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The Lipase Inhibitor Tetrahydrolipstatin Binds Covalently to the Putative Active Site Serine of Pancreatic Lipase*

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Tetrahydrolipstatin (THL) is a selective inhibitor of fat absorption. In animal models, it has anti-obesity and anti-hypercholesterolemic activity and is presently in clinical trials for these indications. THL binds covalently to pancreatic lipase. Complete inhibition of lipolytic activity is obtained concomitant with the incorporation of 1 mol of THL/mol of enzyme. Pancreatic lipase is the best studied lipase, but published results concerning its catalytic mechanism are still controversial. In order to learn more about the inhibitory mechanism of THL, a selective lipase inhibitor interacting at or near the catalytic site, and therefore, to obtain more information on the catalytic mechanism of lipase, we have determined the amino acid residue to which THL is bound. After proteolytic degradation of porcine pancreatic lipase inhibited with radioactively labeled THL, the labeled peptides were isolated and analyzed by quantitative amino acid analysis, N-terminal sequencing, and by mass spectrometry with fast atom bombardment ionization. The data clearly show that THL is bound as an ester to the serine 152 of the lipase.

Lipstatin, an inhibitor of pancreatic lipase, was isolated from *Streptomyces toxytricini* (1). Its hydrogenated derivative, tetrahydrolipstatin (THL)[1] (2), has essentially the same inhibitory activity, blocks fat absorption selectively, and has in several animal models anti-obesity (3–5) and anti-hypercholesterolemic (29) activity. In addition to pancreatic lipase (6), other lipases, such as carboxylester lipase, gastric lipase, and the bile-salt-stimulated lipase of human milk (7), are also inhibited by THL. In contrast, several bacterial lipases (7), phospholipase A_2, liver esterase, trypsin, and chymotrypsin (6) are not inhibited by THL. Therefore, THL can be considered to be the first selective irreversible lipase inhibitor.

Pancreatic lipase is the key enzyme of dietary triacylglycerol absorption. It acts at the surface of emulsified lipid droplets, and this interfacial activation distinguishes lipases as a subclass of esterases (8). Even though porcine pancreatic lipase is the best studied triacylglycerol hydrolase, the lipolytic mechanism of this enzyme is still poorly understood. From inhibition experiments with reagents of low selectivity, the involvement of an essential histidine, one carboxyl group, and a serine in the enzymatic mechanism of lipase was proposed (9), and it has been shown that diethyl-*p*-nitrophenylphosphate reacts with Ser^{152} of porcine pancreatic lipase (10). These results can be interpreted to indicate that pancreatic lipase is a serine type esterase with Ser^{152} as the active site serine. This interpretation is confused by the finding that the hydrolytic activity of pancreatic lipase towards soluble substrates, although very low, is not impaired by derivatization with organophosphates (11). Furthermore, it has been found more recently that the C-terminal fragment Ala^{336}-Lys^{449} of lipase hydrolyzes the soluble substrate *p*-nitrophenylacetate at a rate comparable with that of intact lipase (12). Thus, the other widely accepted view is that Ser^{152} is not the active site serine but is involved in interfacial recognition. In addition, from consideration of the homology between pancreatic lipase, lipoprotein lipase, and lecithin-cholesterol acyltransferase and from inhibition data obtained with these three enzymes, it has been proposed that Ser^{110} is the "active site serine" and Ser^{152} the "substrate-binding serine" (13). On the other hand, the x-ray structure of human pancreatic lipase determined recently shows clearly that Ser^{152} forms a hydrogen bonded triad with His^{263} and Asp^{176} and can be superposed with the triad of serine proteases. Furthermore, the two hydrogen bond donors required to form an "oxyanion hole" can also be identified (14). Several lines of evidence indicate that THL interacts with lipases at or near the catalytic site (6, 7). The purpose of the present study was to determine the amino acid residue in porcine pancreatic lipase to which THL becomes covalently linked, in order to learn more about the mode of binding of this selective lipase inhibitor and to obtain more information on the catalytic mechanism of lipase. Our results show that the β-lactone of THL forms an ester with the side chain hydroxyl group of serine 152. This finding lends strong support to the view that serine 152 is the active site serine of porcine pancreatic lipase.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

To inhibit porcine pancreatic lipase, incubation with THL was performed in the presence of emulsified triolein substrate since THL is essentially insoluble in aqueous buffers. Using this method, a linear relation between the percentage of activity loss and the number of THL molecules incorporated can be obtained (6). Unbound THL can be removed together with the triolein by extraction with chloroform (6).

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[1] The abbreviations used are: THL, tetrahydrolipstatin; CAD, collisionally activated decomposition; FABMS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography.

[2] Portions of this paper (including "Experimental Procedures," Table 1, Figs. 1–3 and 6, and Schemes 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

THL labeled with ^{14}C in the leucine moiety and with ^{3}H in the hydrocarbon side chain was used and both radioactivities were quantified at each step. The molar ratio of ^{14}C to ^{3}H remained constant during all experimental procedures and was identical to the ratio in THL used for the incubation of lipase. This indicates that both moieties of the THL molecule were incorporated into the covalent lipase-THL complex and that the degradation and purification procedures did not cleave the ester bond in THL.

Identification of the position within porcine pancreatic lipase of the labeled peptides obtained after degradation of lipase was based on the primary structure reported by De Caro *et al.* (23).

Mild Acid and Cyanogen Bromide Cleavage—In order to get initial information regarding the binding site of THL to lipase, a commercially available crude preparation of porcine pancreatic lipase inhibited with labeled THL was subjected to mild acid treatment, resulting in the cleavage of the Asp-Pro bonds, followed by cyanogen bromide cleavage at methionines. After gel filtration on a Bio-Gel P-30 column, the fraction containing over 80% of the radioactivity was further analyzed. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis one major band with a molecular mass somewhat below 15 kDa was seen. N-terminal sequencing over 10 cycles yielded as the main sequence *X*-Arg-Lys-Thr-Arg-Phe-Ile-Ile-*X*-Gly. Sequence alignment showed that this peptide containing the labeled THL begins with Asp^{67}. The amino acids at positions 1 (Asp) and 9 (His) could not be unequivocally identified due to the very low amount of peptide available. Since mild acid cleaves Asp-Pro bonds and CNBr splits C-terminal to methionine, the expected peptide resulting by these treatments is Asp^{67}-Asp^{176} (Scheme 2). The calculated molecular mass of this peptide, 12 kDa, is in reasonable agreement with that found by gel electrophoresis.

Tryptic Cleavage–In a second degradation experiment, THL—lipase was cleaved by trypsin. Separation of the products by gel filtration on a Bio-Gel P-10 column gave one radioactive peak in addition to activity in the void volume. N-terminal sequencing over 20 cycles of the radioactive peak, which had a molecular mass below 5 kDa, gave the sequence Ser-Ser-Leu-Gly-Tyr-Ser-Pro-Ser-Asn-Val-His-Val-Ile-Gly-His-Ser-Leu-Gly-Ser. Since trypsin cleaves peptides having Arg or Lys at the P1 position, the fragment of lipase labeled with THL has to be the peptide Ser^{137}-Arg^{163}. The same N-terminal sequence was obtained if mild acid and cyanogen bromide cleavage were followed by treatment with trypsin.

Thermolysin Cleavage—Inspection of the sequence of the peptide identified after trypsin cleavage showed that thermolysin treatment could result in smaller peptides suitable for further analysis. Preliminary experiments showed that the best degradation results could be obtained if lipase was not reduced or precleaved prior to thermolysin treatment. Upon separation of the degradation products by gel filtration on Bio-Gel P-6 the radioactivity eluted essentially in one peak (Fig. 1). If this material was directly applied to a C-4 reversed phase HPLC column, no clear separation could be achieved and upon standing or concentration of the eluted fractions the radioactive material was progressively lost, mainly through adsorption to the surfaces of glass or plastic tubes. Resolubilization in detergents, in 50% formic acid or in organic solvent mixtures, failed, but the radioactivity could be partly recovered upon complete oxidation to $^{14}CO_2$ and $^{3}H_2O$.

From the expected extremely hydrophobic nature of a small peptide bound to THL, it seemed justified to use normal phase chromatography for further separation. Optimal separation conditions were worked out on silica gel thin layer chromatography plates. Chromatographic separation of the peak from the Bio-Gel P-6 column on a silica gel column yielded essentially four radioactive peaks as shown in Fig. 2. The total recovery of radioactivity from the column was always over 90%, but the relative distribution to the four peaks varied in different degradation experiments. In one experiment *peak D* was virtually absent and *peak A* contained two-thirds of the total radioactivity. In retrospect, this variability, which obviously depends on minor changes in the experimental conditions, is not surprising, since *peaks A*, *B*, and *C* represent different modifications of the same peptide-THL adduct and *peak D* degradation products of THL (see below). These peaks from the silica gel column were further purified on a C-4 reversed phase HPLC column, and each yielded essentially one peak containing over 90% of the radioactivity applied (Fig. 3). These four fractions were analyzed for amino acids and by mass spectrometry.

Amino Acid Analysis and Sequencing—Aliquots of the fractions A and B were hydrolyzed in 6 M hydrochloric acid. More than 90% of the ^{14}C radioactivity was dissolved in the hydrolysate, but the recovery of ^{3}H was variable and very low, always below 20%. This result was not unexpected, since ^{14}C was incorporated into the leucine moiety of THL and ^{3}H into a lipophilic side chain. The amino acid composition shown in Table 1 indicates that both fractions contain His, Ser, Gly, Val, Ile, and Leu as the predominant amino acids. Since hydrolysis of THL contributes 1 Leu to the overall composition, the simplest peptide compatible with the result would be a pentapeptide containing 1 residue each of His, Ser, Gly, Val, and Ile. In fraction A the peptide could contain a second Gly or this could belong to a contaminant. Assuming, for both fractions, that THL is bound to a pentapeptide, then based on the amino acid analysis, the sum of the contaminating minor peptides would contribute on a weight basis some 24% in fraction A and 8% in fraction B. With fractions C and D no amino acid analysis was performed due to the low amount of material available.

With the fractions A–D no signals could be obtained on a gas phase sequencer. It was suspected that a THL-bound small peptide would be sufficiently lipophilic to be washed out with the organic solvents used, and indeed radioactivity was detected in the waste. Since it was known that the binding of THL to lipase is quite alkali-labile (6), fractions A and B were treated for 4 h at 100 °C with ammonia prior to N-terminal sequencing. On thin layer chromatography the mobility of the radioactivity changed substantially by this treatment, indicating that the bond between THL and the peptide was hydrolyzed by this treatment. N-terminal sequencing resulted for fraction A in the sequence Val-Ile-Gly-His-Ser. Sequence alignment shows that this pentapeptide can only correspond to Val^{148}-Ser^{152} of porcine pancreatic lipase, as this is the only sequence in the lipase which contains these five amino acids. For fraction B, again, no sequence was found, which could indicate that the N-terminal was blocked.

Fast Atom Bombardment Spectrometry (FABMS)—Fig. 4 shows the upper mass region of the mass spectrum obtained from fraction A. Two major peaks are present which according to general experience with FABMS represent the $(M + H)^+$-ions of the analyte. The masses of 1007.7 Da and 1009.7 Da correspond to the $(M + H)^+$ ions of the pentapeptide Val-Ile-Gly-His-Ser covalently bound to unlabeled THL (M + H, theoretical mass: 1007.7 Da) and to ^{14}C-labeled THL (M + H, theoretical mass: 1009.7 Da). The ratio of about 2:3 of the two peaks corresponds closely to the ratio of ^{12}C to ^{14}C in the labeled THL which was used.

The pentapeptide Val-Ile-Gly-His-Ser contains 2 residues

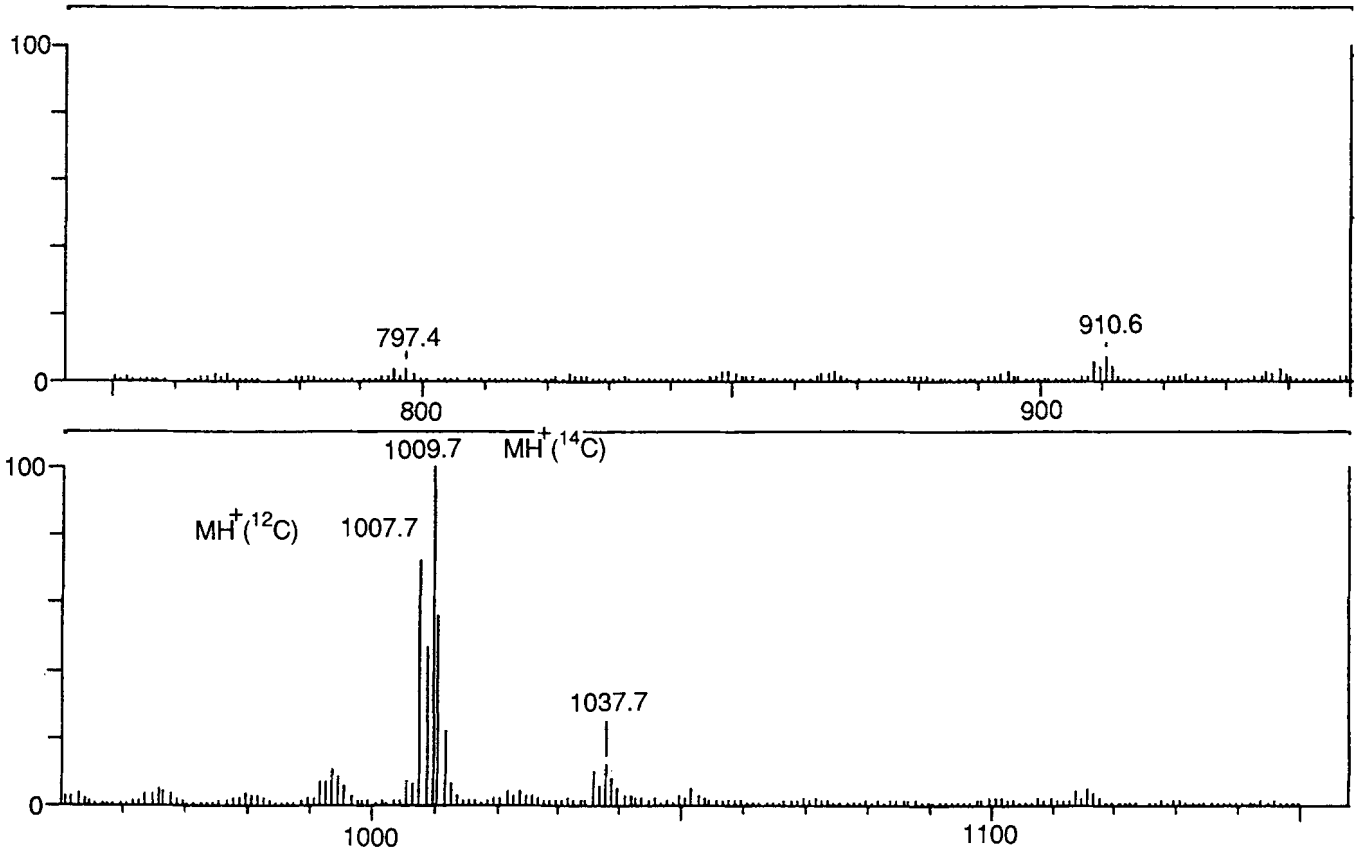


FIG. 4. **Upper mass range of the FAB mass spectrum of fraction A.**

which are potential candidates for a reaction with the β-lactone moiety of THL. Attempts to synthesize a model peptide with THL bound to serine failed, but the reaction of the model dipeptide Z-Val-His-OBn (where Z is benzyloxycarbonyl and OBn is *O*-benzyl) with THL followed by deprotection resulted in a reasonable yield of an adduct in which THL was bound to the imidazole ring of histidine.

In an attempt to localize the binding site of THL within the pentapeptide (fraction A) the fragmentation of the $(M + H)^+$-molecular ions was examined in two ways, first by looking at the (weak) fragmentation occurring in the ion source of the mass spectrometer (Fig. 5) and second by examining the fragmentation of ions collisionally activated by xenon gas in the first field-free region of the mass spectrometer (CAD) (Fig. 6).

Fig. 5 shows the total mass spectrum of fraction A, with 10-fold increased signal amplification with respect to the molecular ion in the mass range between 220 and 1000 to make fragments clearly visible. Among a large number of peaks four doublets with reasonably conserved isotope ratios ($^{12}C/^{14}C$ = 2/3) can be discerned: 908.6/910.6, 795.5/797.5, 738.5/740.5, and 601.4/603.4. The series of these doublets, interpreted in the usual way as C-terminal-containing y″-fragments (24, 25), unequivocally places the THL at the C-terminal serine, as shown in Scheme 3.

Fig. 6 shows the outcome of the experiment with collisionally induced fragmentation of the parent ions and linked scanning of the daughter ions. The spectrum shown is an average of seven scans. With this technique the mass resolution is much poorer than with the conventional scanning technique, as indicated under"Experimental Procedures," therefore the peaks are rather wide and the $^{12}C/^{14}C$-doublets are not resolved. Three of the four peaks indicated as y_{2-4}″ in Fig. 5 are also found in this spectrum at masses near those of the more intense peaks of the doublets at *m/z* 910.5, 797.2, and 740.4 (where *m/z* is mass per number of charges (in mass spectrometry *z* is usually a single positive charge)). Unfortunately the fourth peak, 603.3, is not visible in this analysis. However, there is a peak at 407.3, which must be attributed to the N-terminal fragment generated by the cleavage of the peptide bond between the histidine and the serine residue, commonly designated by b_4 (24, 25). This peak is in full agreement with the conclusion reached above that THL is located at the C-terminal serine. The peak at *m/z* 379.4 contains essentially the same N-terminal peptide portion as the fragment just discussed, less the C=O group of the histidine residue (a_4). This peak further confirms the THL location at the C-terminal amino acid (Fig. 6; Scheme 3).

All other peaks in the spectrum must be ascribed to bond cleavages around or within the THL moiety of the protonated molecule; these fragmentations must be generated by elimination reactions, occurring either in a "quasithermal" mode (26, 27) remote from the site of protonation or somehow assisted by the charged site. Close examination of these peaks on an expanded mass scale shows that they are narrower than the y″-peaks discussed before, which indicates that all of them have lost the ^{14}C-carrying group and therefore are not derived from doublets. Thus the peak at *m/z* 866.2 (Fig. 6) is due to elimination of formylleucine as the ketene and the peak at *m/z* 848.3 to elimination of the same group but as the acid (Scheme 3). In an analogous manner the peak at *m/z* 512.4 is due to loss of the whole THL-group as the β-hydroxyketene and the peak at 494.2 as the β-hydroxy acid (Scheme 3). In both cases the second fragment can be considered as a further piece of evidence that the THL-group is attached to the serine (in contrast to histidine), because only linkage to an oxygen

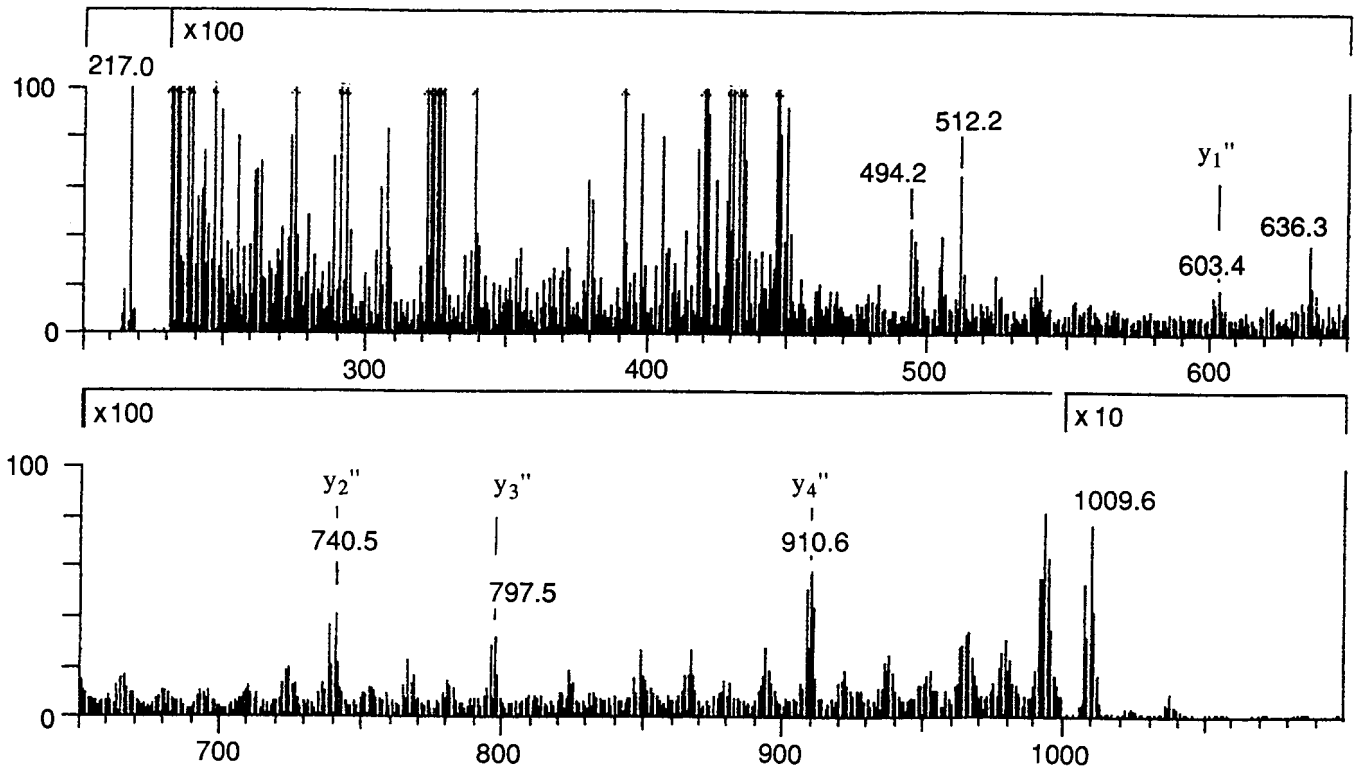
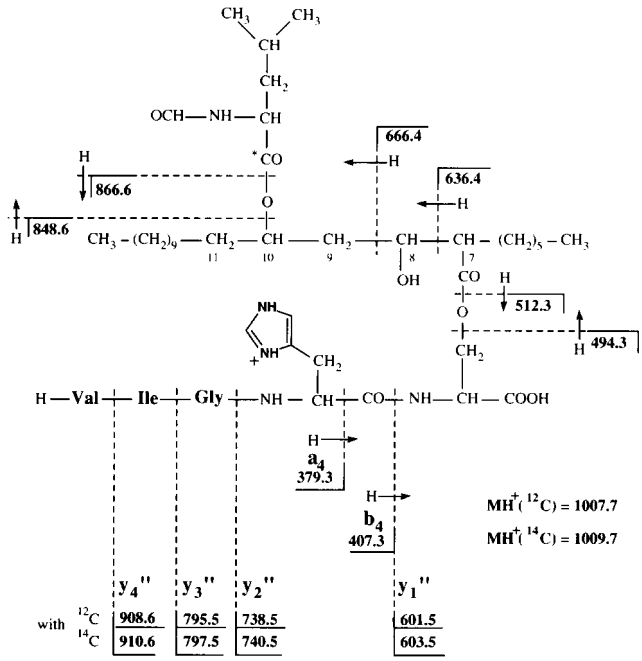


FIG. 5. **FAB mass spectrum of fraction A, amplified × 100 between *m/z* 220 and *m/z* 1000.** The peak at *m/z* 217.0 is the major matrix ion.



SCHEME 3. **Fragmentation scheme of fraction A in the mass spectrometer.** This pattern is deduced from the results shown in Figs. 5 and 6. Protonation at histidine is arbitrary.

atom can give rise to loss of THL as an acid.

The two most remarkable peaks of this series of elimination products are found at *m/z* 636.4 and *m/z* 666.4. These peaks can only be explained by assuming a cleavage of the bonds C-7–C-8 and C-8–C-9, respectively, of the THL main carbon chain, with rearrangement of a hydrogen atom away from the charged peptide-containing fragment (Scheme 3). In the absence of labeling experiments no well founded mechanism can be proposed. One can speculate that the double bonds formed in the chain of the THL-moiety by the elimination of the formylleucine between C-9–C-10 and C-10–C-11 serve as acceptors for the hydrogen atom in an ene-type reaction.

Whatever the exact mechanism, the peak at 636.4 shows that THL is attached to serine by its carboxyl-function, in contrast to its alcohol-function, or, in other words, as an ester in contrast to an ether.

Mass spectrometric analyses of fractions B and C yielded also interpretable results. Fraction B showed an $(M + H)^+$-molecular ion at 1050.8/1052.8, again with the intensity ratio 2/3. The fragmentation examined by conventional and linked scanning with CAD was partly identical with that of fraction A, partly shifted by 43 Da, and corresponded in all details to the interpretation given above (Scheme 3) if 43 Da are added to the N terminus. This strongly indicates that the compound has the same structure as fraction A but is carbamoylated at the N-terminal of valine: H_2N-CO-Val-Ile-Glu-His-Ser(THL)OH. Such a derivative could well have been generated as an artifact during treatment of the peptide mixture with urea.

Fraction C also seems to result from an artifact. The (M + H)-ions at *m/z* 1078.8/1080.8 and the fragmentation pattern are fully compatible with a compound containing the same carbamoylated pentapeptide-THL adduct as fraction B but in addition formylated at the histidine. This product was presumably formed during the chromatography of the sample in 70% formic acid. (The doublet of peaks at *m/z* 1035.7 and 1037.5 in the mass spectrum shown in Fig. 4 can be ascribed to an analogous formylation product of the principal compound of fraction A).

Due to the limited amount of fraction D, no interpretable FAB mass spectra could be obtained, but the low molecular weight peaks point to degradation products of THL. These degradation products must have been formed from the lipase-

THL adduct, because the organic extraction after labeling of lipase would have removed these fragments. On thin layer chromatography the predominant radioactive peak, which still contained equal amounts of ^{3}H and ^{14}C activity, co-migrated with a synthetic δ-lactone analogue of THL, Ro 40–4441 (Scheme 1). The formation of this δ-lactone, a rearranged derivative of hydrolyzed THL with conserved stereochemistry, is only possible if the β-lactone moiety was cleaved by nucleophilic attack on the carbonyl carbon of the four-membered ring (28). Products with inverted stereochemistry, which would arise by addition of a nucleophile in β-position of the lactone, were not observed. This clearly indicates that THL is bound to serine via an ester link and rules out the much less likely possibility of an ether link. This confirms the conclusion obtained by mass spectrometry.

tert-Butyloxycarbonyl Derivatization of the Pentapeptide-THL Adduct—As mentioned before, it was of importance to determine unequivocally whether THL was bound to the histidine or the serine of the isolated pentapeptide. Therefore, in addition to the evidence obtained from FABMS, a chemical derivatization method was sought that could clearly distinguish between the two possibilities. With the synthetic peptide H-Val-Ile-Gly-His-Ser-OH, two *tert*-butyloxycarbonyl groups could be introduced, one at the N-terminal and one at the imidazole of histidine. Using the same reaction conditions, two *tert*-butyloxycarbonyl groups could be introduced into the pentapeptide-THL adduct (fraction A) as was shown by FABMS which yielded a $(M + H)^{+}$-molecular ion doublet at 1207.5/1209.5. This result clearly confirms that THL is bound to the serine 152 of lipase.

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SUPPLEMENTARY MATERIAL
TO
THE LIPASE INHIBITOR TETRAHYDROLIPSTATIN BINDS COVALENTLY TO THE PUTATIVE ACTIVE SITE SERINE OF PANCREATIC LIPASE

by

Paul Hadváry, Walter Sidler, Walter Meister, Walter Vetter, and Helmut Wolfer

Experimental Procedures

Materials

Tetrahydrolipstatin (M_r 496, scheme 1) and [^{14}C] THL with a specific activity of 36.5 mCi/mmol were obtained by chemical synthesis [15]. [^{3}H] THL was prepared from lipstatin, of microbial origin [2], by catalytic reduction with $^3H_2$ gas. The material obtained had a specific activity of 34 Ci/mmol and was diluted to 1.16 mCi/mmol. [^{14}C] and [^{3}H] THL were repurified before use by t.l.c. as described [6]. For double labelling experiments [^{14}C] and [^{3}H] THL were mixed resulting in a final specific activity of 35.3 mCi/mmol for both, ^{14}C and ^{3}H. Thus, 57% of the THL molecules had one ^{14}C isotope incorporated instead of a ^{12}C, which was important for the interpretation of the mass spectra.

A preparation of porcine pancreatic lipase purchased from Sigma (type VI-S, L0382) was used without further purification for the experiments of mild acid, cyanogen bromide, and tryptic cleavage. For the experiments involving thermolysin cleavage, a highly purified porcine pancreatic lipase preparation, generously provided by Professor B. Borgström (Lund, Sweden), was used.

The model pentapeptide H-Val-Ile-Gly-His-Ser-OH was synthesized by the solid-phase technique using *N*-fluorenylmethoxy carbonyl (Fmoc)-amino acids, t-butyl-based side-chain protecting groups and a p-benzyloxybenzylalcohol polystyrene resin [16]. Trypsin and thermolysin were purchased from SERVA (Heidelberg, FRG). All other chemicals were from Merck (Darmstadt, FRG) or from FLUKA (Buchs, Switzerland).

Methods

Inhibition of porcine pancreatic lipase with labelled THL

Incubation of porcine pancreatic lipase with labelled THL was performed in the presence of emulsified triolein as substrate essentially as described [6] but albumin was omitted from the buffer. In a typical experiment, 45 mg (0.9 µmol) of purified lipase was incubated in a total volume of 25 ml with 2.83 µmol ^{14}C and ^{3}H labelled THL added in 1.7 ml DMSO at pH 8.0 for 1 hour at room temperature. With this treatment lipase activity was inhibited by more than 85%. Unbound THL and the lipids of the substrate emulsion were extracted by adding 140 ml methanol followed by 40 ml chloroform, mixing for 2 minutes and centrifuging; the resulting protein precipitate was washed twice with 50 ml chloroform: methanol (1:2). The radioactivity incorporated corresponded to 0.75 mol of THL per mol of lipase.

Carbamoylmethylation of free cysteines of THL-lipase

Based on the method of Hirs [17], 40 mg radioactively labelled dry THL-lipase protein precipitate was dissolved in 2 ml 6 M guanidine-HCl, 150 mM Tris-HCl (pH 7.5) containing 6 mM EDTA and 6 mg dithiothreitol dispersed by ultra-sonication (the reaction vessel was flushed with argon), sealed and incubated overnight at room temperature in the dark. The -SH groups were blocked by adding 17 mg iodoacetamide and incubating the mixture for 1 hour at room temperature in the dark under argon. The reaction was stopped with 2 ml concentrated formic acid, and the protein was subsequently desalted by gel filtration on a Bio-Gel P-2 column (1.8 cm x 15 cm) in free flow, using 70% formic acid as eluent. The protein was monitored at 280 nm.

Combined cleavage of THL-lipase at the Asp-Pro bonds and at methionine

Mild acid cleavage at the asp-pro bonds was performed by incubating the desalted protein solution in 70% formic acid (as it came from the desalting column) at 40°C for 40 h under argon. The protein was cleaved at methionine by addition of 500 mg cyanogen bromide and incubation for 7 h under argon at room temperature. Water (20 ml) was added, and the cyanogen bromide was removed by evaporation, concentrating to a volume of 2 ml. The peptide mixture was then directly applied to a Bio-Gel P-30 column (2 cm x 80 cm) using 50% formic acid as eluent in free flow (hydrostatic pressure 50 cm, flow rate 1-2 ml/h, 2 ml fractions were collected). A 100 µl aliquot was removed from each fraction for radioactivity counting. A 500 µl aliquot from the single peak containing the radioactivity was concentrated and applied to the sequenator, the rest was concentrated under vacuum to 1 ml, diluted with 20 ml water and lyophilized.

Tryptic cleavage of THL-lipase or labelled CNBr-fragment of THL-lipase

Either 40 mg THL-lipase or the lyophilized radioactively labelled CNBr-fragment from THL-lipase was suspended in 2 ml 200 mM ammonium bicarbonate buffer (pH 8.5) and dispersed by ultrasonication. Then 200 µg trypsin dissolved in 200 µl 2 mM HCl was added and incubated for 5 h at 30°C. The reaction was stopped by the addition of 2 ml conc. formic acid. The peptide solution was concentrated in vacuum to 2 ml and directly applied to a Bio-Gel P-6 (1.8 cm x 120 cm) or Bio-Gel P-10 (200-400 mesh) column (1.8 cm x 120 cm) and eluted with 50% formic acid as eluent.

Thermolysin cleavage

In a typical experiment, the radioactively labelled THL-lipase (78 mg) was dissolved in a mixture of 3 ml formic acid and 20 ml water and lyophilized. The fluffy lyophilisate was dissolved in 4 ml 8 M urea (deionized over an Amberlite MB-3 column) and 2 ml of a buffer containing 100 mM HEPES (pH 7.2) and 40 mM $CaCl_2$ was added. Digestion was performed at 40°C by adding thermolysin in portions of 3 mg in 300 µl of the above buffer at times 0, 2 hrs and 18 hrs. After 24 hrs the proteolytic degradation was stopped by adding 2 ml formic acid and the products were directly applied to a Biogel P-6 column.

Chromatographic separations

Gel permeation chromatography was performed using Bio-Gel P (-400 mesh) polyacrylamide beads (Bio-Rad Laboratories, Richmond, CA) swollen and equilibrated in 50% formic acid. The column diameter was 1.5 to 2.0 cm, and the bed height was typically 80 to 120 cm. In order to improve the flow rate of Bio-Gel P-30 in 50% formic acid, the gel was repeatedly suspended in 50% formic acid and the fines were decanted from the settled gel.

Chromatography on silica gel was performed using Kieselgel 60, 0.063-0.2 mm from Merck, Darmstadt, FRG equilibrated with chloroform: methanol: formic acid: water (80: 20: 3: 3). In a typical experiment, following thermolysin cleavage, the radioactive fractions obtained from the Bio-Gel P-6 column were concentrated in a rotary evaporator to about 1 ml, diluted with 9 ml water and lyophilized. The residue (16 mg) was dissolved in 0.5 ml of the eluant, applied to the silica gel column (45 x 1 cm) and chromatographed at a flow rate of 0.9 ml/min.

Reverse phase HPLC was performed using a VYDAC C-4 analytical column of 15 cm length (The Separations Group, Hesperia, CA, Cat. No. 214 TP 5415) at a flow rate of 1 ml/min using as mobile phase 0.1% trifluoroacetic acid in water (solvent A) and n-propanol: solvent A (9:1) (solvent B). Elution was performed for the first 20 min with solvent A:B (75:25), followed by a linear gradient to 80% solvent B over 55 min.

Amino acid analysis and sequencing

Amino-acid analysis was performed on a Biotronic LC-6000 E or on a Labotron Liquimat III amino acid analyzer essentially according to a standard procedure [18]. The samples were lyophilized, 1 ml 6 M hydrochloric acid containing 5 mg/ml phenol was added, and the tubes were flushed with argon and evacuated to less than 1 mbar. After sealing the glass tube, the samples were hydrolyzed for 24 h at 110°C. The hydrolysate (100 µl) was used for radioactivity determination, and the rest was evaporated over potassium hydroxide.

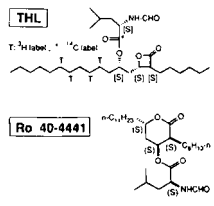
Amino acid sequences were determined on an Applied Biosystems 470 A gas-phase microsequencer following a standard procedure [19]. The phenylthiohydantoin amino acid derivatives were identified using an isocratic system described earlier [20, 21] and a Spherisorb-ODS-2 (5 µm) column (4 mm x 25 mm) at 37°C, as described by Frank [22].

Fast atom bombardment mass spectrometry (FABMS)

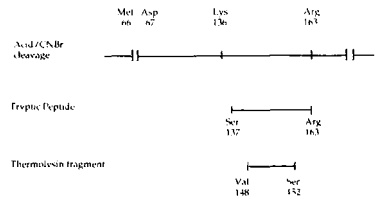
The radioactive fractions were collected, lyophilized and dissolved in 1-thioglycerol at a concentration of 0.5–1 µg/µl. About 1 µl of this solution was put on the FAB target, cooled to 12°C, and exposed to a bombardment by Xenon atoms at about 7 keV. A mass spectrometer MAT 90 from Finnigan MAT was used at an accelerating voltage of 5 kV, with conventional B-scanning, and also with linked (B/E = k) scanning combined with collisional activation in the first field-free region. Excess Xe leaking from the ionization region into the first field-free region served as collision gas. Addition of air did not significantly improve the fragmentation pattern. In the B-scans the scan rate was 10 sec/decade, the resolution about 1/2000, and the accuracy of the mass measurement was ±0.2 Da. In the linked scans, the scan rate was 2 sec/ 100 Da, the range of acceptance for the parent ions was approx. 1/100, and the resolution of the daughter ions approx. 1/400. The accuracy of the mass measurement in these scans was approximately ±0.3 Da.

Derivatisation of the peptide-THL adduct

To 10 µg of the isolated peptide-THL adduct 7 µl dioxane containing di-tert.-butyl-dicarbonate (100 mg in 1 ml) and 7 µl of a buffer (280 mM Na_2HPO_4 and 200 mM KH_2PO_4) were added and stirred for 21 hrs at room temperature. The reaction vessel and the stirring bar were washed with dioxane : water = 1:1. After lyophilization, the product was suspended in 200 µl 5% aqueous $KHSO_4$ and extracted 5x with 200 µl ethyl acetate. After filtration through cotton with $MgSO_4$, the solvent was evaporated under a stream of N_2 and the product dissolved in 1-thioglycerol for analysis by FABMS.



Scheme 1
Structure of THL and of Ro 40-4441



Scheme 2
Proteolytic degradation scheme of THL-Lipase

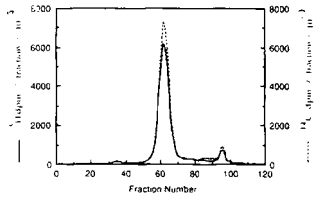


Fig. 1
Gel permeation chromatography on Bio-Gel P-6 after thermolysin cleavage.
The product obtained after thermolysin digestion (see Methods) was loaded onto a column (83 x 2.1 cm), and fractions of 3 ml were collected. The overall recovery of ^{14}C and of ^{3}H radioactivity was 75%.

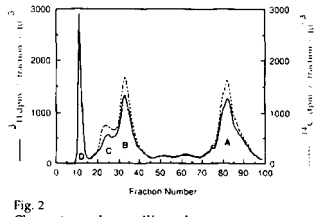


Fig. 2
Chromatography on silica gel
The main peak from the Bio-Gel P-6 column was separated on a silica gel 60 column as described in the Methods. Fractions of 3.25 ml were collected. The eluted main radioactive peaks were designated as A, B, C and D. The overall recovery of radioactivity was 93%.

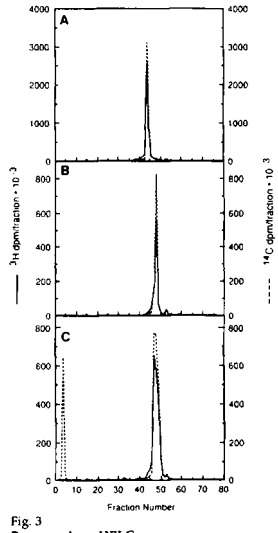


Fig. 3
Reverse phase HPLC
Peaks A, B and C from the silica gel column were further purified on an analytical C-4 reverse phase column using a gradient of n-propanol as described in the Methods. Per run, 1/3 of the respective peak was loaded, and fractions of 1 ml were collected. The overall recovery of radioactivity was always better than 90%.

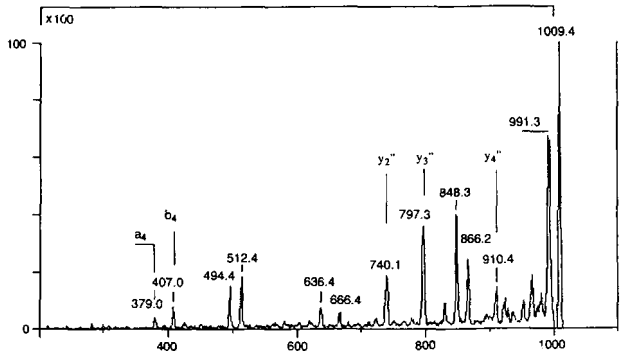


Fig. 6
Daughter ions spectrum of the collisionally activated molecular ions m/z 1009.7 of fraction A.
Summation of 7 scans.

Table 1 **Amino acid composition of the fractions A and B**

Amino acid	Content relative to Ile = 1	
	Fraction A	Fraction B
Lys	0.11	0.00
His	1.49	1.30
Arg	0.07	0.00
Orn	0.13	0.00
Asx	0.25	0.00
Thr	0.08	0.00
Ser	1.41	1.05
Glx	0.34	0.00
Pro	0.00	0.00
Gly	1.93	1.40
Ala	0.16	0.00
Cys	0.00	0.00
Val	1.22	1.11
Met	0.00	0.00
Ile	1.00	1.00
Leu	1.32	1.05
Tyr	0.00	0.00
Phe	0.00	0.00