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Inhibition of human pancreatic lipase by tetrahydrolipstatin: Further kinetic studies showing its reversibility

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ABSTRACT

Tetrahydrolipstatin (THL, Orlistat) is a potent inhibitor of gastrointestinal lipases. Using the pH-stat technique we report that, in the absence of substrate, THL (at a molar excess of 100) inhibits rapidly (after few minutes of incubation) human pancreatic lipase (HPL). Bile salts over their critical micellar concentration (CMC) were found to accelerate the inhibition process.

At variance with the generally accepted model of a covalent and quasi-irreversible acyl-lipase complex, we showed here that the inhibition of HPL could be rapidly and partially reversed in the presence of an emulsion of short- or long-chain triacylglycerols, as indicated by a kinetic reactivation process. The presence of bile salts in the incubation medium, containing THL and HPL, was found to stabilise the covalent complex as reflected by a decrease in the reactivation rate. Paradoxically, the presence of bile salts in the lipase assay enhanced this reactivation process probably by forming mixed micelles between bile salts and THL, which accelerates the deacylation phenomenon.

On the basis of this kinetic study, a general model is proposed to describe the inhibition of lipases by THL in the aqueous phase as well as its partial reactivation process at the lipid–water interface.

1. Introduction

In higher animals, the intestinal absorption of dietary triacylglycerols (TAGs) requires their enzymatic conversion into the more polar fatty acids and monoacylglycerols by digestive lipolytic enzymes. In human, the hydrolysis of dietary TAG begins in the stomach and is catalysed by human gastric lipase (HGL) [1–4]. The partial hydrolysis of TAG occurring at the pH values (ranging from pH 2 to 6) usually prevailing in the stomach rapidly triggers pancreatic lipase activity in the intestine [5,6]. It has been reported [4] that in experiments on healthy human volunteers during the ingestion of a test meal, mean HGL and HPL concentrations of 0.2 μM and 5 μM, respectively, were recorded in the duodenal content (where the pH values ranged from 5 to 7). The bile salts and colipase concentrations were found to amount to around 6 mM and 5 μM, respectively. However, the *in vitro* kinetic experiments performed in this study were not intended to simulate the *in vivo* conditions actually occurring in the human gastrointestinal tract.

The three-dimensional (3D) crystal structures of digestive lipases have been determined during the last decade (e.g. [7–10]). All these 3D structures belong to the α/β-hydrolase-fold family with a catalytic triad similar to the one found in serine proteases. In the so-called closed conformation, the active site is generally buried under a lid or flap, preventing its accessibility to the substrate. The 3D structures of pancreatic lipases complexed with inhibitors or co-crystallised in the presence of micelles show a large rearrangement of the lid, making the active site accessible to substrates [11–13]. In its open conformation, the lid movement exposes a large hydrophobic surface and at the same time the previously accessible hydrophilic domain becomes buried inside the protein.

Conventional treatments for obesity have focused largely on strategies to control energy intake, however, the long-term efficacy of such approaches is limited [14]. A reduction of dietary fat absorption by inhibitors of digestive lipases was thus proposed as an anti-obesity treatment. Tetrahydrolipstatin (THL, Orlistat), a hydrogenated analogue of lipstatin isolated from *Streptomyces toxytricini*, is a potent inhibitor of gastrointestinal lipases [15–21]. Several reports on the clinical application of THL in the treatment of human obesity have been published since 1991 [22–27]. THL was approved, since 1998 in USA and Europe, as a weight loss

Abbreviations: CMC, critical micellar concentration; CVL, *Chromobacterium viscosum* lipase; HCEL, human carboxyl ester lipase; HGLh, uman gastric lipase; HPL, human pancreatic lipase; NaTDC, sodium taurodeoxycholate; OA, oleic acid; PPL, porcine pancreatic lipase; PSO, purified soybean oil; TAG, triacylglycerols; TC4, tributyrin; THL, tetrahydrolipstatin.

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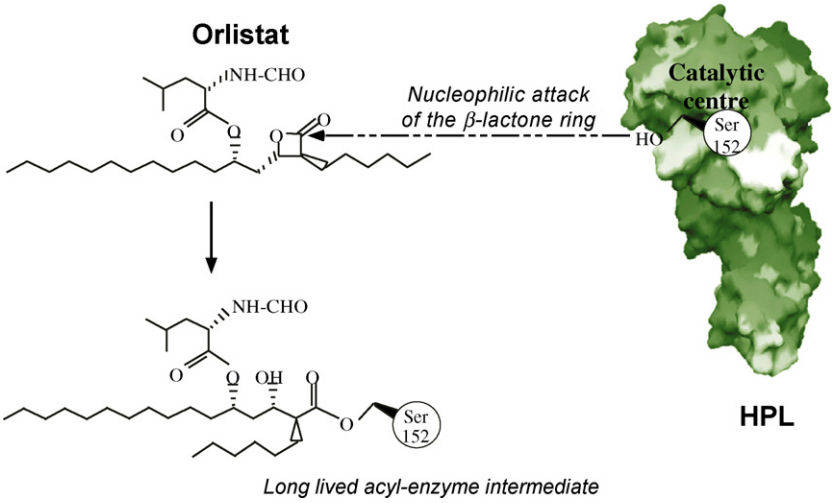

Fig. 1. Schematic illustration of the covalent adduct between the active site serine (152) of HPL and the β-lactone ring of THL in its opened configuration.

medication in the treatment of obesity and it is marketed under the name 'Xenical®'

More recently, THL was found to be a novel inhibitor of the thioesterase domain of fatty acid synthase, an enzyme related to tumour progression [28,29]. Since THL can block cellular fatty acid synthase, the drug has been assessed with success on various tumour cells [30,31].

It has been suggested that a stoichiometric enzyme–inhibitor covalent complex of a long-lived acyl-enzyme type is formed between the open β-lactone ring of THL and the catalytic serine of pancreatic lipase [32–34] (Fig. 1). Nevertheless, inhibition of lipases in general is still poorly understood at the physico-chemical level [35,36]. The partitioning of THL between the micellar and oil phases was performed at our laboratory and THL was found to be mostly associated with the TAG phase, even in the presence of bile salts [37,38]. This interesting physico-chemical property is probably a requirement for prototypic lipase inhibitors to be effective under physiological conditions, *i.e.* in the presence of bile and dietary lipids [39].

From early *in vitro* studies, the inhibition of porcine pancreatic lipase [15,40] was reported to be irreversible whereas the inhibition of human carboxyl ester lipase [18] was found to be reversible and uncompetitive. It was noticed that bile salts above their CMC are needed for THL to be able to effectively inhibit HPL [41] and lipoprotein lipase [42]. The fact that the inhibited HPL could be reactivated by reducing the bile salt concentration below its CMC suggested that, bile salts (above their CMC) may stabilise the acyl-lipase complex [41]. Potthoff et al. [43] reported a reversible and competitive inhibition of *Chromobacterium viscosum* and *Rhizopus oryzae* lipases. Moreover, after inhibition of *C. viscosum* lipase, THL remains chemically unchanged, suggesting the existence of a non-covalent THL/lipase complex.

Although THL is highly effective in blocking the activity of mammalian lipases such as carboxyl ester lipase, originally THL did not appear to inhibit lipases of microbial origin [18]. This author used emulsified tributyrin as substrate to check the inhibition by THL of extracellular lipases from *Staphylococcus aureus* and *Rhyzopus arrhizus* and no effect was observed.

More recently, Mosbah et al. [44] reported the inhibition by THL of a lipase (43 kDa) isolated from *Staphylococcus xylosus*. The authors observed enhanced inhibition in the presence of approximately 4 mM sodium deoxycholate and used inhibition kinetics to classify *S. xylosus* lipase as a serine enzyme. Similarly, the lipase produced by a novel strain of *R. oryzae* WPG was isolated, characterized, and also recognized as a serine enzyme according to its inhibition by THL [45]. Finally, the same group of researchers [46] isolated a novel lipase (50 kDa), from the digestive glands of a scorpion, which was inhibited by THL at a molar ratio THL/lipase of 100. Asler et al. [47] reported a convincing mass spectrometry evidence of a covalently bound THL molecule at the catalytic serine of *Streptomyces rimosus* lipase. Interestingly, these authors reported that although the sample of THL-modified lipase was completely inactivated, both THL-bound and THL-unbound lipase molecules were detected by gel electrophoresis. These observations could be re-interpreted in the light of our present findings by the existence of a partial reactivation process.

The aim of this study was to investigate the inhibition properties of THL on HPL activity, using the pH-stat technique and short- or long-chain TAG as substrates, as well as to systematically study the effects of bile salts on the inhibition efficacy and the reactivation phenomenon.

2. Experimental

2.1. Materials

Tributyrin (TC4) "purum grade" was from Fluka (Buchs, Switzerland). The edible soybean oil was purchased from the company Lesieur (Neuilly-sur-Seine, France) and purified to remove the free fatty acids and partial glycerides on a column of silicic acid (Merck) equilibrated with hexane. The TAG-containing fractions were eluted using stepwise increases in ethyl-ether content, and dried under a vacuum. Using the oil drop tensiometer [48], we checked that no tensioactive contaminants were present in the purified soybean oil (PSO) (interfacial tension variations lower than 0.05 mN m^{-1} h^{-1}). The PSO was stored at −20 °C under an argon atmosphere.

Sodium taurodeoxycholate (NaTDC) was purchased from Sigma® (St. Louis, MO). A stock solution of NaTDC (15 mM) was prepared in 1 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl, 5 mM $CaCl_2$ and was kept at 4 °C. THL (M_w 496) was from Hoffmann-La Roche Ltd., Basle, Switzerland. A stock solution of 10 mM in ethanol was prepared and kept at 4 °C.

Purified HPL [49], and purified porcine pancreatic colipase [50] were a generous gift from J. De Caro (LLE-Marseille). Purified recombinant HPL [51] was a generous gift from F. Carrière (LLE-Marseille) and was used for mass spectroscopy analysis.

2.2. Lipase-inhibitor pre-incubation (methods A and A′).

This method was set up to test in an aqueous medium and in the absence of substrate, the possible direct reactions between lipases and inhibitors [52]. The residual lipase activity was then measured on a mechanically emulsified TAG, using the pH-stat technique. In this study, the pre-incubation of HPL with THL was carried out using two slightly different methods.

2.2.1. Method A

250 μl of HPL solution (0.2 mg ml^{-1} in buffer at pH 8.0) was pre-incubated at 25 °C with 10 μl of THL solution (10 mM in ethanol) at a HPL to THL molar ratio of 1:100, in the presence of a fivefold molar excess of colipase to HPL. The final mixture was continuously stirred using a rotating device at 30 rpm. A control experiment was performed without THL.

2.2.2. Method A′

The experimental conditions were as in *Method A* but NaTDC (4 mM final concentration) was added to the incubation medium in order to create a micellar solution containing THL molecules.

2.3. Lipase activity measurements

The lipase activity was assayed by measuring the fatty acids released from mechanically stirred emulsions of TAG (either TC4 or PSO), using 0.1N NaOH with a pH-stat (Metrohm 718 STAT Titrino, Switzerland), adjusted at an end point value of pH 8. Kinetic assay were performed in a thermostated (37 °C) vessel containing 0.5 ml TAG and 14.5 ml of a 1 mM Tris–HCl buffer, 150 mM NaCl and 5 mM $CaCl_2$. Pure colipase was added in the assay medium at a molar excess of 5 with respect to HPL. When required, 4 mM of NaTDC was added to the assay medium prior to HPL injection.

PSO was not pre-emulsified with gum Arabic as described in earlier publications (e.g. [53]), since we showed recently [54] that gum Arabic can affect the binding as well as the activity of HPL on various TAG, particularly in the absence of bile salts.

2.4. Treatment of the kinetic data

In order to fit the experimental kinetic curves with the general theoretical equation derived from the model proposed by Verger et al. [55] we have used the following simplified equation:

$$P = \frac{C}{B}t + \tau\frac{C}{B}(e^{-t/\tau} - 1) \quad \text{(I)}$$

where P is the product concentration (molecule/volume) and t, time (min). (dP/dt) = C/B when time tends to infinity. C and B are complex parameters resulting from a combination of various individual kinetic rate constants (for their exact definition, see Ref. [55, p. 4025]).

Under steady state conditions, C/B is thus the maximal reaction rate and τ (lag time) is the intercept with time axis of the asymptote from Eq. (I) (see Fig. 2). The values of C/B and τ are given after curve fitting of the experimental data with Eq. (I), using KaleidaGraph 3.0 software.

From Eq. (I), one can see that theoretically the initial rate (at $t = 0$) is equal to zero. However, in order to estimate experimentally the initial activity of HPL we measured the slope of the kinetic recordings 1 min after adding HPL–THL mixture into the lipase assay.

2.5. MALDI-TOF mass spectrometry analysis

MALDI mass spectrometry was performed on a Perceptive Voyager DE-RP (Perceptive Biosystems) time-of-flight mass spectrometer. A solution of sinapinic acid in 60/40 water/acetonitrile was used as a matrix. 100 μl of HPL solution (0.2 mg ml^{-1} in a buffer at pH 8.0) was pre-incubated at 25 °C with 4 μl of THL solution (10 mM in ethanol) at a HPL:THL molar ratio of 1:100. The mixture was continuously vortexed during incubation. A control experiment was performed without THL. A sample of 0.7 μl from a solution of HPL or the HPL–THL incubation mixture was deposited and mixed on the plate target with an equal volume of the above matrix, and the spot was allowed to air-dry. Ions were accelerated with an extraction voltage of 25 kV. Spectra were obtained by accumulation of 256 laser shots.

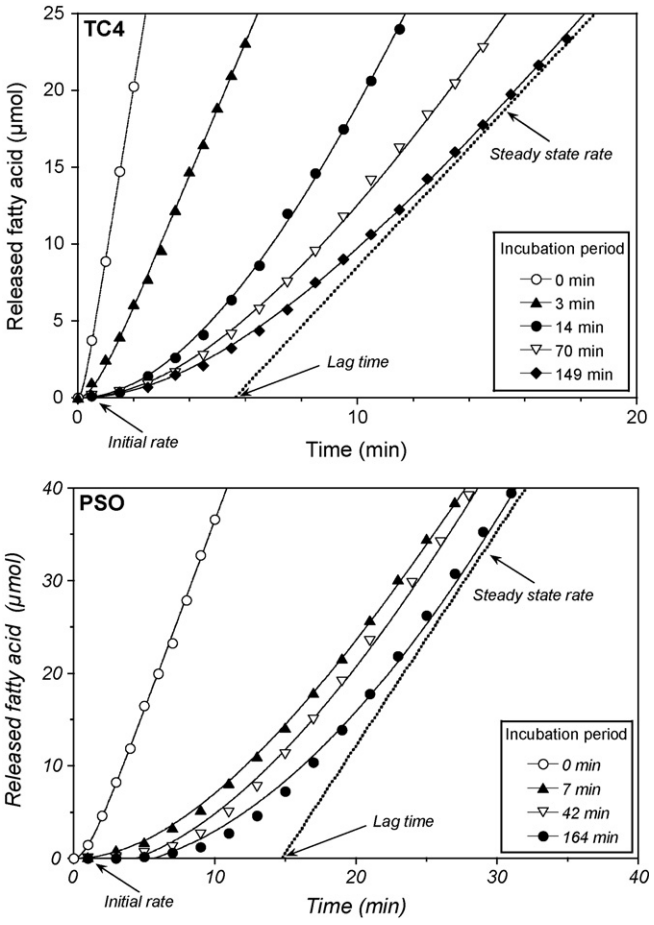

Fig. 2. Typical kinetic recordings of the hydrolysis of TC4 emulsion (upper panel) or PSO emulsion (lower panel) by HPL, using the pH-stat technique. HPL was incubated with THL according to the *Method A′* (in the presence of 4 mM NaTDC). At various time intervals, 10 μl of the incubation medium (around 2 μg of HPL) was injected in the pH-stat vessel, after recording the background hydrolysis for 2 min. Kinetic assays were performed in a thermostated (37 °C) vessel containing 0.5 ml TC4 (upper panel) or PSO (lower panel) mechanically emulsified in 14.5 ml of 1 mM Tris–HCl buffer, 150 mM NaCl and 5 mM $CaCl_2$. Colipase was added to the assay vessel at a fivefold molar excess prior to HPL injection. Kinetic recordings are representative of three independent experiments. The dashed line corresponds to the asymptote reached under steady state conditions obtained by curve fitting the kinetics using Eq. (I) (see Section 2). τ (lag time) is the intercept with time axis of the dashed line, P is the product concentration (molecule/volume) and t, time (min).

3. Results

3.1. Inhibition kinetics of HPL by THL

The pre-incubation of HPL with THL (at a molar ratio of 1–100) was performed in the absence or in the presence of bile salts at 25 °C and pH 8.0. The residual lipase activity was measured with the pH-stat technique either on TC4 or PSO emulsions at 37 °C, using aliquoted samples taken from the incubation medium, as

described under Section 2. We used emulsions of short (TC4) and long (PSO) chain TAG, having very different physico-chemical properties, in order to be able to evidence the intrinsic mechanisms of the interfacial lipase kinetics.

Typical kinetic recordings of the hydrolysis by HPL of a TC4 (Fig. 2 upper panel) or a PSO (Fig. 2 lower panel) emulsion, after various incubation times with THL in the presence of 4 mM NaTDC, were fitted according to Eq. (I) (see Section 2). A good adjustment could be obtained between the experimental data and the above theoretical equation (correlation factor values, $r \geq 0.98$) allowing the measurement of the residual initial activity (at time 1 min) as well as the steady state residual activity and the lag time. However, these lag phases have a totally distinct kinetic origin from the lag phases originally described by Verger et al. [55] and reflecting the rate limiting step of the enzyme penetration into a preformed lipid/water interface.

After few minutes of incubation with THL, HPL initial activity measured either on TC4 (Fig. 2 upper panel) or PSO (Fig. 2 lower panel) emulsions, was drastically reduced. The lipolytic activity, however, progressively increased with time to reach a steady state regime after a lag period of few minutes. Similar kinetic recordings were obtained after 24 h of incubation of HPL with THL (data not shown) or when the kinetic measurements were carried out at pH 6.5. When the inhibited HPL was added to the lipase assay 10 min before adding the TAG substrate (TC4 or PSO), the kinetic recordings (data not shown) showed a similar shape as in Fig. 2. This result indicates that the reactivation process of the HPL is not the consequence of its dilution into the aqueous phase.

Control experiments without THL were run in parallel to each assay, and showed linear kinetics without significant change in hydrolysis rate after 3 h of incubation (data not shown). In the presence of colipase at a molar excess of 5 with respect to HPL, the specific activity (IU/mg) of HPL in the absence or in the presence of 4 mM NaTDC was respectively 7310 ± 258 and 6259 ± 411, when using TC4 as substrate. When using PSO as substrate, these values were 1848 ± 97 and 3145 ± 46, respectively.

The residual HPL activity, measured under the steady state conditions decreased rapidly as a function of the incubation time and reached a plateau of around 40–60% of its initial value when using TC4 (Fig. 3 upper panel) and 50–70% when using PSO (Fig. 3 lower panel) as substrates. These plateau values are not much dependant upon the presence of 4 mM NaTDC either in the incubation medium or in the assay.

No difference in HPL inhibition was noticed as judged by the initial rate or by the steady state residual activity, when the incubation was performed in the absence of colipase (data not shown). Therefore, colipase seems not to be involved in the THL inactivation of HPL in the presence of bile salts in agreement with a previous observation [18].

In the presence of 4 mM NaTDC and colipase in the incubation medium, a rapid and nearly complete reduction of HPL residual initial activity was observed when using either a TC4 emulsion (Fig. 4 upper panel) or a PSO emulsion (Fig. 4 lower panel) as substrate. Namely, after a period of 1 min of incubation with THL only 50% of the initial activity of HPL was detected using either TC4 or PSO emulsions and this value further decreased to less than 20% after 10 min of incubation. This rapid inhibition was observed both in the presence and the absence of NaTDC in the assay.

In the absence of NaTDC in the incubation medium, THL partially inhibits HPL and a plateau value (around 60%) of the residual initial activity is rapidly reached when using TC4 as substrate, both in the absence and the presence of 4 mM NaTDC in the assay (Fig. 4 upper panel). However, a different pattern was observed when using PSO emulsion as substrate (Fig. 4 lower panel). In the absence of NaTDC, both in the incubation medium and the assay, HPL was found to be strongly inhibited by THL although the inhibition rate was slower than found in the presence of bile salts in the incubation medium. The absence of NaTDC in the incubation medium and its presence in the lipase assay result in a moderate inhibitory effect of HPL by THL.

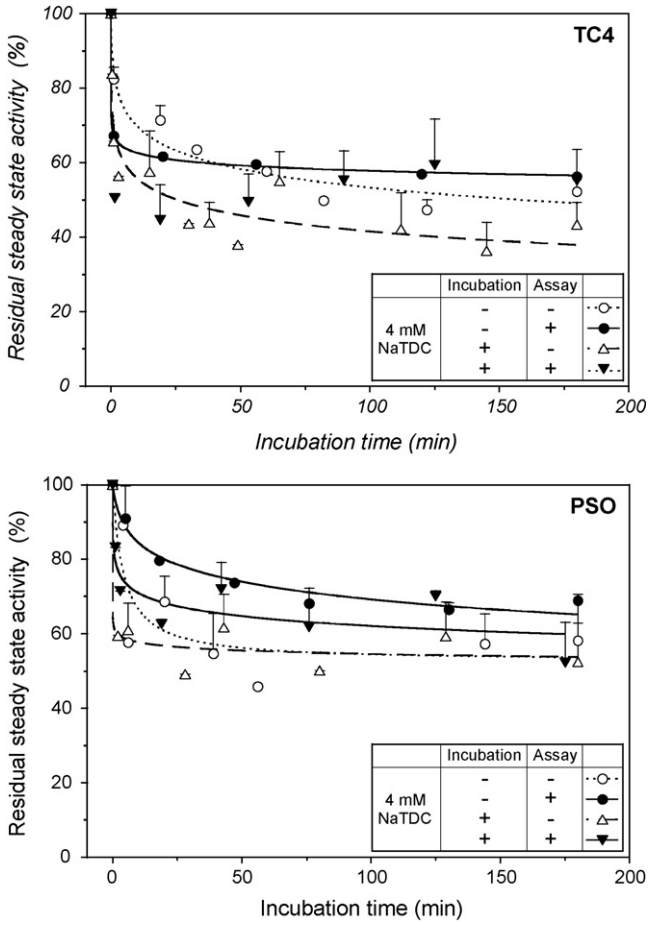

Fig. 3. HPL residual activity, measured using the pH-stat technique, under the steady state conditions using a TC4 emulsion (upper panel) or a PSO emulsion (lower panel), as a function of the incubation time. The incubations were carried out in the absence (circles) or in the presence (triangles) of 4 mM NaTDC in the incubation medium. The full symbols (●, ▼) illustrate the activity assays performed in the presence of 4 mM NaTDC in the assay and the open symbols (○, △) in its absence. The other experimental conditions for the lipase assay are as described in Fig. 2. The values of the steady state residual activity of HPL were determined as described in Section 2. The values are expressed as means ± S.D. ($n \geq 3$ for each activity assay).

3.2. Lag times

The lag times values, ranging from 0.5 to around 10 min, were calculated from the kinetic recordings of the HPL acting on TC4 emulsion (Fig. 5 upper panel) or PSO emulsion (Fig. 5 lower panel).

The presence of 4 mM NaTDC in the incubation medium resulted in long lag times (5–10 min) whatever the substrate used (TC4 or PSO), both in the absence and in the presence of bile salts in the assay. However, when 4 mM NaTDC were present in the lipase assay, a clear reduction (more than 2-fold) of the lag time values was observed. In the presence of 4 mM NaTDC in the lipase assay and no NaTDC in the incubation medium, a negligible lag time was observed (around 1 min) with both substrate emulsions used (TC4 and PSO). This low value of the lag time is comparable to those found in the absence of THL and could be attributed to the mixing time required for the injected lipase sample to be homogeneously distributed.

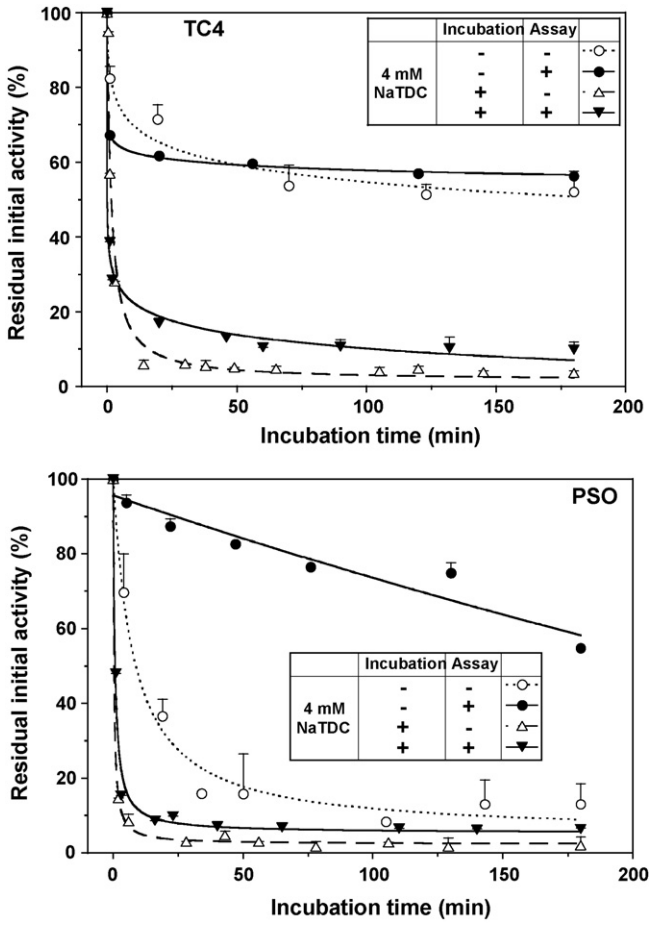

Fig. 4. Residual initial activity of HPL acting on a TC4 emulsion (upper panel) or a PSO emulsion (lower panel), as a function of the incubation time with THL, measured using the pH-stat technique. The incubations were carried out in the absence (circles) or in the presence (triangles) of 4 mM NaTDC in the incubation medium. The full symbols (●, ▼) illustrate the activity assays performed in the presence of 4 mM NaTDC in the assay and the open symbols (○, △) in its absence. The other experimental conditions for the lipase assay are as described in Fig. 2. The values of the residual activity are expressed as means ± S.D. ($n \geq 3$ for each activity assay).

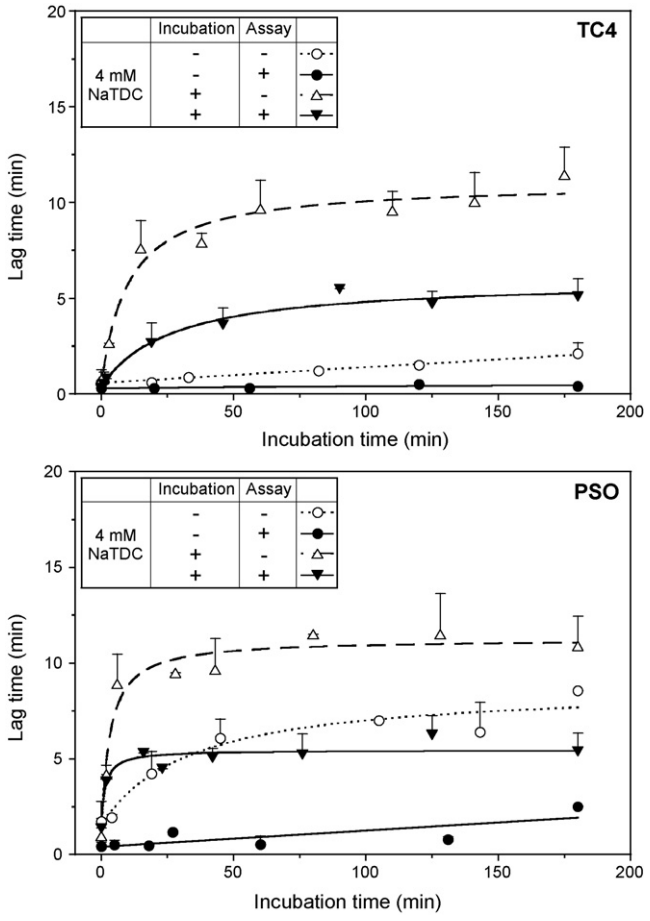

Fig. 5. Variation of the lag time of HPL activity as a function of its incubation time with THL, using a TC4 emulsion (upper panel) or a PSO emulsion (lower panel). The lag times (τ) were determined as illustrated in Fig. 2 and described in Section 2. The incubations were carried out in the absence (circles) or in the presence (triangles) of 4 mM NaTDC in the incubation medium. The full symbols (●, ▼) illustrate the activity assays performed in the presence of 4 mM NaTDC in the assay and the open symbols (○, △) in its absence. The other experimental conditions for the lipase assay are as described in Fig. 2. The values are expressed as means ± S.D. ($n \geq 3$ for each activity assay).

In the absence of NaTDC both in the incubation medium and the assay, a short (1–2 min) lag time was obtained when using TC4 emulsion. However, when using PSO emulsion as substrate, the values of the lag time were higher (a plateau value of around 6 min).

3.3. Mass spectrometry analysis

After incubation of HPL with THL (at a molar ratio of 1–100) at 25 °C and pH 8.0, 0.7 μl of this mixture was sampled at various incubation times ranging from 5 min to 24 h. The corresponding mass spectra were recorded (see Fig. 6), as described under Section 2. In parallel, the lipase activity was measured using the pH-stat technique. As can be seen from Fig. 6, the molecular mass of pure HPL (spectra I) is around 50,660 Da. The presence of three peaks in the spectra is probably due to the heterogeneity in the glycosylation pattern of the HPL sample (F. Carrière, personal communication). The spectra II in Fig. 6 is representative of the spectra recorded between 5 min and 24 h of incubation with a mass around 51,170 Da. The increase of HPL molecular mass is around 500 Da and most probably reflects the covalent binding of THL (*M*w, 496 Da) to HPL. The residual initial activity of HPL, measured on TC4 emulsions was almost completely abolished after a few minutes of incubation (data not shown).

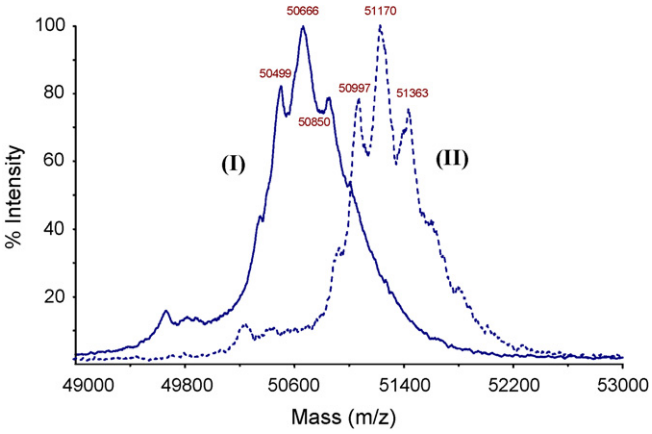

Fig. 6. MALDI-TOF mass spectra of HPL (I) or HPL pre-incubated with THL (II) at a HPL:THL molar ratio of 1:100 (see Section 2 for more details).

4. Discussion

In this study, we showed clearly that, in the absence of substrate, HPL was efficiently inhibited when incubated with a 100-fold

molar excess of THL. This inhibition occurs within a few minutes in the presence of bile salts in the incubation medium (Fig. 4). In the absence of bile salts, however, the inhibition of the lipase activity was slower and only partial when tested on both TC4 and PSO emulsions.

This observed dependence of HPL inhibition by THL upon the presence of bile salts in the incubation medium could be explained either by a possible conformational changes of HPL caused by bile salts or by the formation of THL/bile salt mixed micelles which may give rise to a better 'interfacial quality', improving the lipase adsorption onto these mixed micelles. The increase in the inhibitory efficacy of THL by bile salts over their CMC was reported in the case of pancreatic lipases (in human [41], dog and guinea pig [37]) as well for HGL [20] and lipoprotein lipase [42]. This observation is reminiscent of the inhibition studies of PPL by diethyl *p*-nitrophenyl phosphate (E_{600}). In this latter case, Rouard et al. [56] showed that E_{600} present in mixed micelles of bile salts irreversibly inactivates PPL, whereas aqueous solution of this organo-phosphorous compound does not. Similar effects were reported by Desnuelle et al. [57] when E_{600} was or not emulsified with gum Arabic. Potthoff et al. [43] also reported that the inhibition of *C. viscosum* lipase (CVL) by THL (at a molar excess of 30) was achieved only in the presence of 50% isopropanol.

It appears thus that the availability and/or the organisation of these inhibitors at an interface is one of the most decisive factors on which their efficacy depends. One can imagine that the interfacial properties of the mixed micelles helps the lipase to be in its opened conformation, which probably gives easier access of the inhibitor molecules to the active site. Furthermore, by studying the transfer of radiolabeled THL from an oil drop to the aqueous phase, we found that the transfer rate was increased in the presence of bile salts above their CMC [38], reflecting the solubilisation of THL in the aqueous phase by probably forming mixed micelles with bile salts.

During the lipase assay, an increase with time in the activity of the previously inhibited HPL was observed, as evidenced by the kinetics showing a lag phase both when using short- (TC4) and long- (PSO) TAG substrates (Fig. 2). This is probably due to a slow deacylation of a fraction of the covalent HPL–THL complex during the lipase assay, since we never observed a complete recovery of the activity of the inhibited HPL. Similar observations (reactivation of lipases after their inhibition with THL) were previously reported. Indeed, Borgström [18] reported that THL was found to be a potent inhibitor of human carboxyl ester lipase (HCEL). The activity of the later enzyme increased with time and was almost completely restored after 24 h of incubation with THL, at a molar ratio of HCEL:THL of 1:2.

This reactivation of HCEL was accompanied by a chemical conversion of THL into its hydrolyzed form (the open form of the lactone). This author concluded that the primary hydroxy group of the active serine from HCEL forms an ester with the lactone of THL, which is slowly hydrolyzed in turn and released from the enzyme into the open form of the lactone.

Pottoff et al. [43] reported that after its inhibition by THL, the lipolytic activity of CVL can be partially restored (up to 50% of its initial activity) by extracting the THL–CVL complex with chloroform–methanol. The latter authors, however, concluded that the enzyme does not form a covalent complex with THL molecules.

The presence of 4 mM NaTDC seems to have two opposite and apparently contradictory effects. When present in the incubation medium, NaTDC was found to increase the lag time whereas the presence of 4 mM NaTDC in the lipase assay reduces the lag times (see Fig. 5). These two paradoxical effects of bile salts could be interpreted by firstly taking into account the formation of mixed THL/bile salt micelles and secondly by assuming the coexistence of two forms of inhibited HPL. One can reasonably hypothesised that the covalent binding of THL to HPL happens in two configurations

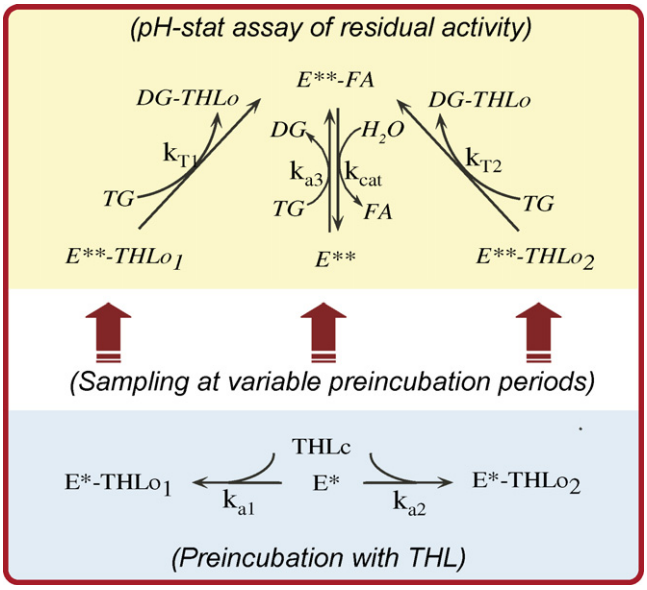

Fig. 7. Kinetic model illustrating the inhibition of HPL by THL in the aqueous phase and its reactivation at a lipid–water interface. Symbols and abbreviations are as follows: *E**, free enzyme (molecule/volume); *E***, interfacial enzyme (molecule/surface); *FA*, fatty acid at the interface (molecule/surface); *E***–FA, interfacial enzyme–fatty acid complex (molecule/surface); THLc, the closed and reactive THL in the bulk (molecule/volume); E*–THLo$_1$, form 1 of the covalent enzyme–THL complex in the bulk (molecule/volume); E*–THLo$_2$, form 2 of the covalent enzyme–THL complex in the bulk (molecule/volume); *E**–THLo$_1$*, interfacial form 1 of the covalent enzyme–THL complex (molecule/surface); *E**–THLo$_2$*, interfacial form 2 of the covalent enzyme–THL complex (molecule/surface). TG, triacylglycerol at the interface (molecule/surface); DG, diacylglycerol at the interface (molecule/surface); DG-THLo, open non-reactive THL trans-esterified on DG (molecule/interface).

as depicted in the lower panel of the model shown in Fig. 7. A first fraction of the lipase molecules will form in the aqueous phase a covalent complex with the open β-lactone ring of THL in a first configuration (*E**–THLo$_1$) whereas the other fraction will also form a covalent complex with THL but in a second configuration (*E**–THLo$_2$). The hypothesis that a fraction of the inhibited lipase molecules forms a non-covalent complex with the β-lactone ring of THL in its closed configuration, as concluded by Potthoff et al. [43] in the case of CVL, can be discarded in our case since the mass spectra (Fig. 6) have shown the presence of only one form of HPL–THL complex. Indeed, if the non-covalent HPL–THLc complex exists, mass spectra should contain, in addition to the covalent HPL–THLo complex, the spectra of pure HPL.

In the assay system, when the mixture (*E**, THLc, *E**–THLo$_1$ and *E**–THLo$_2$) is added to a TAG emulsion, the less stable complex (*E**–THLo$_1$) dissociates and thus the hydrolytic reaction rate increases, as reflected by the existence of lag times (Fig. 5). The dissociation of this less stable complex is enhanced when bile salts are present in the assay, probably due to the formation of mixed THL/bile salt complex. The fact that the reactivation of HPL does not reach 100% at the end of the kinetics (Fig. 3) could be explained by the existence of the second fraction of the enzyme which is more strongly inhibited by THL (*E**–THLo$_2$) and which remains stable, at least during the recording kinetic period. The co-existence of two different forms of THL bound to HPL could be due to two different orientations of this inhibitor molecule in the catalytic cavity of the lipase.

Cygler et al. [58] have reported the 3D structures of covalent complexes of *Candida rugosa* lipase with two enantiomeric (1R and 1S-menthyl hexylphosphonate) transition-state analogs for the hydrolysis of menthyl esters. The 1R enantiomer, derived from the fast-reacting enantiomer of menthol, and the 1S, derived from the

slow-reacting enantiomer, were found to bind to the lipase in two different orientations. Furthermore, in contrast to the fast reacting enantiomer, in the lipase/slow-reacting enantiomer the imidazole ring of the residue His 449 was found to have rotated, thus disrupting its hydrogen bond with the menthol oxygen atom, which probably explains the differences in reactivity between the two enantiomers [58]. Furthermore, Egloff et al. [12] have reported that the two enantiomers of a C_{11} alkyl phosphonate compound, covalently bound to the active serine residue of HPL, occupy two different conformations with a different occupancy levels (57% and 43% for R and S conformations, respectively). As in the case of *C. rugosa* lipase reported above, in one conformation the methoxy oxygen of the first enantiomer forms a hydrogen bond with the residue His 263, whereas in the second enantiomer this hydrogen bond is lacking.

Uppenberg et al. [59] performed structural studies on the lipase B from *Candida antarctica,* which showed the presence of a stereospecific pocket for secondary alcohols. In their conclusion to this study, the authors stated that they could not rule out the possibility that the two enantiomers of the phosphonate inhibitor may bind competitively to the serine. This would give rise to a mixture of conformations in the acyl and alcohol moieties of the inhibitor present in the active site pocket. The enzyme's preference for one of the two enantiomers is therefore mainly governed by the size of the two side chains and their ability to make favorable interactions in the active site of the enzyme.

At our laboratory, Yapoudjian et al. [60] have resolved the 3D-structure of an inactive lipase mutant from *Humicola lanuginosa* co-crystallised with the oleic acid (OA) in mixed micelles with bile salts. In this structure, the OA was found to bind to the catalytic cavity of the lipase in two different orientations. In a lipase molecule, OA occupied a conventional sn-1 binding site, and in a second lipase molecule, OA was trapped in an unexpected fashion, being rotated approximately 180° in respect to the main sn-1 lipase binding site alkyl chain binding site.

In view of the structural studies mentioned above on the various ligand configurations present at the active sites of lipases, the finding that transesterified THL molecules, coupled to DG, were present in their open lactone form would provide direct support for the validity of the kinetic model presented above (Fig. 7).

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