Structural features of a snake venom thrombin-like enzyme: thrombin and trypsin on a single catalytic platform?

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Abstract

The Lachesis muta thrombin-like enzyme (LM-TL) is a single chain serine protease that shares 38\% sequence identity with the serine protease domain of thrombin and also displays similar fibrinogen-clotting activity. In addition, the 228 amino acid residue LM-TL is 52\% identical to trypsin, and cleaves chromogenic substrates with similar specificity. Herein we report a three-dimensional (3D) model validated experimentally for LM-TL based on these two homologous proteins of known 3D structure. Spatial modeling of LM-TL reveals a serine protease with a chymotrypsin fold presenting a hydrophobic pocket on its surface, involved in substrate recognition, and an important 90's loop, involved in restricting the LM-TL catalytic site cleft. Docking analysis showed that LM-TL would not form a stable complex with basic pancreatic trypsin inhibitor and wild-type ecotin since its 90's loop would restrict the access to the catalytic site. LM-TL formed acceptable interactions with fibrinopeptide A and a variant of ecotin; ecotin-TSRR/R in which both the primary and secondary binding sites are mutated Val81Thr, Thr83Ser, Met84Arg, Met85Arg and Asp70Arg. Furthermore, analysis of the primary structures of LM-TL and of the seven snake venom thrombin-like enzymes (SVTLEs) family reveals a subgroup formed by LM-TL, crotalase, and bilincoerin, both closely related to thrombin. Therefore, LM-TL provides an initial point to compare SVTLEs with their counterparts, e.g. the mammalian serine proteases, and a basis for the localization of important residues within the little known SVTLEs family. © 2001 Elsevier Science B.V. All rights reserved.

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Abbreviations: LM-TL, Lachesis muta thrombin-like enzyme; SVTLE, snake venom thrombin-like enzyme; TSV-Pa, plasminogen activator from Trimeresurus stejnegeri venom; BPTI, bovine pancreatic trypsin inhibitor; SCR, structurally conserved region; PDB, Protein Data Bank; RMS, root-mean-square; ecotin-TSRR/R, ecotin with mutations in both primary (Val81Thr, Thr83Ser, Met84Arg, and Met85Arg) and secondary binding sites (Asp70Arg); ecotin-TSRR, ecotin with mutations in primary binding site (Val81Thr, Thr83Ser, Met84Arg, and Met85Arg); FRE, fibrinogen recognition exosite; u-PA, urokinase-type plasminogen activator

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1. Introduction

The Lachesis muta thrombin-like enzyme (LM-TL) is a serine protease purified from L. muta venom, which preferentially cleaves Arg–Gly bonds in fibrinogen α chains [1]. This clotting enzyme (228 residues) releases fibrinopeptide A in the conversion of fibrinogen to fibrin, analogous to thrombin, but hydrolyzes synthetic substrates with specificity similar to trypsin [2]. As expected, the amino acid sequence of this serine protease is 38 and 52\% identical to thrombin and trypsin [3], respectively. The putative
portion of the catalytic site has been previously hypothesized for LM-TL and confirmed as a member of the serine protease family [4]. Enzymes such as LM-TL are called snake venom thrombin-like enzymes (SVTLEs). They are directly involved in the envenomation process with a range of life-threatening activities that cause several deaths per year. Particularly in the rural tropics, snakebite morbidity and mortality has a significant human medical and economic toll. On that account, there is an urgent need to understand the envenomation process and the molecules involved in it.

SVTLEs and thrombin share mechanistic similarities [5,6,7]. In addition trypsin and the serine proteases of snake venoms have a conserved structure and are believed to have evolved from a common ancestor [8]. Since homology studies using the primary sequence could not detect all essential residues for the recognition mechanism, a three-dimensional (3D) structure of LM-TL could assist as a guide for the identification of amino acid residues important for the mechanism of catalysis and biological activity of these snake thrombin-like enzymes. Therefore, in the current absence of any crystal structure of the snake thrombin-like enzymes, we have exploited a combined approach of molecular modeling and fibrinogen-clotting assays, to investigate the structural features of LM-TL. Both thrombin and trypsin crystal structures were used as templates. LM-TL is functionally similar to α-thrombin (256 residues) while trypsin (223 residues) has the conserved disulfide bridge positions and a closer length to that of LM-TL (228 residues), although it does not present the fibrinogen-clotting activity. To test the final LM-TL model, docking simulations with macromolecular inhibitors (bovine pancreatic trypsin inhibitor (BPTI), ecotin, and mutant ecotins) were performed and predictions were analyzed by fibrinogen-clotting assays.

LM-TL also exhibits a high degree of identity (60–66%) with other snake venom proteases, such as batroxobin from Bothrops atrox venom [9] and ancord from Agkistrodon venom [10], proteins which present considerable similarities in their substrate specificity [3]. A multiple sequence alignment of the LM-TL with seven other SVTLEs using the CLUSTAL W 1.7 method was also made and analyzed.

Finally, the LM-TL model was compared with the crystal structure of a serine protease from Trimeresurus stejnegeri venom, a plasminogen activator recently elucidated and called TSV-PA, in order to verify their common structural features [11].

Herein we presented a detailed structural model of a thrombin-like enzyme from snake venom. The LM-TL structure and the analysis using ecotin and mutants provide an initial point to compare SVTLEs and other serine proteases exhibiting a trypsin fold from snake venom.

2. Materials and methods

2.1. Materials

Human fibrinogen was from Kabi Vitrum (Stockholm, Sweden), BPTI was from Sigma (St. Louis, MO, USA), C-terminal hirudin 54–65 peptide (sulfated on Tyr)$^5$ was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). All other chemicals were of the highest grade commercially available.

2.2. General hardware and software

Computer modeling and other graphical approaches were performed on a Silicon Graphics Indigo XZ4000 workstation using the modules Homology, Biopolymer, and Discover of the Insight II (Biosym/MSI, San Diego, CA, USA).

2.3. LM-TL homology modeling

Initially a multiple sequence alignment of LM-TL [3], bovine α-thrombin (entry code 1UCY) [12], and bovine pancreas trypsin (entry code 3PTN) [13] crystal structures, retrieved from The Brookhaven Protein Data Bank (PDB), was made using the Homology module to generate the set of structurally conserved regions (SCRs). Then the coordinates for all conserved residues were transferred to LM-TL and loops were constructed in a single round. Non-conserved loops of LM-TL were modeled with the function 'search loop', which searches a set of selected PDB structures for loops that best fit the given structural environment for non-conserved regions. Candidate loops were picked by visual inspection
of the resulting structure also considering root-mean-square (RMS) deviations. Several rounds of energy minimization were performed in each loop keeping the rest of the molecule restricted. Then, several cycles of constrained energy minimization regularized the structure and geometrical parameters. In subsequent runs, the full model was minimized using the AMBER force field. The model was validated by both the Prostat program of the Insight II and by using the program WHAT CHECK available free on the Internet website (http://biotech.cbi.ac.uk:8400/) [14].

2.4. Docking simulation

We constructed four docking complexes (LM-TL–fibrinopeptide A, LM-TL–BPTI, LM-TL–ecotin-TSRR/R complex A by the ecotin primary binding site and LM-TL–ecotin-TSRR/R complex B by the ecotin secondary binding site) using as templates the following X-ray crystal structures: thrombin–fibrinopeptide A (PDB entry code 1UCY) [12], trypsin–BPTI complex (PDB entry code 1TPA) [15], ecotin–rat trypsin complex (PDB entry code 1SLU) [16] and ecotin–collagenase complex [17], respectively. The ecotin crystal structure (PDB entry code 1ECY) [18] was also used as a template to construct the engineered ecotin-TSRR/R using the Biopolymer module to mutate the specific positions and the Discover module to minimize the mutated molecule.

Initially the complex crystal structure template was superposed with LM-TL guided by the SCRs using the Homology module. Then, the original enzyme from the crystal (thrombin or trypsin) was deleted using the Builder module and the LM-TL–ligand complex was searched for severe steric overlaps. In the LM-TL–BPTI docking attempt, collision of residues of BPTI with part of the LM-TL model impaired subsequent steps. The other docking complexes were submitted to subsequent energy minimization using the AMBER force field with the Discover module. Important interactions were analyzed as hydrogen bonds and electrostatic interactions, hydrophobic effects and complementarity of shapes. All docking complexes were constructed using the same procedure as described by Katz et al. [19].

2.5. Superposition of the LM-TL model with the crystal structure of thrombin, trypsin and TSV-PA

The LM-TL model was superposed with the crystal structure of trypsin [13], thrombin [12] and TSV-PA [11] on the Homology module, guided by the SCRs, using the Cα atoms.

2.6. Multiple sequence alignment of LM-TL and SVTLEs

The primary structures of LM-TL and other SVTLEs were aligned using CLUSTAL W 1.7 [20] and adjusted to account for obvious sequence similarities not detected by the algorithm. The primary sequences of the SVTLEs were obtained from the National Center of Biotechnology Information Protein Database and the accession numbers of the proteins are: thrombin-like enzyme from L. muta (S35689), bilineohin (S65621), batroxobin (AAA48552), ancred (P26324), bothrotoxins (AAB30013), calobin (AAC59906), and flavoxobin (A41456). Crotalase was copied from Pirkle et al. [21].

2.7. Protein purification

Bothrojaracins and bothroalternins were purified from Bothrops jararaca and Bothrops alternatus crude venoms, respectively, as described by Castro et al. [22] using a two-step procedure: gel filtration on a Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) followed by affinity chromatography on a column of α-D-Phe-Pro-Arg-chloromethyl-ketone-α-thrombin coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden). Briefly, fractions of about 30 kDa containing inhibitory activity toward thrombin, obtained from Sephacryl S-200 column, equilibrated with 20 mM Tris–HCl, 150 mM NaCl, pH 7.5, were incubated on a α-D-Phe-Pro-Arg-chloromethyl-ketone-α-thrombin–Sepharose affinity column for 2 h at room temperature in 20 mM Tris–HCl, 150 mM NaCl at pH 7.5. The material retained on the affinity column was eluted using 0.01 N HCl, 0.5 M NaCl at pH 2.0 and neutralized using 1 M Tris. This pool (~6–7 ml) was extensively dialyzed against 2 mM Tris–HCl at pH 7.5 and then tested for its
ability to inhibit thrombin-induced platelet aggregation. A unique band was observed on SDS-PAGE for each protein and both presented thrombin inhibitory activity.

LM-TL was purified from L. muta venom using a two-step procedure: gel filtration on a Sephacryl S-200 column, equilibrated with 0.1 mM NaHCO₃, pH 7.2, followed by affinity chromatography on a Sepharose–benzamidine column from Sigma (St. Louis, MO, USA), equilibrated with 50 mM Tris–HCl, 0.4 M NaCl at pH 9.0 and eluted using 0.1 M CH₃COONa, pH 5.0. The eluted material revealed a unique band on SDS-PAGE presenting thrombin-like activity on fibrinogen-clotting assays. Thrombin was purified according to the Ngai and Chang procedure [23].

2.8. Ecotin mutagenesis, expression and purification

Ecotin and ecotin variants were expressed and purified as described by Wang et al. [24].

2.9. Fibrinogen-clotting assays

Fibrinogen clotting was measured in a Thermomax Microplate ELISA Reader according to the method described by Ribeiro et al. [25]. Briefly, enzyme (2 nM) was incubated in the presence or absence of protease inhibitors serially diluted in 10 mM HEPES buffer, pH 7.4, using ELISA microplates. Assays were started by the addition of 50 μl of fibrinogen 4 mg/ml. The assay was performed at 37°C during 20 min using a Thermomax ELISA reader (Molecular Devices, Menlo Park, CA, USA).

3. Results and discussion

3.1. LM-TL overall structure features

The LM-TL sequence was aligned with thrombin and trypsin sequences providing eight blocks of SCRs comprising 70% of all residues (Fig. 1). The amino acids involved in these serine protease structure motifs appear to be either conserved or replaced by similar residues in LM-TL (Fig. 1) and the hypothesized ββ hydrolase fold typical of several serine proteases from the chymotrypsin family was confirmed (Fig. 2A). Superposition of the Cα atoms of the LM-TL model with thrombin and trypsin crystal structures (Fig. 2B) revealed RMS deviation of 0.78 and 0.98 Å, respectively. A better superposition of this fibrinogen-clotting enzyme was obtained with thrombin, in agreement with their functional homology, although its highest homology was with trypsin.

The arrangement of disulfide bonds of LM-TL is topologically equivalent to that of trypsin, except for the Cys¹²⁸–Cys³² bond of trypsin that is replaced by a stacking interaction between Pro¹²⁸ and Phe³² in LM-TL. This interaction thus helps to stabilize the long Pro¹²⁸–Val¹³⁸ unstructured segment. The C-terminal peptide of LM-TL, contiguous to the 231–241 helix, comprises an extended conformation for the Val²⁴²–Ser²⁵⁰ segment, which lies approximately perpendicular to the axis of the helix. This spatial arrangement observed in our model will ensure the formation of a disulfide bond between Cys⁸ and Cys²⁴⁰ (Fig. 2A), characteristic of venom serine proteases including TSV-PA [11,21,7]. The superposition of the LM-TL model with the crystal structure (TSV-PA) (RMS = 0.78 Å) showed that the overall folds of their β–β-barrel structures, in spite of the accumulation of mutations, are superposable (not shown). The
replacement of the Cys^{128}–Cys^{232} bond of trypsin by one involving the C-terminal cysteine is observed on both proteins and was also predicted by previous theoretical studies of TSV-PA [26]. This model arrangement, confirmed by crystallography studies of TSV-PA [11], reinforces the same disulfide-pairing disposition for other snake venom serine protease such as LM-TL. C-terminal extended conformation of LM-TL lies on a slightly different region when compared to TSV-PA (not shown). Recently, Krem et al. have investigated the possible dominant role of the C-terminal sequence of serine proteases in specifying its function, due to the fact that it makes up most of the surface of the S1–S3 specificity sites [6]. LM-TL and TSV-PA present totally different biological activities, which could reinforce a structure–function relationship to this region for snake venom serine proteases. Therefore LM-TL along with TSV-PA could be used as a basis to investigate the C-terminal functional role in snake thrombin-like enzymes compared to other snake venom serine proteases.

3.2. LM-TL catalytic site

LM-TL presents several important positions to the efficient proteolytic activity conserved as catalytic triad residues (His^{57}, Asp^{102} and Ser^{195}), the primary (S1), secondary (S2) and tertiary (S3) specificity sites (Asp^{189}, Gly^{216} and Gly^{226}) (Fig. 1). Also, the spatial organization of the catalytic triad residues His^{57}, Asp^{102}, and Ser^{195} at the active site cleft between the two subdomains of the β-barrel structure is preserved (Fig. 2A,B). Superposition of the LM-TL 3D model with thrombin and trypsin crystal structures revealed Asp^{189} as the primary specificity site (S1), conserved at the bottom of the pocket (Fig. 2B) positioned to make a salt bridge with basic P1 residues of substrates and inhibitors, in analogy to thrombin and trypsin. Both residues of glycine 226 (S2) and 216 (S3) are also maintained as in thrombin and trypsin, allowing the large substrate side chain access to the base of the LM-TL catalytic site (Fig. 2B).

LM-TL structure lacks amino acid residue insertions around the active site cleft (S1 loop) in compar-
Fig. 3. Analysis of docking complexes of LM-TL with fibrinopeptide A (A), BPTI (B) and ecotin-TSRR/R using primary (C) and secondary (D) binding sites. Enzyme is shown in red, substrate and inhibitors are in blue, hydrogen bond predictions are represented by green dotted lines (A, C, and D) and steric hindrance by gray dotted lines (B). (A) Environment of fibrinopeptide A (blue) in the LM-TL active site cleft (red) shows the R16-V15 bond of fibrinopeptide lying proximal to the catalytic triad (H57, D102, and S195). (B) Part of the hypothetical docking complex formed between LM-TL (red) and BPTI (blue). The BPTI docking is as in the trypsin-Arg15-BPTI complex. Only the cleft region of LM-TL is shown as well as the binding site of BPTI revealing the collision of F95 and R193 of LM-TL with the BPTI structure (gray dotted lines). (C) The ecotin-TSRR/R primary binding site presents most of its mutated residues involved in hydrogen bond interactions with the catalytic site of LM-TL. The R84 of ecotin-TSRR/R makes a salt bridge with the LM-TL S1 site (D189), while R193 and Y172 are involved in hydrogen bond interactions, similar to fibrinogen. The hydrophobic site (F95, W99, F214, W215) of LM-TL partially interacts with the L59 of ecotin-TSRR/R, and S195 of the catalytic triad interact with F214 not with H57. (D) The ecotin-TSRR/R secondary binding site’s most important mutated position (R70) is involved in interactions with D100, K180 and K97. The α-helix C-terminal portion of LM-TL composed of D233, Y234, S241 and I243 also interacts with E65, G66, W67 and G68, which are classical interactions for the trypsin-ecotin complex.

As a consequence, the LM-TL active entrance is a trypsin cleft-like structure, less deep and narrow (Fig. 2B). In addition, the LM-TL S1 loop (Thr190) is as polar as trypsin (Ser190), and in contrast to thrombin which presents one less hydrogen bond acceptor/donor residue at this position (Ala190) (Fig. 1). The feature of the LM-TL active site could provide a suitable explanation for its synthetic substrate specificity similar to that of trypsin, i.e. \( p \)-tosyl-\( \alpha \)-arginine methyl ester-HCl and \( \alpha \)-\( N \)-benzoyl-\( \alpha \)-arginine-\( p \)-nitroanilide-HCl [28]. The superposition of LM-TL with TSV-PA highlighted the significant role of the 193 position, which in TSV-PA (Phe193) is directly involved in preferential catalytic activity upon plasminogen, restricting its interaction with inhibitors such as BPTI [11,29] (not shown). In LM-TL, this position is occupied by an arginine and could have
3.3. LM-TL hydrophobic site

Thrombin acts on fibrinogen cleaving fibrinopeptides A and B using two distinct hydrophobic recognition sites [1,27]. The LM-TL S2 pocket abuts the unique hydrophobic pocket (Phe8, Trp9, Tyr172, Phe214, and Trp215) comparable to that found in the thrombin crystal structure (PDB entry code 1FPH) involved in binding of fibrinopeptide A (segment 97–99, Ile7,14, and Trp215) (Fig. 1) [30]. Therefore, the LM-TL hydrophobic pocket should be participating in the cleavage of fibrinogen as a consequence of the accommodation of the apolar residues usually found in fibrinopeptide A (Phe8 and Leu9) and at N-Bz-Phe-Val-Arg-pNA and H-Phe-Pip-Arg-pNA (LM-TL chromogenic substrates) [1]. This region, called the argy-binding site, is conserved on thrombin of several species, apparently pointing to the importance of this site for LM-TL thrombin-like activity [31]. On that account, we built a LM-TL–fibrinopeptide A docking complex, which predicted several interactions. First, our molecular studies revealed the Arg16 (P1) of fibrinopeptide A making a salt bridge with Asp189 (S1) of LM-TL and lying proximal to its catalytic triad (Fig. 3A). Second, as expected, the Phe8, which is believed to be critical for efficient thrombin-catalyzed proteolysis of fibrinogen [32], and Leu9 of fibrinopeptide A occupy the hydrophobic pocket of LM-TL (Phe85, Trp99, Tyr172, Phe214, and Trp215), similar to the thrombin mechanism. In addition, hydrogen bond interactions are observed between fibrinopeptide A (Phe8, Gly13, and Arg16) and LM-TL (Tyr172, Asp189, Thr189, Arg193, Ser195, and Gly216) revealing a complex network of interactions involved in the recognition of fibrinopeptide A (Fig. 3A). Our predictions showed Arg193 directly participating on the substrate recognition by LM-TL reinforcing the importance of this position on substrate binding that should be further investigated in mutational studies.

3.4. LM-TL has a fibrinogen recognition exosite (FRE)?

Interestingly enough, the LM-TL surface has a positively charged patch, comprised of arginine (60, 81, 82, 110, and 113) and lysine (73, 76, 85, 86, and 87) residues, located in a spatial region analogous to the thrombin FRE, composed of arginine (35, 67, 73, 75, 77, and 110) and lysine (36, 70, 81, and 109) residues (Fig. 1). FRE is a key site for thrombin molecular recognition and subsequent catalysis of its natural substrate [33]. Since the LM-TL positive site presents variations in some positions, we used three thrombin FRE-dependent inhibitors in order to verify the possible functional similarities between...
this positively charged region of LM-TL and the thrombin FRE.

Bothrojaracins, bothroalternins and the COOH-terminal domain of hirudin called hirugen are FRE-dependent inhibitors purified from B. jararaca and B. alternatus venoms and Hirudo medicinalis saliva, respectively [34,22,35]. They act by blocking the FRE, thus decreasing catalysis of its natural substrates with different \( K_\text{f} \). Our experimental assays showed that none of these thrombin inhibitors was able to inhibit LM-TL fibrinogen-clotting activity in the concentration tested, in contrast to their effects upon thrombin (Table 1). Our data are in agreement with that observed for crotalase [7]. This fibrinogen-clotting enzyme isolated from Crotalus adamanteus venom, although of a lesser extent than that observed for LM-TL, also presents a positively charged patch on its structure. This enzyme was also insensitive to hirudin, a FRE-dependent inhibitor. In view of these results and of the substituted positions on this region of both crotalase and LM-TL, in comparison to thrombin, our result reinforced the hypothesis that this region could not be capable of interaction with these FRE-dependent inhibitors or at least of being affected by them. The participation of this patch in SVTLEs fibrinogen recognition still must be further investigated.

3.5. LM-TL residue 225 and \( \text{Na}^+ \) binding site

Recent studies have shown that the nature of residue 225 specifies an important property in serine proteases: binding of \( \text{Na}^+ \), the most abundant cation in the extracellular fluids where most of serine proteases act in vivo, including snake venom serine protease. \( \text{Na}^+ \) enhances allosterically thrombin catalytic activity and might have been evolutionarily advantageous [36]. Tyrosine at position 225 enables a \( \text{Na}^+ \) binding near the S1 site on thrombin (Fig. 2B), whereas proline at the same position abrogates this function on mutated (Tyr225Pro) thrombin and on trypsin (Pro225) [36]. LM-TL shows a proline at that position and presents the same architecture found in all proteases with Pro225, i.e. trypsin [37] (Fig. 2B). Pro225 of these enzymes does not interfere in the water channel connecting S1 to the aperture at the bottom of the molecule, in contrast to thrombin Tyr225 (Fig. 2B). In addition, the orientation of the carbonyl oxygen atom of residue 224, shifted by the presence of Pro225, reduces the depth of the primary specificity site around Asp189 similar to that shown by the LM-TL model (Fig. 2B).

Therefore, our theoretical data infers that \( \text{Na}^+ \) does not allosterically regulate LM-TL proteolytic activity. The analysis of the effect of increasing concentrations of \( \text{Na}^+ \) upon LM-TL fibrinogen-clotting activity confirmed our predictions. \( \text{Na}^+ \) did not modulate LM-TL activity (0.03 M NaCl), in contrast to thrombin results (not shown). Hence our results reinforced this conserved architecture found for proteases with Pro225, including SVTLEs.

3.6. Interactions with macromolecular serine protease inhibitors

LM-TL does not present either the typical Tyr-
Pro–Pro–Trp characteristic insertion in the 60 loop or the 148 restriction loop of thrombin. Both the 60’s and 148 loops of thrombin exert a strong steric hindrance on thrombin interactions with trypsin inhibitors such as ecotin and BPTI [38,39]. Ecotin is a dimeric macromolecule that inhibits serine proteases of chymotrypsin folding by two different binding sites. The ecotin primary binding site (P1 = Met84) interacts with the catalytic site of the target enzyme while the secondary binding site (P1 = Arg20) binds to the C-terminal region [40]. BPTI is a Kunitz-type trypsin inhibitor with a single binding site (P1 = Lys17) that shares with the ecotin primary site a similar canonical mechanism [41].

The absence of typical thrombin 60 and 148 loops in the LM-TL structure could conceivably suggest an interaction of this enzyme with trypsin inhibitors. However, our docking studies using the LM-TL model predicted unfeasible interactions, since the extended 90’s loop that borders the LM-TL active site cleft dramatically restricts the BPTI or ecotin access to the catalytic site. The attempted docking of BPTI (Fig. 3B) or ecotin (not shown) with the LM-TL model resulted in a severe collision of the inhibitor-binding region with part of the 90’s loop (Phe95 and Trp99) as well as sterically hindering other residues (Lys89, Arg193, and Ser250) of the enzyme. This data is similar to that described for attempted docking of BPTI with thrombin [27]. Our experimental results corroborate the predicted inefficacy of these trypsin inhibitors against LM-TL, since neither ecotin nor BPTI were able to interfere with LM-TL or thrombin fibrinogen-clotting activities (Table 1).

3.7. Interactions with engineered serine protease inhibitors

In order to validate the overall folding of the LM-TL structure, we decided to use a macromolecular inhibitor with a broader specificity. Hence we used an ecotin variant constructed to inhibit thrombin, trypsin and other more specific serine proteases such as urokinase-type plasminogen activator (u-PA) (T. Takeuchi, personal communication). This variant called ecotin-TSRR/R presents mutations in both primary (Val81Thr, Thr83Ser, Met84Arg, and Met85Arg) and secondary binding sites (Asp70Arg) to increase the affinity for thrombin and u-PA. First of all, in order to predict a hypothetical binding mechanism between LM-TL and ecotin-TSRR/R, we constructed the engineered ecotin using the wild-type ecotin as a template, as described in Section 2. Secondly, two complexes (A and B) of LM-TL–ecotin-TSRR/R were built: complex A involving the protein–protein interaction by the primary binding site of ecotin-TSRR/R, and complex B, constructed for the secondary binding site.

The analysis of complex A showed P1 (Arg84) from the primary binding site of ecotin-TSRR/R, inserted into the pocket and presenting electrostatic interactions with S1 (Asp109) of LM-TL (Fig. 3C) as is observed for thrombin (not shown). There is extensive, though not complete, surface complementarity between TSRR/R and the LM-TL, pocket, and this allows a high density of van der Waals contacts. Analyzing the mutated positions it is possible to observe hydrogen bond contacts between ecotin-TSRR/R (Arg54, Thr51, Ser83, Arg84, Arg85, and Ala86) and LM-TL (Leu41, His57, Thr96, Arg193, Asp199, Ser195, and Trp215) amino acid residues as expected (Fig. 3C). The critical 90 loop, where part of the hydrophobic site is located, partially interacts with Leu20 of ecotin-TSRR/R avoiding its steric hindrance.

Analogous to complex A, the hypothetical complex B involving the secondary binding site of the ecotin-TSRR/R and the C-terminal of LM-TL revealed several van der Waals contacts. Presumed salt bridge interactions including those between Arg70 of the ecotin secondary binding site and Asp100 of LM-TL could also be observed (Fig. 3D). Examination of the structure shows that the loop at the distal end of ecotin relative to the reactive site, comprising residues Glu65, Gly66, Trp67, Tyr69, and Arg70, showed favorable contacts to residues Lys97, Asp100, and Glu101, and the C-terminal helical tail of LM-TL (residues Asp233, Trp237, Ser241, and Ile243) (Fig. 3D). The number of hydrogen bonds is average for protein–protein recognition sites and the whole structure is comparable to the trypsin–ecotin complex, which presents nine hydrogen bonds [42]. Results obtained by fibrinogen-clotting assay showed an agreement with the analysis of these docking complexes (Table 1). The inhibitory effects of ecotin-TSRR/R upon macromolecular substrate recognition by LM-TL were the same order of magnitude as that
observed for thrombin inhibition, confirming their 3D structural similarity.

This data is also in agreement with the isolation of LM-TL from the *L. muta* venom using an ecotin-TSRR/R affinity column. This technique was used to evaluate the utility of ecotin and ecotin variants as affinity chromatography reagents for the identification of proteases from several biological sources. It provided us with a novel and rapid method to purify LM-TL from its original source (H. Castro, unpublished data).

In order to establish the significance of the secondary binding site on the ecotin-TSRR/R–LM-TL interaction, we used another ecotin variant called ecotin-TSRR, which presents the same mutations on the primary binding site but keeps the wild-type secondary binding site (Asp79). Our experimental results showed that LM-TL is more affected than thrombin by ecotin-TSRR (Table 1). The inhibition of thrombin was almost insensitive to changes at the secondary binding site of the TSRR mutant, suggesting the dominant role of electrostatic interactions at the thrombin primary binding site. Regardless, the mutation at the 70 position had a dramatic effect on the inhibition of fibrinogen-clotting activity for LM-TL, since ecotin-TSRR was at least 20-fold and one order of magnitude less effective than ecotin-TSRR/R.

The efforts to identify the role of the secondary site of ecotin upon the target enzyme revealed different inhibitory effects of mutated ecotins depending on the target enzyme [43,44]. Our results for LM-TL can be compared to those obtained for rat trypsin or u-PA. Mutated ecotin for the secondary site weakened its interaction with u-PA and rat trypsin by over three orders of magnitude [44]. In contrast, thrombin data is similar to that described for bovine trypsin, where a minimal impact of the mutated secondary binding site was observed [43,44].

In that way, our results using engineered ecotins determined at once the maintenance of a serine protease common fold for LM-TL as well as the importance of the secondary binding site of ecotin on its inhibitory mechanism as a key element in LM-TL recognition by mutant ecotin. As far as the secondary binding site of ecotin interacts with the C-terminal portion of the target enzyme, these findings also strengthen the several and substantial differences among the C-terminal surface region of serine proteases from the chymotrypsin family [6].

3.8. Comparison of LM-TL structure with other SVTLEs

Analysis of the multiple sequence alignment of the eight SVTLEs (Fig. 4) indicated remarkable preservation of serine protease structural features including cysteine residues, and significant elements of secondary structure (α-helices, β-strands and loops). Other positions important to efficient catalytic activity such as catalytic triad, S1 and S2 sites (His57, Asp102, Ser195, Asp189, Gly216) are highly structurally conserved in SVTLEs (Fig. 4).

In the course of divergence, several interesting mutations are observed in the flanking sequence of LM-TL such as Gln56, Arg193, and Phe214, which are unusual residues on serine proteases of the chymotrypsin family. Nevertheless, this is a common feature of SVTLEs, which generally present a variety of residues (Fig. 4) other than the usual Ala56, Gly193 or Ser214 of thrombin and trypsin (Fig. 1). Due to their location, some of these substituted residues could be involved in the limited recognition and specific cleavage of macromolecular substrates by snake venom serine proteases. This is reinforced by the docking complex of LM-TL–fibrinopeptide A, which shows Arg193 and Phe214 directly involved in the cleavage mechanism (Fig. 3A).

The SVTLE surfaces may show considerable shape differences given the wide variations in loop sequence and length. The 90 loop, the most distinct loop of the LM-TL structure, is not strictly conserved in the SVTLEs family (Fig. 4). The typical 90 loop is only observed in crotalase, a fibrinogen-clotting enzyme isolated from *C. adamanteus* venom [7] and bilineocin, purified from *Agkistrodon bilineatus* venom [45], in spite of the high degree of sequence identity of the SVTLEs aligned (60–66%) (Fig. 4).

Analyzing the regions probably involved in recognition and cleavage of fibrinogen in the SVTLEs primary sequence, we observed that once more only crotalase and bilineocin present both an aryl-binding site and a positively charged region (Fig. 4). The finding of lack of activity of FRE-dependent inhibitors upon LM-TL proteolytic activity is similar to that obtained with crotalase [7], and is also in accor-
dance with the hirudin insensitivity of SVTLEs [21]. Therefore our data reinforces the idea that fibrinogen recognition by these SVTLEs may occur in a complex manner, which could use both aryl and positively charged sites, but in a way different from that observed in thrombin.

4. Conclusion

The present study provides insights into LM-TL structure and, by analogy, into thrombin, trypsin and other snake venom serine proteases. We have shown that LM-TL is able to unify the activities of mammalian thrombin and trypsin on a single catalytic platform due to its particular structure. On the level of the overall topology of the model, a realistic picture may have been obtained, since it summarizes currently available experimental observations in a concise and organized fashion. Therefore this model provides a basis for the rational design of experiments to study the structural features of SVTLEs and a logical starting point for detailed investigations of the relationship of structure to function of serine proteases with chymotrypsin folds.

Also, this paper showed for the first time the inhibition of a SVTLE using a bidentate macromolecular inhibitor, ecotin. The macromolecular serine protease inhibitors described until now, such as BPTI, are not able to affect SVTLE activity. The engineered ecotin-TSRR/R was able to inhibit LM-TL fibrinogen-clotting activity in agreement with model predictions. Since both catalytic and C-terminal regions, which are conserved in LM-TL and other SVTLEs, appear to be important sites of interaction with ecotin-TSRR/R, our results could point to the application of ecotin as a tool to identify the importance of the variant residues to SVTLEs macromolecular recognition. Improving the affinity of ecotin-TSRR/R to other SVTLEs, could allow us to distinguish important characteristics of this important family of enzymes.

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