymes leads to 97% inactivation.

Table II: Interfacial Binding to a Tributyrin Emulsion of Various Pancreatic Lipases in the Absence (–) or Presence (+) of 4 mM NaTDC[a]

lipases	NaTDC	total lipase activity recovered in aqueous supernatant (%)	total lipase activity recovered in tributyrin phase (%)	partitioning coefficient[b]
HPL	–	25	22	1.1
	+	6	66	0.1
DPL	–	30	nd	nd
	+	5	nd	nd
r-GPL	–	78	1	78.0
	+	34	12	2.8

[a] The lipase activity in the supernatant and that in the tributyrin phase were determined on tributyrin as substrate as described in Materials and Methods. [b] The partitioning coefficient was calculated as the ratio between the total lipase activity recovered in the aqueous supernatant and the total lipase activity recovered in the tributyrin phase. Partial recovery of lipolytic activity (ranging from 46% to 79%) was probably due to interfacial denaturation during the experimental protocol, as observed previously (Moreau et al., 1991).

After treatment with C_{12}-TNB (method A), we checked that both DPL and r-GPL were completely unable to hydrolyze tributyrin at any of the substrate concentrations tested (Figure 3). SH_{II} modification induced a total loss of lipase as well as esterase activity. The distinction between the activities was originally established by Sarda and Desnuelle (1958). The results of this experiment strongly support the idea that the SH_{II} group may be involved in the functioning of the catalytic sites of pancreatic lipases.

Method A corresponds to the incubation of lipase and inactivator in the absence of substrate, i.e., without an interface. In the case of E_{600}, a well-known water-insoluble inactivator of pancreatic lipase, it has been clearly established that the presence of colipase and mixed micelles including E_{600} enhanced the inactivation of the enzyme (Rouard et al., 1978). We therefore investigated the influence of the presence of bile salt micelles during DPL and r-GPL inactivation by THL and C_{12}-TNB (see Table III). Despite the presence of a micellar interface, these experiments are still classified under method A because of the absence of substrate. Bile salts above their CMC have opposite effects on the inactivating power of THL and C_{12}-TNB. In the absence of bile salts, C_{12}-TNB is a more potent pancreatic lipase inactivator than THL. The respective inactivating powers of these two substances were reversed in the presence of bile salts. Furthermore, in the absence of bile salts, we observed that C_{12}-TNB no longer reacted with THL-treated r-GPL (THL/r-GPL) (see Table III).

Method B. In order to compare the inactivatory capacities of THL and C_{12}-TNB during lipolysis, we performed two series of kinetic experiments: with and without bile salts. The lipase was first added to the emulsified substrate. Once the lipolysis had reached a steady state, we injected the inactivator at an enzyme molar excess of 50 000 relative to the enzyme.

In the absence of bile salts, tributyrin hydrolysis by r-GPL was immediately stopped by injecting C_{12}-TNB or THL (267 μM final concentration) (Figure 5A). Likewise, in the case of DPL, the THL injection (53 μM final concentration)

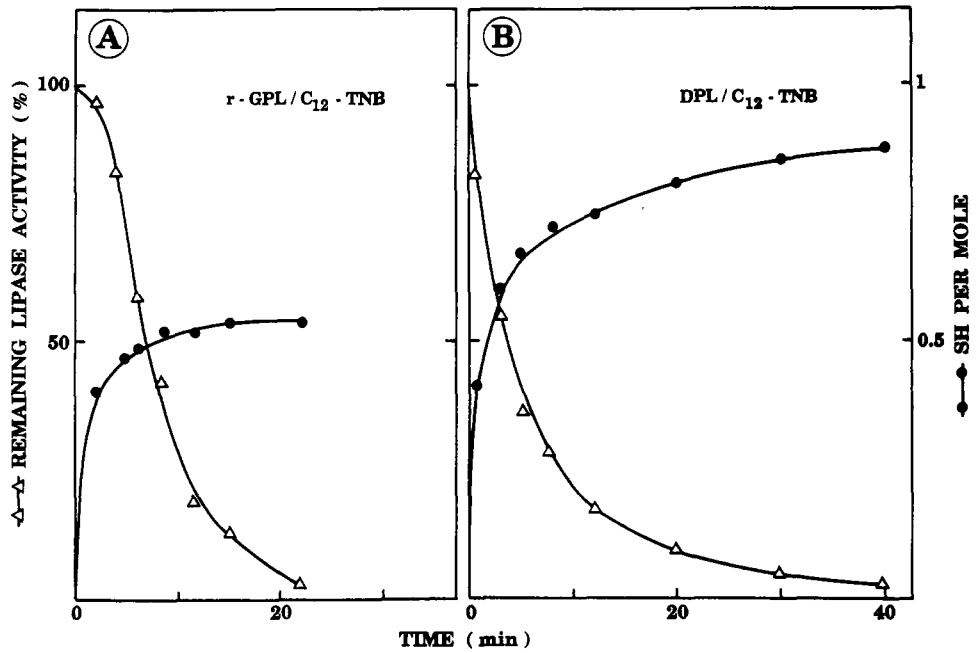

FIGURE 4: Time course of modification of sulfhydryl group induced by C_{12}-TNB: (A) 12 nmol of r-GPL incubated at pH 8.0 (30 °C) with 240 nm of C_{12}-TNB; (B) 8 nmol of DPL incubated at pH 8.0 (30 °C) with 160 nmol of C_{12}-TNB. The TNB released was measured spectrophotometrically at 412 nm (ϵ = 13 600 M^{-1} cm^{-1}). The number of sulfhydryl groups modified per lipase molecule was calculated at various incubation times (●). The residual lipase activity was estimated in parallel by using the tributyrin assay (△).

Table III: Reaction Times Required To Reduce the Initial Lipase Activity by 50% (Method A)[a]

lipases	NaTDC	THL	C_{12}-TNB
DPL	–	>24 h	5 min
	+	11 min	20 min
r-GPL	–	37 min	8 min
	+	<1 min	6 h
THL/r-GPL	–		>24 h[b]

[a] 1.5 nmol of each lipase was incubated with 30 nmol of THL or C12-TNB. The incubations were performed in the absence (–) or presence (+) of 4 mM NaTDC in 100 μL of 0.1 M Tris-HCl buffer (pH 8). The lipase activity was determined on tributyrin substrate as described in Materials and Methods. [b] Reaction time of C_{12}-TNB with inactive THL-treated r-GPL. One microliter of a 0.3 M ethanolic solution of THL (molar excess of 57 to r-GPL) was added to 600 μL of 0.1 M Tris-HCl (pH 8) and 1 mM EDTA containing 0.3 mg of r-GPL. After a 3-h incubation, C_{12}-TNB (molar excess of 20 to r-GPL) was added to the reaction medium. The optical density at 412 nm was recorded over 24 h and never reached a value higher than 0.015. Under the above experimental conditions, the theoretical optical density value for a stoichiometric reaction of r-GPL with C_{12}-TNB is 0.136.

immediately and completely stopped tributyrin hydrolysis (Figure 5C). However, C_{12}-TNB injection (final concentrations ranging from 53 to 212 μM) led to a reduction of only 67% in the initial DPL velocity (see Figure 5C). With higher molar excesses (up to 200 000 relative to the enzyme) of C_{12}-TNB, we systematically obtained the same inactivation levels as the ones obtained with lower molar excesses of C_{12}-TNB (data not shown).

In the presence of bile salts (4 mM NaTDC final concentration), the C_{12}-TNB no longer inactivated either r-GPL (Figure 5B) or DPL (Figure 5D), even when molar excesses of up to 250 000 were used (data not shown). THL injection during tributyrin hydrolysis by r-GPL was just as efficient in both the presence and absence of bile salts. However, the presence of bile salts reduced the inactivation level of DPL by THL. When the concentration of THL was increased (265 μM final concentration), the DPL inactivation levels also increased (data not shown).

Partitioning of THL and C_{12}-TNB between Micellar and Oil Phases. C_{12}-TNB and THL are amphiphilic compounds that are insoluble in water and form stable monomolecular films at the air/water interface (Ransac et al., 1991). We checked that C_{12}-TNB and THL are soluble in tributyrin up to 10% (w/v): after 10 mg of each compound was added to 100 μL of pure tributyrin, the solution was optically clear, and no aggregates were detected as judged by optical density measurements at 500 nm (data not shown). We investigated the behavior of C_{12}-TNB and THL at NaTDC concentrations ranging from 0 to 20 mM, after injecting known amounts of either C_{12}-TNB or THL solutions into a tributyrin emulsion. After centrifugation and separation of the oil from the micellar phases, we observed that C_{12}-TNB could be completely solubilized into NaTDC micelles (Figure 6A). In both the presence and absence of tributyrin, 95% of the initial amount of C_{12}-TNB was detected in the clear micellar supernatant (Figure 6A). Quite the opposite behavior, in terms of phase partitioning, was observed with THL. In the absence of tributyrin, only 15% of the initial amount of THL was solubilized in a 20 mM NaTDC micellar solution. In the presence of tributyrin, THL partitioned preferentially with the oil phase, and negligible amounts of THL were detected in the micellar phase (Figure 6B).

DISCUSSION

The decrease in the activity of a chemically modified lipase on an emulsified substrate can be attributed either to changes in the interfacial adsorption process or to catalysis. In order to determine which of these two elementary steps was involved in the lipase mechanism, we checked the catalytic activity of C_{12}-TNB-treated lipases on emulsified as well as molecularly dispersed tributyrin solutions. We deliberately avoided the use of traditional chromogenic esters, such as *p*-nitrophenol acetate, as models for soluble lipase substrates. Interpretation of kinetic results obtained with *p*-nitrophenol acetate is difficult, first because of its chemical structure, which is unrelated to triglycerides, and second because of its high water reactivity as an activated phenolic ester. We have observed

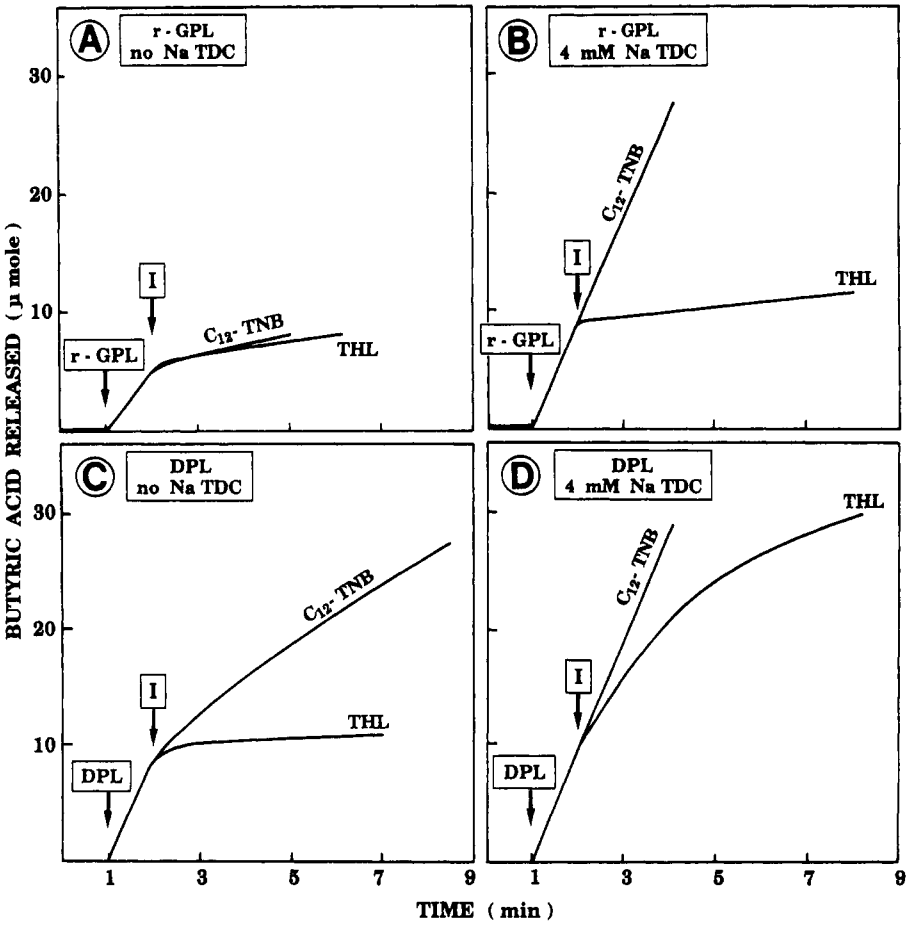

FIGURE 5: Effects of THL and C_{12}-TNB (0.267 mM final concentration) on the rate of hydrolysis of tributyrin by r-GPL (5.3 nM) in the absence (A) and presence (B) of NaTDC (4 mM). Effects of THL and C_{12}-TNB (53 μM final concentration) on the rate of hydrolysis of tributyrin by DPL (1.1 nM, in the presence of a 5-fold molar excess of colipase) in the absence (C) and presence (D) of NaTDC (4 mM). Assays were carried out titrimetrically at 37 °C and pH 8.0 using 0.5 mL of emulsified tributyrin in 14.5 mL of 150 mM NaCl and 4 mM $CaCl_2$. Each kinetic recording is a representative experiment.

that r-GPL, unlike DPL, is not "activated" by a tributyrin/water interface (see Figure 3). In fact, interfacial activation of classical lipases may be viewed as a depressed action on monomeric esters rather than an increased interfacial activity on aggregated substrates. In the presence of a tributyrin emulsion, the question arose as to how r-GPL partitions between the triglyceride/water interface and the monomeric tributyrin solution. As shown in Table II, r-GPL behaved atypically as compared to "classical" pancreatic lipases: the guinea pig lipase was found to be preferentially located in the aqueous phase of a tributyrin emulsion. This behavior is apparently paradoxical in view of the fact that r-GPL was very efficiently inactivated during lipolysis (method B) by water-insoluble compounds such as THL and C_{12}-TNB (see Figure 5A), while the enzyme was mainly located in the aqueous phase (see Table II). It is possible that, due to a rapid enzyme exchange between the water-soluble and the adsorbed forms [E $\leftrightarrow$ E* equilibrium, according to Verger and de Haas (1976)], the inactivation process may have occurred mainly with the transiently adsorbed lipase molecules.

During this study, we observed that the single SH_{II} of DPL and r-GPL was not accessible to water-soluble sulfhydryl reagents such as DTNB and 4-PDS under nondenaturing conditions. The titration of the single cysteine residue of DPL and r-GPL was possible only using the amphiphilic sulfhydryl reagent C_{12}-TNB. This difference in reactivity may be attributable to the hydrophobic character of C_{12}-TNB and its potential capacity to form aggregates. Figure 4 shows the concomitant loss of activity of both lipases with SH group titration. The fact that C_{12}-TNB-treated DPL and r-GPL have lost their enzymatic activity on monomeric tributyrin solutions strongly suggests that the catalytic mechanism is affected by SH_{II} modification. Upon observing an unmodified lipid binding capacity to a tributyrin emulsion of C_{12}-TNB-treated HPL, Gargouri et al. (1992) reached the same conclusion indirectly.

The three-dimensional structure of HPL, published by Winkler et al. (1991), shows an active site buried under a 23 amino acid loop (the "flap") delimited by the disulfide bridge between Cys237 and Cys261. Another lipase from *Rhizomucor miehei*, the structure of which was solved by Brady et al. (1991), also possesses an inaccessible active site. Brzozowski et al. (1991) cocrystallized the latter lipase with *n*-hexylphosphonate ethyl ester and noted that the flap covering the active site had moved, exposing the catalytic triad to the solvent. We took advantage of the "open" three-dimensional structure of the new pancreatic lipase/procolipase complex recently determined by van Tilbeurgh et al. (1993), which has a phospholipid molecule located at the enzyme's active site. Drastic structural changes in the pancreatic lipase architecture occurred upon lipid binding. For instance, a considerable conformation-induced fit led to the creation of the oxyanion hole, which is known to be essential for serine esterase activity. The β-5 loop (residues 76–85) partly covering the active site in the "closed" HPL structure moved away toward the β-strand containing Cys103 to allow the formation of the oxyanion hole (van Tilbeurgh et al., 1993). The presence of a bulky dodecyl chain linked by a disulfide bond to the SH_{II} (Cys103)

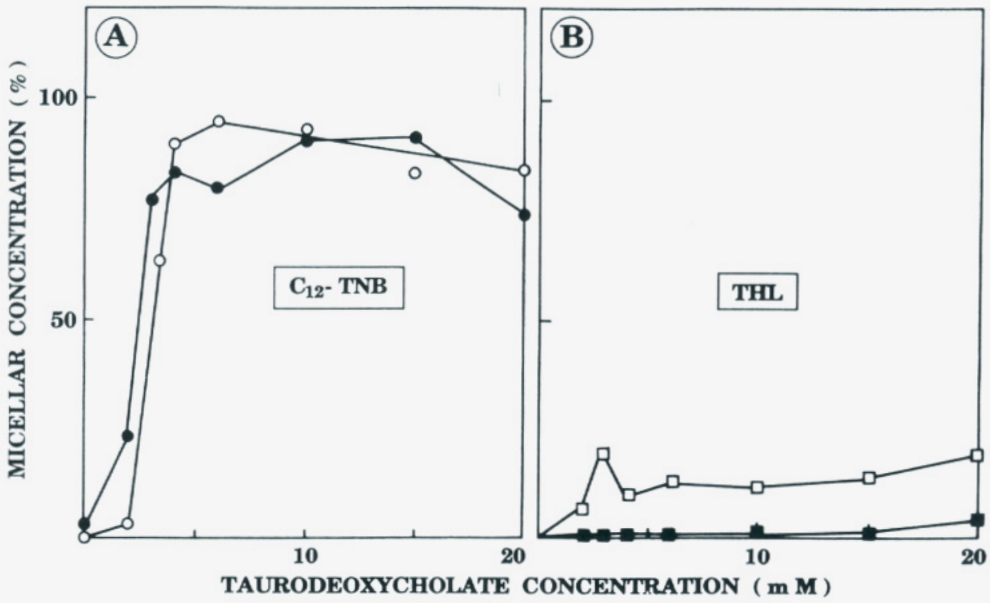

FIGURE 6: Partitioning of C_{12}-TNB (A) and THL (B) between tributyrin and the micellar phases as a function of NaTDC concentration. C_{12}-TNB (1.9 mM final concentration) and [^{14}C]THL (specific radioactivity = 9.96 μCi/mg; 1.3 μM final concentration) were added to an assay containing 0.5 mL of tributyrin and 14.5 mL of 0.3 mM Tris-HCl (pH 8), 1.4 mM $CaCl_2$, and 150 mM NaCl. After incubation at 37 °C under vigorous stirring, the emulsion was centrifuged at 6500*g* for 10 min. The amount of micellar C_{12}-TNB was estimated after adding 10 μL of pure β-mercaptoethanol to 1 mL of the aqueous supernatant. The TNB ions released were measured spectrophotometrically at 412 nm (ϵ = 13 600). The amount of micellar THL was estimated by counting 1 mL of the aqueous supernatant with 10 mL of a scintillation cocktail using a scintillation spectrometer (Beckman LS 3800). Open and closed symbols refer to the absence and presence of tributyrin, respectively.

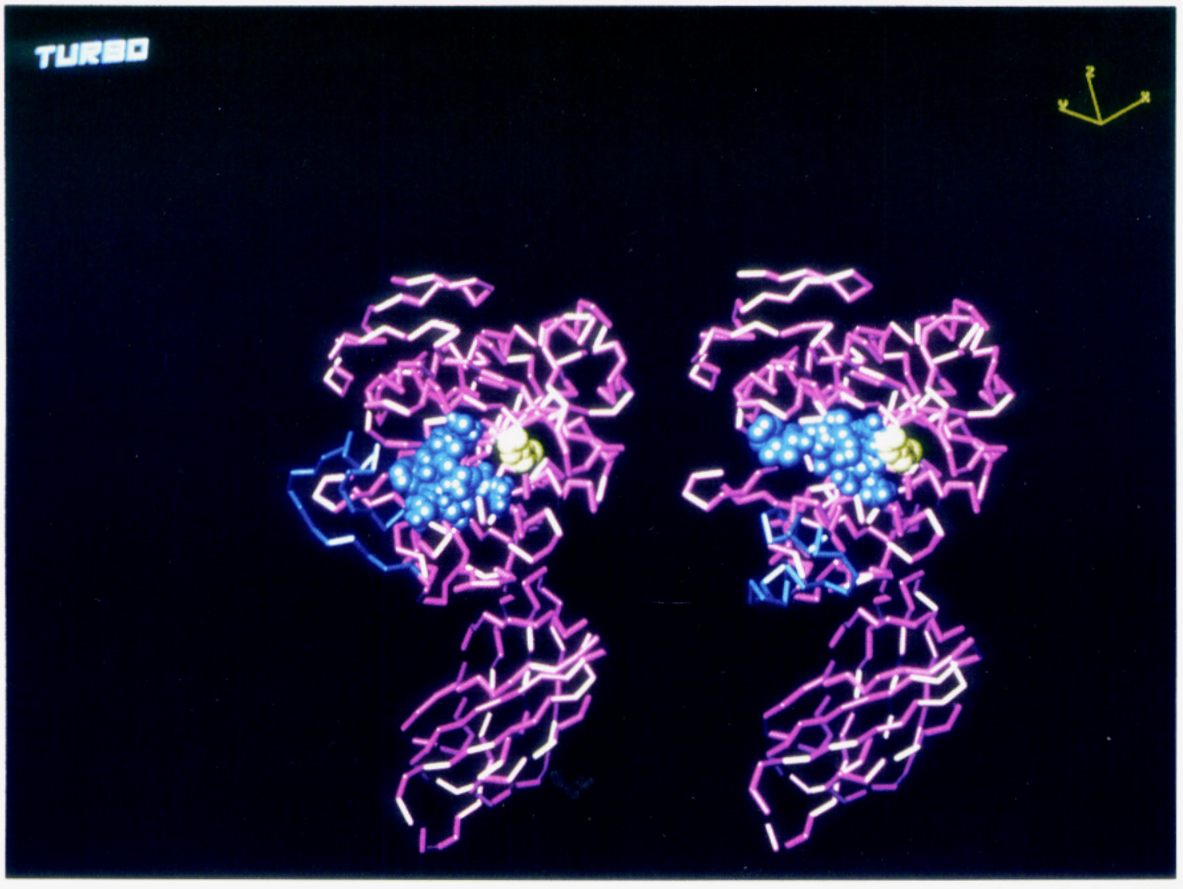

FIGURE 7: C$^{\alpha}$ tracing of the "closed" (left) and the "open" (right) forms of HPL as determined by X-ray crystallography (van Tilbeurgh et al., 1993). The β-5 surface loop (residues 76–85) that undergoes a conformational change upon substrate binding is shown as blue spheres. The flap (residues 238–261) is also in blue. A model of thiododecyl substituent bound to Cys103 is represented as green spheres.

may have hampered the β-5 loop movement due to steric hindrance. The oxyanion hole therefore could not be formed, so that the modified lipase remains inactive on monomeric as well as emulsified substrates. As illustrated in Figure 7, a steric conflict was found to exist between Gly81 of the β-5 loop and Cys103 after its modification by a thiododecyl substituent, whereas this latter substituent can be accommodated into the "closed" structure of HPL.

Table IV: Schematic Summary of the Effects of NaTDC upon Lipase Inactivation by THL or C_{12}-TNB using Method A or Method B[a]

	NaTDC effect	
method	THL	C_{12}-TNB
A (no substrate)	↑	↓
B (during lipolysis)	→↓	↓

[a] Symbols: ↑, increase; ↓, decrease; → no effect on lipase inactivation levels.

Lüthi-Peng and Winkler (1992) have shown that during reaction of the active-site serine 152 with THL large changes in the intrinsic tryptophan fluorescence were observed. These authors concluded that the lipase undergoes a conformational transition upon THL binding. It is thus likely that THL labeling of pancreatic lipases may lead to considerable conformational changes analogous to those described by van Tilbeurgh et al. (1993) in the presence of mixed phosphatidylcholine/bile salt micelles. This suggests that the β-5 loop, in the "open" conformation, could protect the SH_{II} residue against modification by C_{12}-TNB. In fact, we observed that r-GPL inactivated by THL does not react with C_{12}-TNB (see Table III).

In view of the high sequence homologies between r-GPL (Hjorth et al., 1993), DPL (Kerfelec et al., 1986), and HPL (Lowe et al., 1989), and given the drastic and unique conformational changes occurring in the lipase/colipase complex upon lipid binding, we propose a structural explanation for the paradoxically important role of a cysteine residue that is remote from the active site: 16.1 Å is the distance between the γ-sulfur of Cys103 and the β-oxygen of the active Ser152. Up to now, all of the known pancreatic lipase primary sequences included a conserved Cys residue at position 103. We therefore demonstrate here that steric hindrance around the inaccessible SH group (SH_{II}) of pancreatic lipases is deleterious for the expression of lipolytic activity and that this does not depend upon either the existence or the labeling of the accessible SH_I group.

We then focused on the effects of bile salts when comparing the inactivatory capacities of THL and C_{12}-TNB (two amphiphilic compounds) using the two different methods: in the absence of substrate (method A) and during lipolysis (method B). Table IV qualitatively summarizes the effects of bile salts on lipase inactivation under these conditions.

Method A. Lipase inactivation by E_{600}, a classical specific serine esterase inactivator, is known to be favored by the presence of bile salts due to the formation of mixed micelles (Rouard et al., 1978). The reaction of THL with DPL and r-GPL was also enhanced by the presence of bile salts (see Table III). Similar results were previously obtained by Borgström (1988), Gargouri et al. (1991), and Lüthi-Peng and Winkler (1992). The data presented in Figure 6B show that THL is partly solubilized in NaTDC solutions. According to the interpretation given by Lüthi-Peng and Winkler (1992), the THL molecules present in the mixed bile salt micelles probably have easier access to the enzyme's active site than pure THL molecules. Furthermore, as recently demonstrated by van Tilbeurgh et al. (1993), the consequence of the flap opening is the β-5 loop movement, inducing oxyanion hole formation. In turn, this electrophilic area may have stabilized the acylated catalytic Ser152 linked to the open lactonic form of a THL molecule. This would explain why the inactivation of pancreatic lipases by THL, as well as by E_{600}, is more efficient in the presence of bile salts (see Figure 7). In contrast, the inactivation of r-GPL and DPL by C_{12}-TNB was reduced in the presence of bile salts (see Table III). In that latter case, the mixed C_{12}-TNB/bile salt micelles may have also triggered the β-5 loop movement, but due to steric hindrances, this opening may have prevented the SH_{II} from reacting with the sulfhydryl reagents (see Figure 7, right). Thus, the existence of an "open" conformation of the β-5 loop, induced upon binding to bile salt micelles, could explain the opposite effects of these detergents on the inactivation rates of inhibitors, which were targeted either toward the essential serine 152 or the cysteine 103 residue.

Method B. It should be noted that, with method B (inactivation during lipolysis), several kinetic events take place simultaneously. The inactivator must diffuse and be adsorbed at the oil/water interface. The fact that the rate of diffusion and the partitioning coefficient between the various phases contribute to the overall inactivation process as much as the rate of the chemical reaction *sensus stricto* is confirmed by the present data. Since THL and C_{12}-TNB are water-insoluble amphiphilic compounds, they may also affect the physicochemical properties of the interface. This type of inhibition is usually detected by increasing the substrate concentration, i.e., the specific surface of the assay medium. In our assays no such recovery of lipase activity was noted upon further additions of tributyrin, indicating that irreversible covalent inactivation processes had occurred (data not shown).

When THL was injected during lipolysis (method B), the rate of inactivation of r-GPL was not affected by the presence of NaTDC (see Figure 5A,B), whereas the inactivation of both lipases by C_{12}-TNB was completely abolished by NaTDC (4 mM final concentration) (see Figure 5). These drastic and completely opposite effects of bile salts upon the THL and C_{12}-TNB inactivation rates can be partly explained by their respective micellar partitioning. When C_{12}-TNB was added to tributyrin emulsions, its concentration in the micellar phase increased with the bile salt concentration (see Figure 6). At the 4 mM NaTDC concentration used in the inactivation studies described above, 95% of the C_{12}-TNB was detected in the micellar phase. Thus, C_{12}-TNB was desorbed and washed off of the oil/water interface by a micellar solution of NaTDC. Whereas THL was barely solubilized by bile salts, in the presence of a tributyrin emulsion no THL was recovered in the micellar phase.

In conclusion, due to its unique partitioning behavior in the bile salt micellar phase, the inactivatory capacity of C_{12}-TNB was abolished in the presence of NaTDC. As a result, in the presence of an emulsified triglyceride substrate and bile salts THL is by far the most potent inactivator. Tetrahydrolipstatin can even be said to be a prototypically active agent under physiological conditions, i.e., in the presence of bile and dietary lipids (Hadvàry et al., 1978; Meier et al., 1989; Hauptman et al., 1992).

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