Volume 299, number 1, 111–115 FEBS 10778 March 1992

Identification of the active-site serine in human pancreatic lipase by chemical modification with tetrahydrolipstatin

Qiaoqian Lüthi-Peng, Hans Peter Märki and Paul Hadváry

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

Received 3 December 1991; revised version received 15 January 1992

A chemical modification approach was used in this study to identify the active site serine residue of human pancreatic lipase. Purified human pancreatic lipase was covalently modified by incubation with [^{3}H], [^{14}C]tetrahydrolipstatin (THL), a potent inhibitor of pancreatic lipase. The radiolabeled lipase was digested with thermolysin, and the peptides were separated by HPLC. A single THL-peptide-adduct was obtained which was identical to that obtained earlier from porcine pancreatic lipase. This pentapeptide with the sequence VIGHS is covalently bound to a THL molecule via the side chain hydroxyl group of the serine unit corresponding to Ser-152 of the lipase. The selective cleavage of the THL–serine bond by mild acid treatment resulted in the formation of the δ-lactone Ro 40-4441 in high yield and clearly proves that THL is attached via an ester bond and with retention of stereochemistry at all chiral centers to the side chain hydroxyl group of Ser-152 of the lipase. The results obtained for human pancreatic lipase corroborate the inhibition mechanism of THL found on the porcine enzyme, and are in full agreement with the identification of the Ser-152...His-263...Asp-176 catalytic triad in the X-ray structure of human pancreatic lipase.

Human pancreatic lipase active-site serine; Tetrahydrolipstatin (THL); Structure of lipase-THL adduct

1. INTRODUCTION

Pancreatic lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) is one of the two predominant lipolytic enzymes secreted by the pancreas. The preferred substrates for pancreatic lipase are emulsified triglycerides. The high activity of pancreatic lipase at an oil–water interface requires several properties that are not found in enzymes acting on soluble substrates [1]. Numerous studies have identified amino acids or regions of the protein that may be important for the interfacial activation or the catalytic activity of lipase. Most studies have used chemical modification experiments or spectral methods [2–5]. However, the reliability of both approaches is unknown. Although several groups have demonstrated that porcine pancreatic lipase (pPL) is a serine-type esterase with Ser-152 as the active-site serine [5–9], the lipolytic mechanism of human pancreatic lipase (hPL) is still only based on the functional and structural similarities, as well as on the high degree of sequence homology, to the porcine enzyme. In addition, it was postulated from the lipase crystal structure determination that Ser-152[1] would be the acylable serine in the enzyme and participate in the catalytic activity of hPL [10].

Tetrahydrolipstatin (THL[2]), a potent inhibitor of pancreatic lipase [11–14], blocks fat absorption selectively, and has been shown in several animal models to have anti-obesity and anti-hypercholesterolemic activity [6]. THL was used to identify the active-site serine in pPL [6]. Since THL inhibits hPL with similar characteristics as the pPL, we have used this inhibitor to experimentally verify the postulated role of Ser-152 in hPL. The mechanism of inhibition of hPL by THL is of special interest, since its understanding is a prerequisite for the design of structurally new lipase inhibitors as potential anti-obesity drugs.

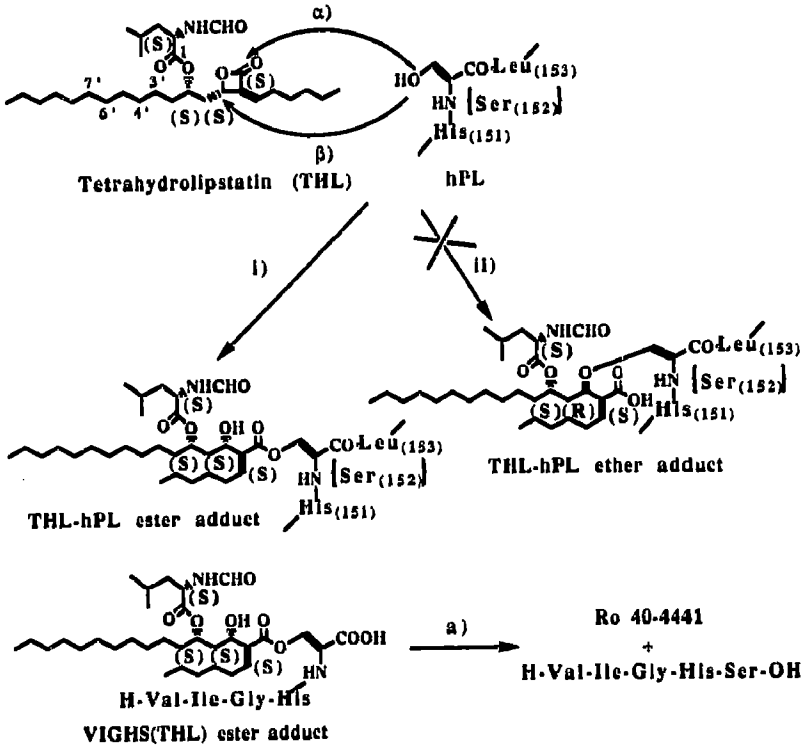
Our results clearly identify the THL binding site on hPL and precisely define the binding mode of the inhibitor to the side chain hydroxyl group of Ser-152. The data presented are in full agreement with the identification of the Ser-152, His-263, Asp-176 trypsin-like catalytic triad in the X-ray structure of hPL.

Abbreviations: CZE, capillary zone electrophoresis; FABMS, fast atom bombardment mass spectrometry; FHHA, 5-formylleucyloxy-2-hexyl-2-hexadecenoic acid; hPL, human pancreatic lipase; pPL, porcine pancreatic lipase; TFA, trifluoroacetic acid; THL, tetrahydrolipstatin. The residues of human pancreatic lipase are numbered according to the sequence of porcine pancreatic lipase [10,17].

Correspondence address: H.P. Märki, F. Hoffmann-La Roche Ltd., PRPM 15/330, Grenzacherstrasse 124, CH-4002 Basel, Switzerland. Fax: (41) (61) 691 93 91.

2. EXPERIMENTAL

Tetrahydrolipstatin (THL, M_r 495.75, Scheme 1), [^{3}H] and [^{14}C]THL ([^{3}H] at C(3′), C(4′), C(6′), C(7′); [^{14}C]: C(1)) as well as the pentapeptide VIGHS were obtained as described in [6]. hPL was obtained from Prof. B. Borgström, Lund [15]. The model compounds Ro 40-4441 and Ro 43-3042 were synthesized according to [16]. Ro 42-7476 (THL–butanol ester adduct) was prepared from THL and lithiumbutanolate. Thermolysin was a product of Serva. All other reagents were of the highest grade obtained from Merck, Fluka and Sigma.



Scheme 1. Schematic representation of the formation of an ester bond between the side chain hydroxyl group of Ser-152 of human pancreatic lipase (pathway i, nucleophilic attack of type α) and the excluded alternative formation of an ether bond (pathway ii, nucleophilic attack of type β). Chemical degradation of VIGHS(THL). The identification of the reaction products Ro 40-4441 (t.l.c., 91.5% conversion) and VIGHS (CZE) proves the ester structure of VIGHS(THL). a) 1% *p*-toluene-sulfonic acid monohydrate in acetonitrile, 24 h, room temperature.

2.1. *Isolation and characterization of [^{3}H], [^{14}C]THL modified peptides*

The purified hPL was modified by [^{3}H], [^{14}C]THL similarly as described for pPL [6]. The lipase activity was assessed using triolein as substrate [14]. The radioactively labeled THL-lipase was extracted with chloroform:methanol (1:2.5) to remove unreacted THL and then digested using thermolysin in 5 M urea at 47°C for 26 h. The thermolysin-digested peptides were chromatographed on HPLC (Vydac C4 × 0.46 cm, 5 μ). Two solvents were used. A: 0.1% TFA in H_2O; B: 0.1% TFA in 70% acetonitrile in H_2O. The pooled radiolabeled fractions were further purified on a RP C8 column (12 × 0.4 cm, 5 μ, Merck) using a gradient of 20 mM sodium phosphate in H_2O, pH 7.0, and 20 mM sodium phosphate, pH 7.0, in 60% acetonitrile. The radiolabeled fractions were finally desalted on a Vydac C4 column.

The amino acid analysis and FABMS characterization of the isolated THL-bound peptides, as well as the derivatization of the peptide adduct B, were carried out similarly as described in [6]. N-Terminal sequencing was carried out on an Applied Biosystems 475A Protein Sequencer. Due to the long alkyl chains present in THL, a covalent sequencing protocol was derived, employing immobilization of the sample via an acrylamine linker to a Sequelon-AA (Millipore, Bedford, USA), polyvinylidentrifluoride (PVDF) membrane.

2.2. *Acid hydrolysis of the THL-peptide adduct A*

20 μl of 1% toluene-sulfonic acid in acetonitrile was added to 6 μg of the isolated THL-peptide A, and the mixture was incubated for 24 h at room temperature. 20 μl of triethylamine was then added, and the mixture was evaporated. 1 nmol of the product was dissolved in 10 μl of water (0.05 mg/ml) for CZE. The rest of the contents was then developed on silica gel t.l.c. in CH_2Cl_2:$(CH_3CH_2)_2O$ (9:1).

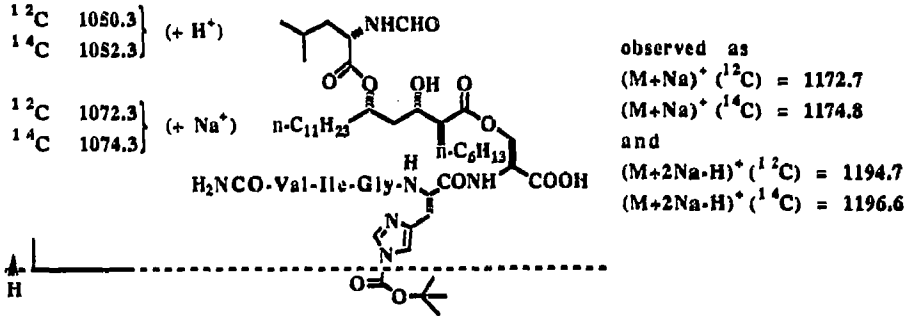
2.3. *Capillary zone electrophoresis (CZE)*

The experiments were carried out on an apparatus Model 270A, Applied Biosystems. Samples were loaded hydrodynamically by applying vacuum into a fused-silica capillary column. Each load was carried out for 1 s. The capillary column (50 μm × 72 cm) was primed with 200 mM sodium phosphate, pH 2.5, and the samples were separated at 25 kV with a current of 100 μA for 25 min, maintaining the capillary column at 30°C. Separated components were monitored at 200 nm.

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of [^{3}H], [^{14}C]THL-modified peptides*

In the current study, the incubation of hPL with THL resulted in incorporation of 0.89 mol of THL per mol of the lipase (M_r 52,000). This almost 1:1 stoichiometry of the interaction with hPL was in good agreement with the results reported for pPL and the lipases from other species [11,14]. The [^{3}H], [^{14}C]THL-labeled protein was then subjected to thermolysin cleavage. After purification on HPLC, two [^{3}H], [^{14}C]THL-peptides, A and B, were obtained. The results of amino acid analysis and N-terminal sequencing of the THL-peptide A, combined with the finding that a molecular ion doublet at 1,007.6/1,009.6 Da for peptide A and at 1,050.4/1,052.4 Da for peptide B is found in the FABMS (data not shown), indicate clearly that a H-Val-Ile-Gly-His-Ser-OH pentapeptide is covalently bound to THL in both the isolated fractions, A and B, the only difference being



Scheme 2. Mass spectrometric fragmentation scheme of peptide B derivatized with a tert.-butyloxycarbonyl group at the imidazole of His.

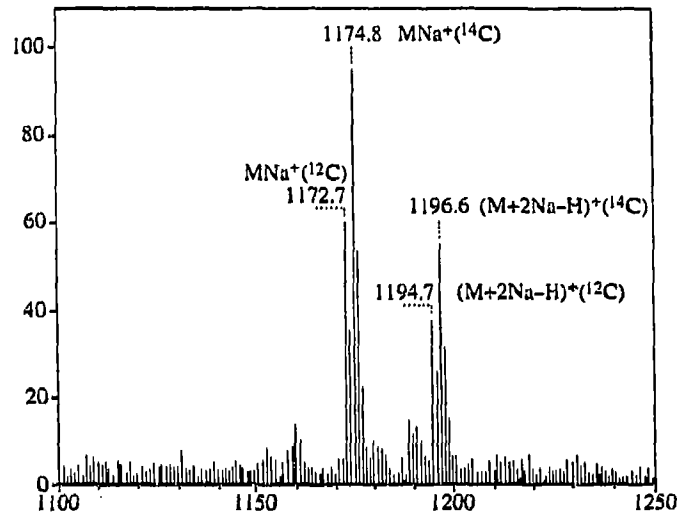


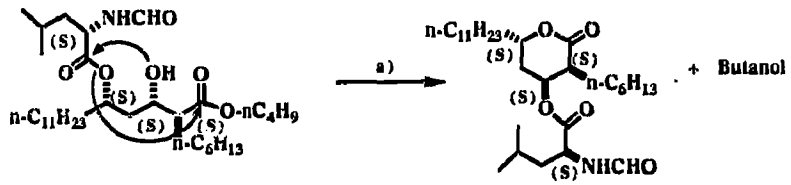
Fig. 1. Upper mass range of the FAB mass spectrum of the isolated peptide B derivatized with a tert.-butyloxycarbonyl group.

an extra carbamoyl group at the N-terminal valine of peptide B (Scheme 2). Such a derivative could easily have been generated as an artifact during the thermolysin digestion in the presence of urea. All these data indicate that the two THL-peptides isolated are identical to those obtained with pPL [6]. Sequence alignment based on the primary structure of the lipase reported by Winkler et al. [10] shows that the pentapeptide can only correspond to Val-148–Ser-152 of hPL, as this is the only sequence in the lipase which contains these five amino acids.

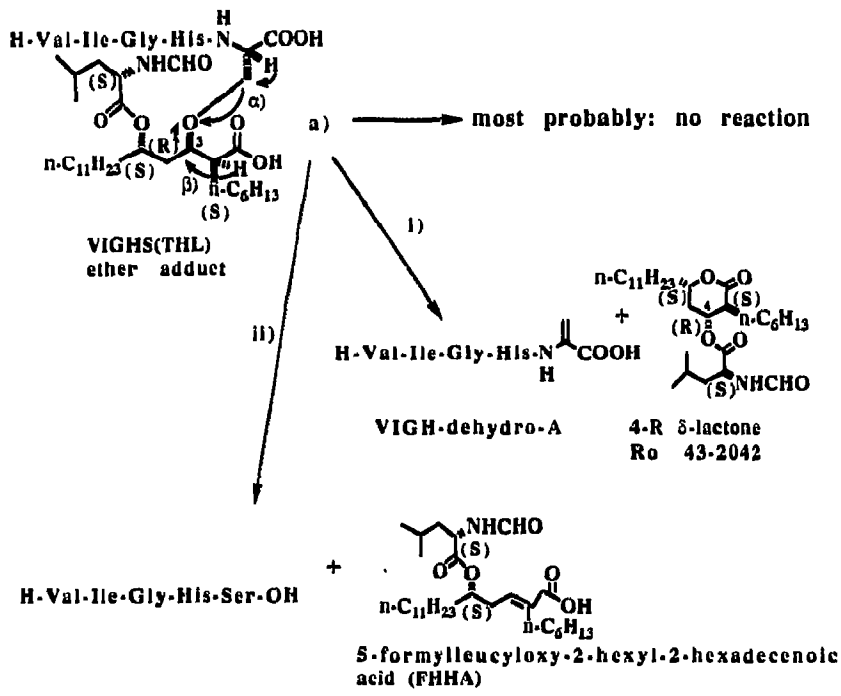
3.2. *Determination of THL binding site and binding mode*

Theoretically, the pentapeptide, VIGHS, contains two residues which can covalently react with the β-lactone moiety of THL, namely the imidazole of histidine and the hydroxyl of serine. With the synthetic pentapeptide VIGHS, two tert.-butyloxylcarbonyl groups can be introduced, one at the N-terminal and one at the imidazole of histidine. Using the same reaction conditions, one tert.-butyloxylcarbonyl group has been introduced into the THL-pentapeptide B whose N-terminal is blocked by a carbamoyl group as shown by FABMS, which yielded a $(M+Na)^+$-molecular ion doublet at 1,172.7/1,174.8 Da, and $(M+2Na)^+$-molecular ion doublet at 1,194.7/1,196.6 (Fig. 1 and Scheme 2). This experiment clearly demonstrates the presence of a free imidazole unit in histidine and therefore confirms that THL is bound to Ser-152 of the lipase.

A covalent attachment of a THL molecule to Ser-152 of hPL is possible either via an ester bond as already postulated in the case of pPL [6], or via an ether link (Schemes 1 and 4). The THL-butanol adduct, Ro 42-7476, has been synthesized as a model compound with an ester bond between a THL unit and an alcohol moiety. Under mild acidic conditions, butanol is liberated and the THL rearranges cleanly and in high yield (78% isolated) to the δ-lactone Ro 40-4441 with S-stereochemistry at all 4 chiral centers (Scheme 3). The treatment of the THL-pentapeptide adduct A under identical conditions (Schemes 1 and 4) leads to the cleavage of the THL-pentapeptide bond and to the almost exclusive formation of Ro 40-4441. 91.5% of the



Scheme 3. Rearrangement of the THL-butanol ester adduct, Ro 42-7476, to the δ-lactone Ro 40-4441 and butanol; model reaction for the VIGHS(THL) ester adduct cleavage. a) 1% p-toluene-sulfonic acid monohydrate in acetonitrile, 6 h, room temperature, 78% isolated yield.



Scheme 4. Postulated degradation of a hypothetical VIGHS(THL) ether adduct. This pathway can be excluded, since no Ro 43-2042 and at most 8.5% (t.l.c.) of FHHA was observed under degradation conditions a). a) 1% *p*-toluene-sulfonic acid monohydrate in acetonitrile, 24 h, room temperature. Pathway i) represents the possible reaction products induced by elimination of type α; pathway ii) represents the possible reaction products induced by elimination of type β.

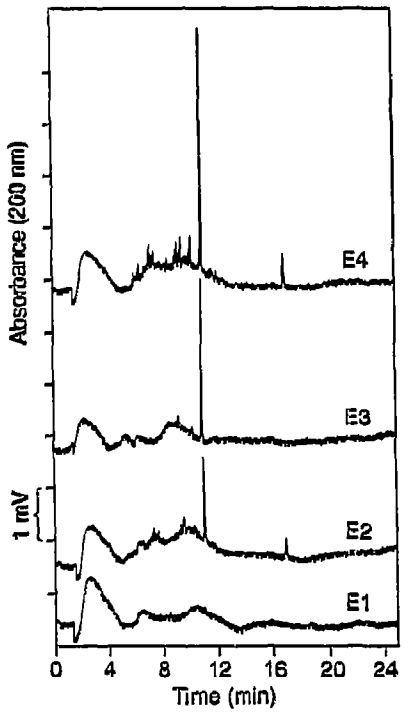


Fig. 2. Comparison of CZE electropherograms of untreated THL-peptide A (E1), acid hydrolysis products of THL-peptide A (E2), synthetic VIGHS pentapeptide reference (E3) and the mixture of both acid hydrolysis products of THL-peptide A and the VIGHS reference (E4). The experimental conditions are described in section 2.

radioactivity in the degradation mixture co-migrated with a synthetic reference of δ-lactone Ro 40-4441 on t.l.c. (R_f 0.31), whereas 8.5% of the radioactivity was observed in the R_f region 0.00–0.05. The material recovered from the t.l.c.-spot with a R_f 0.31 showed a ^{3}H-to-^{14}C ratio similar to that of the originally used THL, which is in full agreement with the structure of Ro 40-4441. The formation of the isomeric δ-lactone Ro 43-2042 (R_f 0.25) can be excluded.

A THL-pentapeptide with an ether link must have an inverted stereochemical center at position 3. Such a compound is expected to be stable under the mild acidic conditions used (Scheme 4). Forced degradation under acidic conditions could only lead to elimination processes producing either Ro 43-2042 and VIGH-dehydro-A (type α) or giving the unsaturated acid FHHA together with VIGHS (type β). The formation of Ro 43-2042 and of substantial amounts of FHHA have been excluded, thus confirming that the THL molecule is attached to Ser-152 via an ester link and with retained S-stereochemistry at all 4 chiral centers with a structure as drawn for the VIGHS(THL) ester adduct in Scheme 1.

The peptide products of the acid hydrolysis of the THL-peptide A were identified by CZE. Fig. 2 shows four electropherograms (E1–E4). Electropherogram E1 (of untreated THL-peptide A) is only poorly resolved, probably due to the hydrophobicity of the THL-adduct

and its poor solubility in the electrophoretic buffer system. E2, however, produced after acid hydrolysis of THL-peptide A, is well resolved and shows one major peak. Electropherogram E3 shows the migration pattern of the synthetic reference VIGHS pentapeptide. In E4 the acid hydrolysis product of THL-peptide A and the reference VIGHS pentapeptide were co-injected in a molar ratio of 1:2. Based on migration–time comparison, the major peak at ca. 10.6 min in E2 could clearly be identified as newly formed VIGHS pentapeptide as a result of the acid hydrolysis. The second compound formed (Scheme 1), the THL-degradation product, Ro 40-4441, cannot be seen by this technique, but has separately been identified by silica gel t.l.c. as shown above.

This study clearly demonstrates that covalent modification of lipase by THL followed by thermolysin digestion leads to the formation of the same peptide-THL adduct in the case of hPL as described earlier for pPL [6]. The present work provides in addition further evidence that THL is bound to the side chain hydroxyl group of Ser-152 via an ester bond to the carbonyl function of the β-lactone moiety (Scheme 1) and with retention of stereochemistry at all 4 chiral centers. Taken together, this study fully confirms the hypothesis that hPL follows a serine hydrolase mechanism with Ser-152 as the active site serine.

Acknowledgements: We wish to thank Dr. Hans-Werner Lahm, Michael Manneberg, and Urs Roethlisberger for their contributions in amino acid analysis and amino acid sequence determinations, Dr. Walter Vetter and Walter Meister for the FABMS measurements, Arno Friedlein for carrying out CZE experiment, Dr. Henri Stalder for the synthesis of reference compounds, Dr. Niklaus Flück and Dr. Josef Würsch for the preparation of radioactively labeled THL, Prof. Bengt Borgström (Lund, Sweden) for purified human pancreatic lipase, and Arnold Trzeciak for the synthesis of VIGHS reference pentapeptide. Thanks also to Dr. Nigel Gains for linguistic advice.

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