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 concomitantly 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) (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 A₂, 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 from 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-nitrophenyl phosphate reacts with Ser₁⁵² of porcine pancreatic lipase (10). These results can be interpreted to indicate that pancreatic lipase is a serine type esterase with Ser₁⁵² 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 Ala₃₅₆-Lys₄₄₉ 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 Ser₁⁵² 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 Ser₁⁵² is the “active site serine” and Ser₁⁵² the “substrate-binding serine” (13). On the other hand, the x-ray structure of human pancreatic lipase determined recently shows clearly that Ser₁⁵² forms a hydrogen bond triad with His₁⁸⁰ and Asp₁⁷⁶ 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

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).
THL labeled with $^{13}$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 $^{13}$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 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 from these treatments is Asp$^{67}$-Asp$^{76}$ (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-Gly-Leu-Ser. Since trypsin cleaves peptides having Arg or Lys at the P1 position, the fragment of lipase labeled with THL could have been Arg$^{58}$ or Lys$^{65}$ at the P1 position, giving the sequence His-Ser-Leu-Gly-Ser. Since trypsin cleaves peptides having Ser-Ser-Leu-Gly-Tyr-Ser-Pro-Ser-Asn-Val-His-Val-Ile-Gly-His-Ser, the amino acids at positions 1 and 9 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 from these treatments is Asp$^{67}$-Asp$^{76}$ (Scheme 2). The calculated molecular mass of this peptide, 12 kDa, is in reasonable agreement with that found by gel electrophoresis.

**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$_2$O.

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 $^{13}$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 $^{13}$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 radioactive 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 $A$ 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 $^{13}$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 $^{13}$C to $^{14}$C in the labeled THL which was used.

The pentapeptide Val-Ile-Gly-His-Ser contains 2 residues...
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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 benzyloxy-carbonyl 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 (14C/12C = 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. 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, 603.5, 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 b₄ (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 (a₄). 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 14C-carrying group and therefore are not derived from doublets. Thus the peak at m/z 866.2 (Fig. 6) is due to elimination of formyleucine 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...
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FIG. 5. FAB mass spectrum of fraction A, amplified \( \times 100 \) between \( m/z \) 220 and \( m/z \) 1000. The peak at \( m/z \) 217.0 is the major matrix ion.

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 formyleucine 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-
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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.

**tet-Butyloxy carbonyl 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-OMe, two tet-butyloxy carbonyl groups could be introduced, one at the N-terminal and one at the imidazole of histidine. Using the same reaction conditions, two tet-butyloxy carbonyl 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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**REFERENCES**

Materials

Tetradroplastatin (M, 46,000; scheme 1) and \(^{14}C\) TRL with a specific activity of 34.5 mCi/mmol were obtained by chemical synthesis [15]. \(^{14}C\) TRL was prepared from lipoproteins, of internal origin [15], by catalytic reduction with \(^{3}\)H gas. The material obtained had a specific activity of 12 mCi/mmol and was dried to 1 e 10 mCi/mmol. \(^{14}C\) TRL and \(^{3}\)H TRL were repurified before use as described (8).

For double labeling experiments \(^{3}\)H and \(^{14}C\) TRL were mixed prior to a final specific activity of 3.5 mCi/mmol for both \(^{3}\)H and \(^{14}C\). Thus, 5% of the TRL molecules had one or isotope incorporated to different extent. The volume of the mass was measured.

A preparation of porcine pancreatic lipase purchased from Sigma type VLS, 2500 was used without further purification for the experiments of mild acid, cysteine bromide, erythro cyclic ascorbic acid in the presence of varying hydrophobicity, temperature and ionic strength. A high purity porcine pancreatic lipase preparation, generously provided by Professor B. Bergström (London, Sweden), was used.

The model enzyme H-Val-Leu-Glu-His-Asp-Val-CH\(_2\)COOH was synthesized by the solid-phase method using on a support of (S)-mesitylene-2,3-dicarboxamide side-chain presenting groups and a poly-(2-mercaptoethyl)polyethylene terephthalate (PMPET) and thermolysis was performed in a 1:1 molar ratio (PMPET: PMPET) with a 75:25 molar ratio of PMPET to PMPET (PMPET: PMPET).

Methods

Inhibition of porcine pancreatic lipase with labelled TRL

Inactivation of porcine pancreatic lipase with labelled TRL was performed in the presence of free cysteine as the substrate essentially as described (15). In brief, 20 mg of albumin was added to the buffer. In a typical experiment, 45 mCi \(99\) mCi of purified lipase was incubated in a total volume of 15 ml with 2.43 mCi \(99\) mCi and \(^{14}C\) labelled TRL, added in a final volume of 0.8 ml of buffer (pH 8.4) in a 1.8 cm diameter glass vessel and at 30°C. After 1 hour of reaction, the mixture was centrifuged (5000 g, 10 min) and the supernatant was removed and stored frozen. The radioactive lipase activity was quantitated by inhibition of the lipase activity at 37°C. The mixture was incubated with 150 l of 0.05 M Tris-HCl buffer with 0.5 mCi of \(^{14}C\) TRL and 0.5 mCi of \(^{14}C\) TRL (100:1) as substrate. The lipase activity was determined in the presence of varying amounts of lipase. The mixture was incubated with 50 mCi of \(^{14}C\) TRL and 50 mCi of \(^{14}C\) TRL (1:1) for 20 min. The radioactive lipase activity was determined in the presence of varying amounts of lipase.

Concurrent cleavage of TRL lipase-at the Arg-Pro bond site and methylthiolation

The lipase was used as a substrate for methylthiolation. The radioactivity was determined in the presence of varying amounts of lipase. The mixture was incubated with 150 l of 0.05 M Tris-HCl buffer with 0.5 mCi of \(^{14}C\) TRL and 0.5 mCi of \(^{14}C\) TRL (100:1) as substrate. The lipase activity was determined in the presence of varying amounts of lipase.

Thermolysis (cleavage)

In a typical experiment, the radioactively labelled TRL (30 mg) was dissolved in a mixture of 3 ml of formic acid and 2 ml of water and lyophilized. The lyophilized mixture was dissolved in 4 ml of 8 M 2-propanol solution and 5 ml of 2-propanol, then the mixture was heated at 80°C for 10 min. The mixture was then diluted with 50 ml of water and lyophilized again. The desired product was obtained by the addition of 0.05 mCi of \(^{14}C\) TRL to the reaction mixture. The mixture was centrifuged (100,000 g, 1 h) and the supernatant was removed and stored frozen.

Chloroformysis (cleavage)

The mixture was incubated with 50 mCi of \(^{14}C\) TRL and 50 mCi of \(^{14}C\) TRL (1:1) for 20 min. The radioactive lipase activity was determined in the presence of varying amounts of lipase. The mixture was incubated with 50 mCi of \(^{14}C\) TRL and 50 mCi of \(^{14}C\) TRL (1:1) for 20 min. The radioactive lipase activity was determined in the presence of varying amounts of lipase.

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Fig. 5
Daughter ions spectrum of the collisionally activated molecular ions m/z 1099.7 of fraction A. Summation of 7 scans.

Table 1  
Amino acid composition of the fractions A and B

<table>
<thead>
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<th>Amino Acid</th>
<th>Content relative to Leu = 1</th>
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<tr>
<td></td>
<td>Fraction A</td>
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<tr>
<td>Lys</td>
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