Biochimica et Biophysica Acta, 962 (1988) 308–316
Elsevier

BBA 52929

Mode of action of tetrahydrolipstatin: a derivative of the naturally occurring lipase inhibitor lipstatin

Bengt Borgström

Department of Physiological Chemistry, University of Lund, Lund (Sweden)

(Received 23 May 1988)

Key words: Pancreatic lipase; Gastric lipase; Carboxyl ester lipase; Cholesterol esterase; Uncompetitive inhibition; (*R. arrhizus*)

Tetrahydrolipstatin is a specific lipase inhibitor derived from lipstatin, a lipid produced by *Streptomyces toxytricini*. In addition to pancreatic lipase, it is shown in the present study that tetrahydrolipstatin also inhibits human gastric lipase, carboxyl ester lipase (cholesterol esterase) of pancreatic origin and the closely related bile-salt-stimulated lipase of human milk. It does not inhibit the exocellular lipase from *Rhizopus arrhizus* or a lipase recently isolated from *Staphylococcus aureus*. In the presence of a water-insoluble substrate, such as tributyrin, the inhibition has the characteristics of an irreversible inactivation of the uncompetitive type, thus indicating that an enzyme · substrate · inhibitor complex is formed, which cannot undergo further reaction to yield the normal product. This reaction probably takes place at the aqueous / oil interface of the substrate. In aqueous solution, in the absence of substrate, the inhibition of carboxyl ester lipase by tetrahydrolipstatin has the characteristics of being reversible, and finally becomes of a temporary nature analogues to the trypsin-trypsin inhibitor system. It is suggested that an enzyme-inhibitor complex of an acyl-enzyme type is formed that is slowly hydrolysed, with water as the final acceptor, leaving an intact enzyme and an inactive form of the inhibitor. The enzyme thus consumes the inhibitor, which undergoes a chemical conversion, as indicated by a change in mobility in an appropriate thin-layer chromatographic system, indicating an increase in hydrophilicity. Evidence is presented that the reaction product is an acid and that the functional group of tetrahydrolipstatin is the β-lactone reacting with the active site of the enzyme.

Introduction

Lipstatin is a pancreatic lipase inhibitor of microbial origin [1,2] which, by catalytic hydrogenation, gives tetrahydrolipstatin (THL). These compounds are biologically active, and their chemical structure has been worked out and proved by chemical synthesis [3,4]. THL has been reported to inhibit pancreatic lipase in vitro [1], and when fed to mice, reduces fat absorption and lowers plasma cholesterol [5]. The experiments presented in this paper were undertaken to elucidate the mode of action of THL in vitro with pancreatic lipase and possibly other lipolytic enzymes inhibited by this factor.

Abbreviations: HCEL, human carboxyl ester lipase (EC 3.1.1.13); HGL, human gastric lipase (EC 3.1.1.-); HPL, human pancreatic lipase (EC 3.1.1.3); THL, tetrahydrolipstatin.

Correspondence: Bengt Borgström, Department of Physiological Chemistry, Box 94, S-221 00, Lund, Sweden.

Materials and Methods

Materials

Human pancreatic lipase (HPL) and colipase were prepared from pancreatic tissue as previously described [6,7]. The specific activity of HPL was around 4000 U/mg when tested against tributyrin as substrate in the presence of colipase in buffer B (see below). Human pancreatic carboxyl ester lipase (HCEL) was prepared from pancreatic juice (Sternby, unpublished data). The specific activity was 220 U/mg using tributyrin as substrate in buffer C. The molecular weight of HCEL was considered to be 100 000 [8]. Human gastric lipase (HGL) was prepared from pancreatic juice essentially as described [9], and was a gift from S. Bernbäck, University of Umeå, Sweden. The specific activity was 665 U/mg when assayed against tributyrin as substrate in buffer B at pH 6.0. Bile-salt-stimulated lipase from human milk was a gift from L. Bläckberg, University of Umeå, Sweden. *Staphylococcus aureus* lipase was purified and characterized as described [10], and was a gift from Jan Rollof University of Lund, Sweden. The exocellular lipase from the mold *Rhizopus arrhizus* was obtained from Societe d'Etudes et d'Applications Biochimiques, Jouys-en-Josas, France.

Chemicals. THL was obtained from Hoffmann-La Roche, Basle, Switzerland. The structure and scheme of numeration of the carbon atoms of THL is given according to Kitahara et al. [11], as seen in Fig. 1. The chemical name of THL is (2*S*,3*S*,5*S*)-5(*N*-formyl-L-leucyloxy)-2-hexyl-3-hydroxyhexadecanoic lactone [3]. [2-^{14}C]THL and [1''-^{14}C]THL were also obtained from Hoffmann-La Roche, Basle, Switzerland: the specific activities were 4.1 MBq/mg. The M_r of THL is 496 and the compound is soluble in organic solvents, such as heptane and chloroform: it is also soluble in dimethyl sulfoxide. It is stable in these solvents at +4° C for long periods of time.

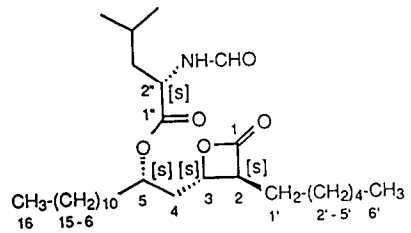

Fig. 1. Structural formula of THL [1–4] and scheme for numeration of the carbons as proposed in Ref. 11.

Taurodeoxycholate and taurocholate were synthesized in our laboratory according to standard methods and were more than 98% pure by thin-layer chromatography (TLC).

Experimental procedures

Kinetic studies were undertaken using a Mettler automatic pH stat titration system (DK 10, DK 11, DV 11, 13 and 201 with a 1 ml byrette and a two-channel recorder). The cumulative as well as the rate of fatty acid production in μmol/min were registered [12]. Titrations were performed in 10–15 ml volumes contained in 20 ml glass vials, and stirring was accomplished with a stirring rod at a rate of 1500 rpm. The temperature of the incubations was controlled by water circulating outside the vessel. Different buffers were used, depending on which enzyme was assayed: buffer A for experiments with pancreatic lipase was 0.2 mM Tris-HCl (pH 7.0)/150 mM NaCl/0.2 mM $CaCl_2$. Buffer B, in addition, was 4 mM in taurodeoxycholate while buffer C contained 5 mM taurodeoxycholate and taurocholate. Three different types of experiments were performed using tributyrin as substrate. (1) THL dissolved in dimethyl sulphoxide was pipetted into the titration vial and the solvent was evaporated under a stream of nitrogen. Tributyrin was then added in a volume of 0.2–2.0 ml and mixed well, followed by 15 ml of buffer. The pH was adjusted and the suspension was stirred for a few minutes. The enzyme was then added and release of fatty acid was determined as described above. (2) THL dissolved in dimethyl sulphoxide was added last in the sequence, i.e., after the enzyme. (3) Enzyme was added to 15 ml buffer followed by THL dissolved in dimethyl sulphoxide under stirring. After a 5 min incubation in the titration vessel at constant pH, substrate was added, and the residual enzyme activity was determined. In some experiments in which THL and enzyme were incubated over long periods of time, the enzyme concentration was high and the residual enzyme activity was measured by taking aliquots of the incubation and adding this to a titration vessel containing buffer and substrate.

para-Nitrophenyl acetate was used as substrate for HCEL to study the kinetics of the reaction with THL. In these experiments, a modification of a method previously described for the hydrolysis of *para*-nitrophenyl acetate [16] was used. THL and HCEL were mixed in a volume of 1.4 ml for 5 min, and the substrate was then added in a volume of 0.1 ml. The amount of residual HCEL activity was then measured by recording the increase in absorbance at 400 nm over a 10 min period and correcting for blank values.

In experiments to study the possible conversions of THL as a consequence of the incubation with enzymes, [2-^{14}C]THL was used and the lipid-soluble product(s) were extracted with 16.25 vol. of methanol/chloroform/heptane (1.41 : 1.25 : 1) [13]. The phases were split with 5.25 ml 2% aqueous acid phosphate solution, and the lower phase was evaporated. The lipids were separated on TLC DC-Alufolien (Kieselgel 60 20 × 20 cm, Merck, Darmstadt). Two solvent systems were used to develop the plates: a relatively non-polar system composed of chloroform/acetone/acetic acid (96 : 4 : 0.5 (system A)) and a more polar system of the same solvents (70 : 30 : 0.5 (system B)). The plates were developed using phosphomolybdate and cut into strips, which were scraped into vials. 1 ml 95% ethanol and 10 ml scintillation fluid (ES 299, Packard) were added and the radioactivity was determined.

The reaction product obtained was also partitioned in an alkaline system in which a borate/carbonate buffer (pH 10.5) was used instead of the acid phosphate solution described above [13].

The reaction product was treated further with a methylating agent (5% acetyl chloride in dry methanol) overnight followed by partitioning in the alkaline two-phase system described above.

The surface pressure at the aqueous/air interphase of THL was measured using a surface balance [14]. A measure of the hydrophobicity of THL was obtained by partitioning [^{14}C]THL between aqueous buffer (pH 7.4) and octanol and tributyrin, respectively.

Calculations

If log v (lipase activity in μmol/min) is plotted over the incubation time, straight lines are obtained, from which the first-order inactivation constants can be calculated [12].

Results

The surface activity of THL was measured at the aqueous/air interface at 25 °C using a surface balance and the equilibrium spreading pressure was found to be 24 dynes/cm. The subphase was 0.15 M NaCl. This compares to 13 dynes/cm for triolein and 45 dynes/cm for dipalmitoylphosphatidylcholine. The partition coefficient for THL between octanol or tributyrin and aqueous buffer (pH 7.4) was for both greater than 10000.

Inactivation of different lipases by THL

Fig. 2 shows the inactivation of human pancreatic lipase with THL dissolved in the tributyrin substrate in increasing amounts. The experiments

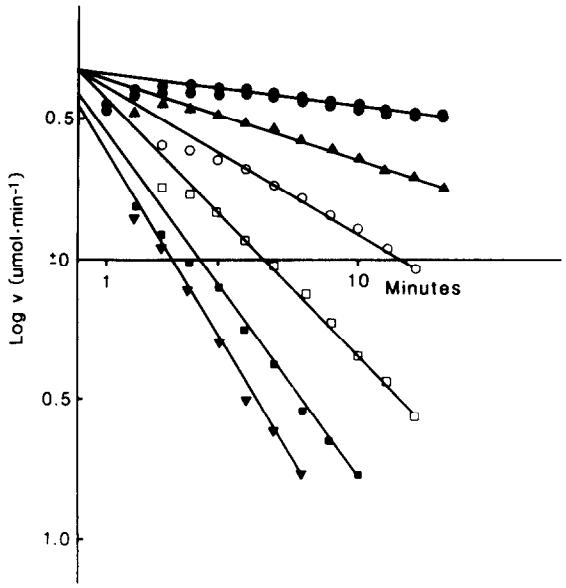

Fig. 2. Effect of THL on the rate of inactivation of tributyrin with HPL. 0–200 nmol of THL (●, 0; ▲, 10; ○, 20; □, 50; ■, 100 and ▼, 200) dissolved in chloroform were pipetted into glass vials. The solvent was evaporated and 0.5 ml tributyrin was added. After mixing, 15 ml of buffer A were added and the system was equilibrated at 10 °C using a magnetic stirrer for emulsification. At zero time, 20 pmol of HPL were added and the rate of fatty acid release, v, in μmol/min, was determined. Log v was plotted over time of incubation. When extrapolated to zero time, $c_{cat} \cdot E_0$ is obtained [12]. E_0 was 4.73 μmol/min. The slope of the curves is $-K_i/2.3$ where K_i is the first-order inactivation constant [12].

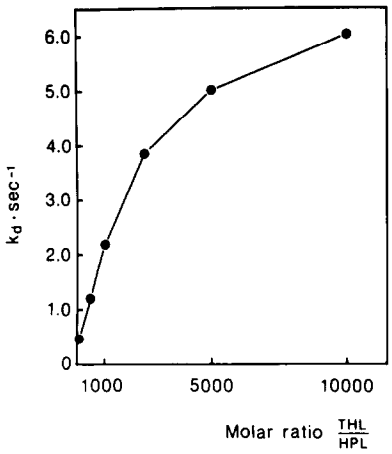

Fig. 3. First-order inactivation constants calculated from the values in Fig. 1 plotted over the molar THL/HPL ratio.

were performed in buffer (pH 7.0) at 10 °C to minimize surface denaturation of lipase [12].

Increasing the amount of THL dissolved in the substrate leads to an increased rate of inactivation. Fig. 3 shows a plot of the first-order inactivation constant over the molar ratio THL/HPL, and shows a rapid increase followed by a tendency towards saturation. Repeated addition of enzyme resulted in similar rates of inactivation. No difference in the effect of THL was noted if the reaction with HPL was performed in buffer A or in bile salt buffer (buffer B) in the presence of colipase. Colipase, therefore, is not involved in the inactivation reaction. The course of inactivation with THL, of HGL and of HCEL was very similar. The rate of inactivation (surface denaturation) in the absence of THL was most rapid for HPL, even though this assay was performed at 10 °C, compared to 20 °C for the other two enzymes.

Bile-salt-stimulated lipase from human milk behaved very similarly to HCEL. Potent exocellular lipases from *S. aureus* and *B. ahrrizus*, when tested with tributyrin as stubstrate, were not affected by 100 nmol of THL.

The importance of the order of addition of the components on the effect of THL

In the experiments previously described, THL was dissolved in the substrate, which was emulsified in the buffer prior to the addition of the enzyme. The results of three different experiments, A, B and C, in which the order of addition of the components was varied, are given in Fig. 4. In the text to the figure, the conditions of the experiments are given in detail. In Fig. 4A, the substrate tributyrin was emulsified with the buffer, followed by the addition of HCEL (125 pmol). The initial rate of hydrolysis extrapolated to zero time was 1.5 μmol/min, and the decay of activity was almost negligible, indicating that the activity of the enzyme was stable under these conditions. Addition to the same incubation of another 0.5 ml of tributyrin did not change the rate of hydrolysis significantly, an indication that the enzyme already worked at a saturating level with 0.5 ml tributyrin. Addition of 100 nmol of THL (dissolved in dimethyl sulphoxide) at this stage resulted in a rate of inactivation of the enzyme which corresponded to 10 $nmol \cdot min^{-2}$. When the same amount of THL was dissolved in 0.5 ml tributyrin and this was emulsified in the buffer, followed by addition of the the enzyme, the rate of inactivation of the enzyme corresponded to 87 $nmol \cdot min^{-2}$ (Fig. 4, BI). When the same amount of enzyme was again added, it was inactivated at a

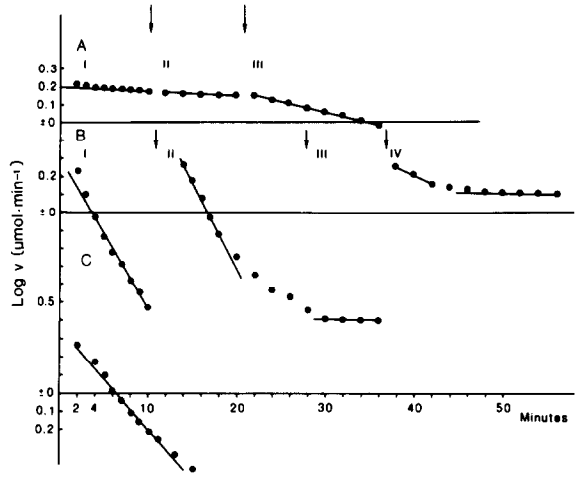

Fig. 4. Effects of the order of addition of the components on the rate of inactivation of HCEL by THL. (A) I: (0.5 ml tributyrin + buffer) + HCEL. II: + 0.5 ml tributyrin. III: + THL added dissolved in dimethyl sulphoxide. (B) I: (0.5 ml tributyrin + THL) + buffer + HCEL. II: + additional HCEL. III: + 0.5 ml tributyrin. IV: + additional HCEL. (C) (1.0 ml tributyrin + THL) + buffer + HCEL. The buffer was C at 25 °C and each addition of HCEL was 125 pmol and of THL, 100 nmol. The arrows mark the additions to the incubations.

similar rate (BII). This is explained by the fact that THL was present in the substrate in a large excess over the amount of enzyme added (800-fold). When an additional 0.5 ml tributyrin was added (BIII), low rates of hydrolysis and inactivation took place, as the major part of the enzyme added in the previous step had been inactivated. Addition at this stage of still another dose of enzyme (BIV) resulted in a high rate of hydrolysis with a low rate of inactivation (about 25% compared to BI). This can best be explained if no exchange of THL took place between the old and the freshly added substrate, while the added enzyme had distributed over the whole substrate interface. Finally, 1 ml of tributyrin containing 100 nmol of THL, when emulsified prior to the addition of the enzyme (expt. C), gave a rate of inactivation of HCEL that was 54 nmol $\cdot$ min^{-2}, or half that when the substrate was 0.5 ml (BI). These experiments indicate that the rate of inactivation of the enzyme is related to the concentration of THL in the substrate or at the aqueous/substrate interface. This is also demonstrated in experiments (not shown) in which three different levels of substrate, at constant HPL concentrations, were reacted with and without THL. The curves for log v vs. time in the absence of THL show little increase in log v when tributyrin was increased from 0.5 to 2.0 ml. Therefore, the enzyme was already saturated with substrate at the lowest level used. When THL was present, the rate of inactivation decreased with increases in substrate concentration, indicating that the surface concentration of THL decreases.

Kinetics of the HCEL / THL / tributyrin system

HCEL and THL were added to 10 ml of buffer C to final concentrations of $5 \cdot 10^{-10}$ and $2 \cdot 10^{-9}$ M, respectively. After a 5 min incubation, 10–100 μl of substrate were added, followed by emulsification and titration. The rates of hydrolysis compared to those obtained in the absence of THL were recorded and plotted as $1/v$ versus $1/s$. The regression lines were calculated and the equations for the lines in the absence and presence of THL were: $1/v = 9.93 \cdot 1/s + 0.97$ and $1/v = 8.54 \cdot 1/s + 2.13$, respectively, indicating that the lines are close to parallel. This behaviour indicates that the inhibition is of the uncompetetive type and that a non-reactive enzyme $\cdot$ inhibitor $\cdot$ substrate complex is formed [15].

Interaction of THL with HCEL and HPL in aqueous solution

In the experiments with HCEL, the enzyme was diluted in 15 ml buffer C to a concentration of $3.33 \cdot 10^{-10}$ M, and varying amounts of THL, dissolved in dimethyl sulphoxide, were added. After a 5 min incubation with stirring, the residual HCEL activity was determined by titration after the addition of 0.2 ml tributyrin. The concentration of THL resulting in 50% inhibition was $1.3 \cdot 10^{-9}$ M. In these experiments, the ratio of THL to HCEL was close to 1, while in the inactivation studies with THL dissolved in tributyrin, as previously discussed, a THL/HCEL ratio of 100–1000 was necessary for THL to be active. In similar studies using HPL and HGL, the concentration of THL required to obtain 50% inhibition was almost 200-times that compared to with HCEL.

As the presence of the insoluble substrate (tributyrin) complicates the measurement of residual enzyme activity in incubations of enzyme and inhibitor, kinetic studies were also performed using a water-soluble substrate for HCEL in the form of *para*-nitrophenyl acetate. In these experiments, enzyme and inhibitor were mixed, and the residual enzyme activity was measured spectrophotometrically. Fig. 5 shows a plot of HCEL activity as measured with *para*-nitrophenyl acetate over THL concentrations. From these figures, the dissociation constant, k_i, for the reaction can be approximated, as discussed [17]. In the case studied, the course of the curve is concave, which indicates that the inhibition is reversible and that K_i is of the same order of magnitude as the total enzyme concentration, i.e., approx. $3 \cdot 10^{-9}$. A prerequisite for this conclusion is that the incubation time is sufficient to obtain complete association between enzyme and inhibitor. If equimolar concentrations of enzyme and inhibitor are reacted, the half-life of inhibition can be approximated [17]. We reacted HCEL, at a concentration of $3.3 \cdot 10^{-9}$ M, with *para*-nitrophenyl acetate and followed the increase in absorbance at 400 nm for 4.5 min. At this time, THL was added at an equimolar concentration, and the optical density was recorded further (see Fig. 6). The curve allowed an ap-

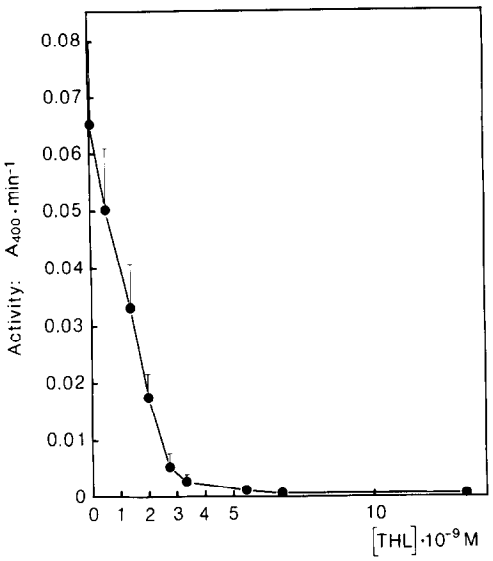

Fig. 5. Effect of different concentrations of THL on the residual activity of HCEL as measured by *para*-nitriphenyl acetate. *para*-Nitrophenyl acetate was $3.3 \cdot 10^{-4}$ M, HCEL, $3.3 \cdot 10^{-9}$ M (pH 7.4). Points are means of six determinations.

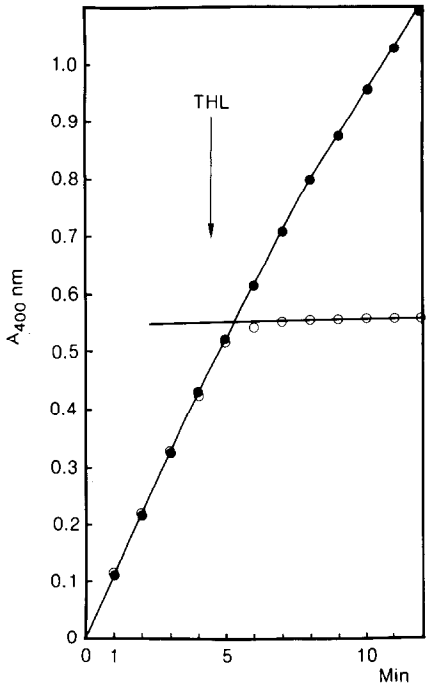

Fig. 6. Rate of association of HCEL and THL at equimolar concentrations ($= 3.3 \cdot 10^{-9}$ M). HCEL was mixed into 1.4 ml Tris-HCl buffer (pH 7.4) and added at zero time with 0.1 ml *para*-nitrophenyl acetate solution: the A_{400} was followed with time (●). In another sample, 5 µl THL in dimethyl sulphoxide was added after 4.5 min: the sample was rapidly mixed and the reading was continued (○).

proximation of the half-live of association to 75 s and the association rate constant, k_{on}, was calculated as $4 \cdot 10^{-7}$ $M^{-1} \cdot s^{-1}$ [17]. These results indicate that the 5 min incubation time used in the experiments in this study is sufficient to achieve complete association.

HCEL activity returns with time when incubated with THL. Indications for a change in the chemical structure of THL

When HCEL (10^{-8} M) was incubated in buffer (pH 7.0) at room temperature with THL ($2 \cdot 10^{-8}$ M), the enzyme activity was almost completely abolished. If the incubation was left, the enzyme activity, however, returned with time (see Fig. 7), and was almost completely restored after 24 h. The curve had the shape of an autocatalytic reaction and indicated that THL was converted to an inactive form.

In order to study the possible chemical conversion of THL during incubation with HCEL, [2-^{14}C]THL was used. In a typical experiment, 10 ml of a 10^{-5} M solution of buffer C in THL containing $3 \cdot 10^{6}$ dpm labeled THL and $5 \cdot 10^{-7}$ M in HCEL was incubated at 37 °C. Samples of 200 µl of the incubation medium were taken after 1, 24 and 48 h and pipetted into 3.25 ml of a solution composed of methanol/chloroform/heptane (1.41 : 1.25 : 1). After mixing, 1.05 ml of 2%

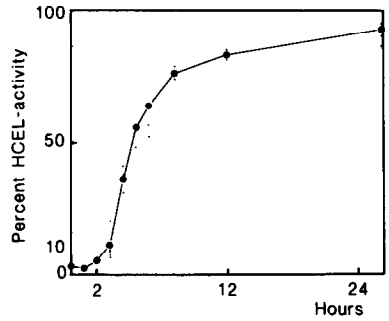

Fig. 7. HCEL, 10^{-8} M and THL, $2 \cdot 10^{-8}$ M in buffer C were incubated at room temperature. Samples were taken at different times to measure HCEL activity with tributyrin as substrate. The figures are given as percent enzyme activity in relation to zero time in the absence of THL. Corresponding solutions of HCEL in buffer are stable at room temperature for the time periods used. The small dots represent the results of single experiments.

TABLE I

[^{14}C]THL was incubated with HCEL as described in the text. After incubation for 1, 24 and 48, the lipids were extracted and the samples were separated by TLC using the non-polar solvent system A, and with the 48 h sample, also with the more polar system B. Appropriate standards were run and identification was carried out using phosphomolybdate. The strips were cut into seven and eleven pieces, respectively, for A and B, and the radioactivity was counted. The percent activity distribution in the different fractions are given below. A and B refer to the solvent system. OHC_{16}, β-hydroxyhexadecanoic acid.

A

Hours	0	1	24	48
Front 7	–	–	–	0.7
6	–	–	–	0.9
5	–	–	1.1	2.9
4	98 - 100	96.3	1.3	1.3
3	± 0.6	–	–	0.7
2	–	–	13.8	0.8
1 Start	± 0.8	3.7	84.0	92.9

B

	48H	Spots	Standard
Front 11	4.8	•	•
10	0.1	•	
9	0.1	•	
8	0.1		
7	93.4	•	•
6	1.3	•	
5	0.1		
4	–	•	
3	–		
2	–		
1 Start	–		THL OHC_{16}

aqueous acid phosphate was added and the two phases were allowed to separate. No significant radioactivity was found in the upper aqueous/methanol phase. The lower phase was evaporated and dissolved in 25 μl chloroform: 10 μl of this solution was separated by TLC with cold THL added to the application spot using the less polar elution system A. After running the plates, they were developed with phosphomolybdate, and the alumina strips were cut by scissors into seven pieces and the radioactivity was counted. With this procedure, THL was found in spot 4 and was close to 100% pure. Table I shows the activity distribution obtained after 1, 24 and 48 h incubation. It is obvious that THL is converted to more polar compound(s) remaining close to the site of application.

The remaining incubation medium of close to 10 ml was partitioned (extracted) in a way similar to that stated above, the lower phase was evaporated and the content was taken up into 1 ml of chloroform. 10 μl of this solution was separated by TLC with solvent system B. With this system, THL moved with the front and was responsible for some 5% of the radioactivity. The main part of the radioactivity was then found in fraction 7 and moved close to a 3-hydroxyhexadecanoic acid standard. Several spots appeared, which coloured with phosphomolybdate, but which were without radioactivity. Their origins are not clear, but extraction of the enzyme solution used in the same way indicated the presence of lipid-soluble substance therein. The reaction product isolated from fraction 7 had no inhibitor activity.

When partitioned in a two-phase alkaline solvent system [13], 60% of the labeled reaction product of THL was found in the upper phase compared to less than 1% in an acid system. This behaviour is similar to that seen for oleic acid [13], and strongly indicates that the reaction product is an acid. This conclusion is further strenghtened by the finding that methylation of the reaction product shifted it to the lipid phase in the alkaline two-phase partition system.

When [1''-^{14}C]THL (labeled in the 1-formyl-leucine moiety) was used in a similar experiment with HCEL, no radioactivity was found in the upper phase, indicating none of this part of THL had been split off.

Using bile-salt-stimulated lipase from human milk as an enzyme source, an identical conversion of THL to a more polar product was seen. Using HPL, even at 10-times the enzyme concentration, no polar reaction product was indicated using the same procedure.

Discussion

Lipstatin belongs to a family of 'esterase inhibitors' isolated from streptomyces strains [1,11,20]. The first inhibitor of this kind was isolated and its structure was determined by Umezawa et al. [20]. It was named esterastin and was reported to exhibit a strong inhibition of pancreatic esterase. It was followed by lipstatin [1,2] and valilactone [11]. Chemically, these compounds are derived from the group of mycolic acids, which are hydroxy fatty acids possessing a 2-alkyl substitution. (Mycolic acids were first isolated and named from lipid extracts from human tubercle bacilli [19].) The mycolic acid moiety of the esterase inhibitors contains hydroxy groups in the 3- and 5-positions, the 3-hydroxy group forming a β-lactone and that in

the 5-position being esterified with an amino acid: *N*-acetyl-1-aspargine, *N*-formyl-1-leucine and *N*-formyl-1-valine for esterastin, lipstatin and valilactone, respectively. These compounds are found in the cellular membrane of the mycelia and nothing is known about their biological function.

In the present study, it is shown that THL, the hydrogenated product of the naturally occurring lipstatin, not only inhibits pancreatic lipase but also the two other lipolytic enzymes secreted into the gastrointestinal tract, i.e., the acid lipase of human gastric content and the carboxyl ester lipase of pancreatic juice.

The enzyme–THL–substrate interactions are rather complex, and cannot at present be described with any accuracy. Lipases by definition work in the aqueous/oil interface of their substrate, to which the enzyme has a high affinity [21]. THL has a low hydrophilicity and distributes in a two-phase aqueous/oil partition system in favour of the lipid phase: it is surface active and can be classified as an insoluble non-swelling amphiphile [22], forming stable monolayers at interfaces. It can also be expected to form mixed micelles with bile salts. THL, therefore, can be assumed to be present at the substrate/water interface of the lipase substrates. In this study, THL has been added either to the oil (substrate) phase or to the aqueous phase dissolved in dimethyl sulphoxide and in most of the experiments performed, especially those with HCEL, the aqueous solution has contained bile salts over the critical micellar concentration. THL in the latter experiments, therefore, can be thought to be in a mixed bile salt micellar solution. HPL has been suggested to be activated at the interface of the substrate, forming an activated enzyme-substrate complex [21]. In the presence of bile salts, colipase is necessary for the binding of the enzyme to the substrate. The results indicate that colipase is not involved directly in the THL inhibition.For HCEL to interact with water-insoluble substrates, bile salts are necessary but the mechanism of action is complex [23]. An acyl-enzyme mechanism for HCEL catalysis has been previously indicated [24]. With respect to HGL, nothing is known about the mechanism of its binding to the substrate. In pure systems, however, HGL is very sensitive to surface denaturation and is protected by the presence of amphiphilic substances, such as bile salts and albumin [25].

The interaction between THL and enzyme is different, depending on whether a water-insoluble substrate is present or not. In the former case, enzyme and inhibitor react at the substrate/aqueous interface and the enzyme is irreversibly inactivated, probably by the formation of an enzyme · inhibitor · substrate complex which does not undergo further reaction. In this reaction, the inhibitor/enzyme molar ratio has to be high and the different enzymes, gastric lipase, pancreatic lipase and carboxyl ester lipase, react in a similar way. The way of addition of the inhibitor is important for the reaction (Fig. 4), and inhibitor added to the aqueous phase (in dimethyl sulphoxide) after the enzyme only slowly becomes reactive. This may be explained as either due to a slow equilibration of the inhibitor between the aqueous phase and the oil phase or due to the relative unavailability of the enzyme when bound to the substrate. Furthermore, when present in the substrate interface, the exchange of inhibitor to new emulsion droplets is slow. These factors are important and have to be recognized to obtain an efficient mode of action of the inhibitor.

When enzyme and inhibitor, on the other hand, are both added to an aqueous (or micellar) solution, they interact in a different way. In these experiments, the residual enzyme activity was measured by the addition of substrate after equilibration of enzyme and inhibitor had occurred. With HCEL, the concentration of inhibitor needed for 50% inhibition is only 1/200 that of the two other enzymes. There is no known explanation for these differences, but they may be due to differences in the microenvironment of the reactants.

The residual enzyme activity in the experiments with HCEL has been measured using both a water-insoluble substrate (tributyrin) and a water-soluble substrate (*para*-nitrophenyl acetate). In both cases, the results have been similar, indicating a reversible binding, and have allowed the calculation of an inhibitor constant and an association rate constant.

Another feature of the THL-HCEL complex in aqueous solution is that in the absence of substrate, enzyme activity returns with time. This may

be a case of temporary inhibition, previously described for the trypsin-trypsin inhibitor system [26,27], and in which case the covalent complex is cleaved by the action of trypsin. By analogy, a covalent complex may be formed between THL and HCEL that is cleaved by the enzyme, leaving an inactive product of THL. This idea is supported by the results of this study, in which a polar reaction product of THL could be shown to appear parallel with the return of enzyme activity. The results furthermore suggest that the reaction product is an acid, and we might speculate that the enzyme and the inhibitor form a covalent bond of the acyl-enzyme type involving the opening of the β-lactone ring of the inhibitor. The enzyme-acyl bond then is hydrolysed by the enzyme, leaving an inactive hydroxycarboxylic acid. The identity of the reaction product of THL is currently under investigation.

Acknowledgements

This work was supported by grants from Hoffmann-La Roche, Basle, Switzerland and in part by grants from the Swedish Medical Research Council.

References

1 Weibel, E.K., Hadvary, P., Hochuli, E., Kupfer, E. and Lengsfeld, H. (1987) J. Antibiot. 40, 1081–1085.
2 Hocuhuli, E., Kupfer, E., Maurer, R., Meister, W., Mercadal, Y. and Schmidt, K. (1987) J. Antibiot. 40, 1086–1091.
3 Barbier, P. and Schneider, F. (1987) Helv. Chim. Acta 70, 196–202.
4 Barbier, P., Schneider, F. and Widmer, U. (1987) Helv. Chim. Acta 70, 1412–1418.
5 Lengsfeld, H., Hadvary, P., Fleury, A., Barbier, P., Hochuli, E., Kupfer, E., Meier, M.K., Schneider, F., Weibel, E. and Widmer, U. (1986) IX International Symposium on Drugs Affecting Lipid Metabolism. Florence, Abstr. p. 34.
6 Sternby, B. and Borgström, B. (1979) Biochim. Biophys. Acta 572, 235–243.
7 Sternby, B. and Borgström, B. (1981) Comp. Biochem. Physiol. 688, 15–18.
8 Lombardo, D., Guy, O. and Figarella, C. (1978) Biochim. Biophys. Acta 527, 142–149.
9 Tiruppathi, C. and Balasubramanian, K.A. (1982) Biochim. Biophys. Acta 712, 692–697.
10 Rollof, J., Hedström, S.Å. and Nilsson-Ehle, P. (1987) Biochim. Biophys. Acta 921, 364–369.
11 Kitahara, M., Asano, M., Naganawa, H., Meade, K., Hamada, M., Aoyagi, T. and Umezawa, H. (1987) J. Antibiot. 40, 1647–1650.
12 Borgström, B. (1982) Biochim. Biophys. Acta 712, 490–497.
13 Belfrage, P. and Vaughan, M. (1969) J. Lipid Res. 10, 341–344.
14 Verger, R. and De Haas, G.H. (1976) Annu. Rev. Biophys. Bioeng. 5, 77–117.
15 Lehninger, A.L. (1976) Biochemistry, 2nd edn., p. 200, Worth, New York.
16 Erlanson, C. (1970) Scand. J. Gastroenterol. 5, 333–336.
17 Bieth, J.G. (1980) Bull. Eur. Physiopathol. Resp. 16 (Suppl.), 183–195.
18 Kondo, S., Uotani, K., Miyamoto, T., Hazoto, T., Naganawa, H., Aoyagi, T. and Umezawa, H. (1978) J. Antibiot. 31, 797–800.
19 Stodola, F.H., Lesuk, A. and Anderson, R.J. (1938) J. Biol. Chem. 126, 505–513.
20 Umezawa, H., Aoyagi, T., Hazato, T., Uotani, K., Kojima, F., Hamada, M. and Takeuchi, T. (1978) J. Antibiot. 31, 639–641.
21 Verger, R. (1984) in Lipases. (Borgström, B. and Brockman, H.L., eds.), pp. 83–150, Elsevier, Amsterdam.
22 Borgström, B. (1974) in Biomembranes, Vol. 4B (Smyth, D.H., ed.), pp. 555–620, Plenum Press, New York.
23 Erlanson, C. (1986) in Molecular and Cellular Basis of Digestion. (Desnuelle, P., ed.), pp. 297–308, Elsevier, Amsterdam.
24 Stout, J.S., Sutton, L.D. and Quinn, D.M. (1985) Biochim. Biophys. Acta 837, 6–12.
25 Gargouri, Y., Pieroni, G., Lowe, P.A., Sarda, L. and Verger, R. (1986) Eur. J. Biochem. 156, 305–310.
26 Laskowski, M. and Feng Chi Wu (1953) J. Biol. Chem. 204, 797–805.
27 Laskowski, M. and Sealock, R.W. (1971) in The Enzymes III, 3rd edn. (Boyer, P., ed.), pp. 375–473, Academic Press, New York.