Structure-based Design of a Potent Chimeric Thrombin Inhibitor*

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The action of thrombin is central to coagulation (1). Physiologically, its activity is regulated by serpins such as antithrombin III, heparin cofactor II, and protease nexin I as well as the general proteinase scavenger α2-macroglobulin (2). Inhibition by antithrombin III and heparin cofactor II is strongly accelerated by the acidic glycosaminoglycan heparin. Thrombin is inhibited only weakly by other typical serine proteinase inhibitors such as the Kunitz inhibitor BPTI (3). The structure of human α-thrombin (4, 5) reveals an unusually deep and narrow active site cleft, which is a major determinant of its restricted specificity. Although the number of endogenous thrombin inhibitors is small, various hematophagous parasites have developed potent antithrombotic agents, of which hirudin from the medicinal leech Hirudo medicinalis is presently the best known (6). The structure determination of the hirudin-thrombin complex (7, 8) revealed primarily a two-site interaction, namely limited penetration into the active site cleft and extensive electrostatic interaction between the acidic carboxyl-terminal “tail” of hirudin with the basic fibrinogen recognition exosite of thrombin. More recent structure elucidations of complexes of thrombin with rhodniin (9) (from the assassin bug Rhodnius prolixus (10)) and ornithodorin (11) (from the soft tick Ornithodoros moubata) also show this two-site interaction. Despite its Kunitz-like fold, ornithodorin binds thrombin in a manner that is completely different from that of the well known BPTI-serine proteinase interaction (12); contacts are made between the amino terminus of ornithodorin and residues at the active site, thus resembling the interaction of hirudin with thrombin.

Rhodniin is composed of two Kazal-type domains (9). The first domain binds in a canonical manner (12), with its reactive site loop occupying the active site of thrombin as would a substrate. The unconventional disulfide bridge arrangement of rhodniin, which it shares with the plasmin inhibitor bedellin B-3 (13) and the trypstatine inhibitor LDTI (leech-derived trypstatine inhibitor) (14), allows a particularly narrow reactive site loop, which is able to fit into thrombin’s restrictive active site canyon. The second acidic domain binds at the fibrinogen recognition exosite. Despite the close structural homology of LDTI to rhodniin (15, 16), the leech-derived inhibitor does not inhibit thrombin. This prompted us to attempt modification of the LDTI molecule to convert it into a thrombin inhibitor.

The synthetic gene for rLDTI, expressed in Saccharomyces cerevisiae (17), facilitates the construction of mutants to probe aspects of specificity and selectivity for serine proteinases. The volume of structural data available for thrombin (18) allows a rational approach to the design of specific inhibitors, for which its restricted active site canyon poses particularly stringent conditions. In this paper, we show that trimming the reactive site loop of LDTI to reduce collisions with residues lining the active site cleft of thrombin can produce an inhibitor with an inhibition constant in the 10 μM range. An inhibitor with similar affinity is obtained upon introduction of a hirudin tail fragment to untrimmed rLDTI, showing that thrombin can accept unfavorable substituents in its active site cleft upon favorable binding at the fibrinogen exosite. A mutant combining both favorable properties inhibits thrombin with a Ki value in the 10 pM range, indicating that the interactions at the active site and exosite are additive.

MATERIALS AND METHODS

With the exception of the modifications outlined below, all reagents and methods were used or carried out as described in the accompanying paper (16). Human thrombin was kindly provided by M. Otte (LMU, München, Germany); thromboplastin was purchased from Dade (Unterschleißheim, Germany). The substrate Tos-Gly-Pro-Arg-pNA was purchased from Sigma, and the reagent for measuring prothrombin time was purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

For design of the variants, coordinates of the thrombin-fibrinopep-
tide A-hirugen complex (19), the solution structure of rLDTI (15), the rhodnin-thrombin complex (9), and the rLDTI-trypsain complex (16) were superimposed and displayed using the program O (20).

Variants were obtained by cassette mutagenesis. Substitutions and insertions of the desired sequences were performed with the cloning vector pRM 5.1.5 harboring the synthetic rLDTI gene (17). After digestion of the vector with AgeI/HindIII, SphI/NsiI, or ClaI/HindIII, respectively, the fragments coding for the reactive area of rLDTI (Ala3–Ile26) and the fragment coding for the C terminus of rLDTI (Thr32–Asn46) were deleted. The vector fragments were isolated by agarose gel electrophoresis and religated with the appropriate hybridized oligonucleotides.

The oligonucleotide sequences for the AgeI/HindIII fragment of rLDTI-var1 and rLDTI-var2 were 5'-CCG GTG AAC CAG ACG AAG CAC ACG TTT AAT A and 3'-A CTG GGT CTT CTT CTG CTA ATT ATT CGA or 5'-CCG GTG ACT CGG TCG AAT CCC AAT A and 3'-A CTG AAG CTT CTT TAA GGT CTT CTT ATG AAT ATT CGA; sequences for the SphI/NsiI fragment of rLDTI-var3 were 5'-CCA AGA ACA CCA CCA CCG TGG CCA CTG AAG CTT CTT TAA GGT CTT CTT CTG GGT TTC CGA AAC GTG TCT CAG ACA CCA AGA GTG TGT GGT GCT GGT CTT ACA AGT GGT CTA AGC CCA TGC and 3'-GCT AGG GTG TTC CGG AAG ATG CTC AAG GGT TCT ATC GGT CTT CTA TGC TCG AAG AAT ATT CGA or 5'-GCT AGG GTG TTC CGG AAG ATG CTC AAG GGT TCT ATC GGT CTT CTA TGC TCG AAG AAT ATT CGA; sequences for the ClaI/HindIII fragment of rLDTI-var4 were 5'-CCG GTG AAC CAG ACG AAG CAC ACG TTT AAT A and 3'-A CTG GGT CTT CTT CTG CTA ATT ATT CGA or 5'-CCG GTG ACT CGG TCG AAT CCC AAT A and 3'-A CTG AAG CTT CTT TAA GGT CTT CTT ATG AAT ATT CGA; sequences for the SphI/NsiI fragment of rLDTI-var5 were 5'-CCA AGA ACA CCA CCA CCG TGG CCA CTG AAG CTT CTT TAA GGT CTT CTT CTG GGT TTC CGA AAC GTG TCT CAG ACA CCA AGA GTG TGT GGT GCT GGT CTT ACA AGT GGT CTA AGC CCA TGC and 3'-GCT AGG GTG TTC CGG AAG ATG CTC AAG GGT TCT ATC GGT CTT CTA TGC TCG AAG AAT ATT CGA or 5'-GCT AGG GTG TTC CGG AAG ATG CTC AAG GGT TCT ATC GGT CTT CTA TGC TCG AAG AAT ATT CGA.

For expression in S. cerevisiae, the modified rLDTI genes were isolated by XbaI/HindIII cleavage and ligated into yeast shuttle vector pVT102U/a (17). The resulting expression vectors pMH 2.1.1 (rLDTI-var1), pMH 1.1.3 (rLDTI-var2), pRM 14.1.2 (rLDTI-var3), pHB 4.1.1 (rLDTI-var4), and pHB 5.1.1 (rLDTI-var5) were used to transform S. cerevisiae S-78 (21). Standard yeast expression experiments were performed as described previously (17).

Yeast culture broth was harvested after 168 h of fermentation (6000 × g for 20 min at 4 °C). The crude supernatant was additionally centrifuged at 9000 × g for 10 min at 4 °C and concentrated using an ultrafiltration membrane with a 3-kDa cut-off value (YM3 membrane, Amicon). The buffer was exchanged by dialysis (1-kDa cut-off value, Spectra-Por 6 Membrane; Spectrum, Houston, TX) against 20 mM NaH2PO4, pH 7.8 (rLDTI-var3) or against 20 mM Tris/HCl, pH 7.2 (rLDTI-var1, -var2, -var4, and -var5). The variant rLDTI-var3 was purified by cation exchange chromatography (Fractogel® EMD SO3–650(S) column 150–10, Merck) similar to wild-type rLDTI (17). Yellow pigments in the dialyzed supernatants of rLDTI-var1, -var2, -var4, and -var5 were separated using anion exchange chromatography (Fractogel® EMD TMAE 650(Q) column) with a flow rate of 1.5 ml/min. The flow-through fraction harboring the rLDTI variants was dialyzed against 20 mM sodium phosphate buffer, pH 4.0, and purified by cation exchange chromatography (Fractogel® EMD SO3–650(S) column 150–10, Merck) at a flow rate of 1.5 ml/min with a linear gradient from 0 to 500 mM NaCl.

Equilibrium dissociation constants (K) for the complexes of rLDTI variants with human thrombin were determined as described in the accompanying paper (16). In the case of rLDTI-var3 and rLDTI-var4, K values were determined using conditions for "classical inhibitors" (22) and the "specific velocity plot" of Ref. 23.

For determination of prothrombin time, 0.1 ml of thromboplastin and 0.1 ml of inhibitor (dissolved in 25 mM CaCl2, 5% ethanol) were incubated at 37 °C for 2 min. Coagulation was initiated by the addition of 0.1 ml of citrated human plasma. For determination of activated partial thromboplastin time, citrated human plasma was incubated at 37 °C with 0.1 ml of prothrombin time reagent. After 3 min, 0.1 ml of inhibitor (dissolved in 25 mM CaCl2, 5% ethanol) was added. For determination of thrombin time, 0.1 ml of citrated human plasma was mixed with 0.05 ml of inhibitor dissolved in 0.154 M NaCl, 5% ethanol, and coagulation was started by the addition of 0.05 ml of thrombin (10 units/ml). Clotting times were determined in duplicate using the coagulometer Thrombotrack 8 (Immuno GmbH, Heidelberg, Germany). Inhibitor concentrations required to double the respective clotting times were determined using semilogarithmic plots of clotting times versus inhibitor concentrations.
RESULTS

The rLDTI variants, displayed schematically in Fig. 1, were constructed by cassette mutagenesis using the cloning vectors pRM 5.1.5 and pHB 2.1.2, harboring the synthetic rLDTI and rLDTI-var\textsuperscript{u4} genes, respectively. The initial mutants were designed on the basis of the solution structure of rLDTI (15) and the sequence of rhodniin (10) only, i.e. prior to the crystal structure elucidations of the complexes of rhodniin-thrombin (9) and rLDTI-trypsin (16). The rhodniin-derived peptide EPDEDEDV, presumed to bind to the fibrinogen recognition exosite, was added to the flexible native C terminus of LDTI (rLDTI-var\textsuperscript{u1}), as was the hirudin tail sequence DFEE-IPEEYLQ (rLDTI-var\textsuperscript{u2}).

Three residues of the reactive site loop (Ile\textsuperscript{9}, Lys\textsuperscript{11}, and Ser\textsuperscript{24}) were identified as yielding potential clashes with the characteristic thrombin 60-loop (Fig. 2) and were therefore replaced with the corresponding residues of rhodniin (Ala, His, and Pro, respectively). Furthermore, Pro\textsuperscript{12} was replaced by its rhodniin counterpart Arg to avoid any possible adverse main chain conformational rigidity. This resulted in mutant rLDTI-var\textsuperscript{u3}.

Subsequent elucidation of the rLDTI-trypsin complex crystal structure (16) and superposition with the ternary complex thrombin-fibrinopeptide A-hirugen (19) revealed the need for a polyglycine spacer between the rLDTI C terminus and the hirudin tail peptide, leading to variants rLDTI-var\textsuperscript{u4} and rLDTI-var\textsuperscript{u5} (Fig. 3). Cloning vectors pHB 6.1.1 (rLDTI-var\textsuperscript{u1}), pHB 1.1.1 (rLDTI-var\textsuperscript{u2}), pRM 13.1.1 (rLDTI-var\textsuperscript{u3}), and pHB 2.1.2 (rLDTI-var\textsuperscript{u4}) were used to transform E. coli TG1, and the corresponding DNA sequences were confirmed. For expression in yeast, the single Xba\textsuperscript{I}-Hin\textsuperscript{dIII} gene cassettes were subcloned in the yeast shuttle vector pVT102U\textsuperscript{a} (24), and S. cerevisiae strain S-78 was transformed with the resulting expression vectors pMH 2.1.1 (rLDTI-var\textsuperscript{u1}), pMH 1.1.3 (rLDTI-var\textsuperscript{u2}), pRM14.1.2 (rLDTI-var\textsuperscript{u3}), pHB 4.1.1 (rLDTI-var\textsuperscript{u4}), and pHB 5.1.1 (rLDTI-var\textsuperscript{u5}). Transformed yeast cells cultivated under standard conditions produced recombinant material. For each variant, trypsin-inhibitory activity was detectable in the culture broth as well as a distinct protein band migrating at a Mr corresponding to the theoretical mass as analyzed by SDS-polyacrylamide gel electrophoresis (data not shown).

Yields of the variants were $\text{5.3 mg/liter}$. The isolated material of each variant was homogeneous and $>95\%$ pure as judged by SDS-polyacrylamide gel electrophoresis, isoelectric focusing, and HPLC analysis (data not shown). Automated N-terminal sequencing verified the correct processing of the mating type leader fusion protein. With the exception of rLDTI-var\textsuperscript{u5}, mass spectroscopy of the variants yielded molecular masses in agreement with those calculated (given in parentheses): 5326.8 (5325.9) Da for rLDTI-var\textsuperscript{u1}, 5791.3 (5790.5) Da for rLDTI-var\textsuperscript{u2}, 4773.5 (4779.5) Da for rLDTI-var\textsuperscript{u3}, 5863.6 (5864.6) Da for rLDTI-var\textsuperscript{u4}, and 5772.9 (5900.6) Da for rLDTI-var\textsuperscript{u5}. The lower mass of 127.7 Da for rLDTI-var\textsuperscript{u5} is probably due to truncation of the C-terminal Gln\textsuperscript{56} by endogenous yeast proteinases.

The trypsin-specific inhibitory activity of the isolated variants was found to be $>40\%$ of the theoretical value, which is comparable with recombinant wild-type LDTI (17). Equilibrium dissociation constants ($K_i$) were determined for the complexes of recombinant LDTI (17) and its variants with human $\alpha$-thrombin (Table I). The acidic C-terminal extension in rLDTI-var\textsuperscript{u1} and rLDTI-var\textsuperscript{u2} failed to produce the anticipated increase in affinity for human $\alpha$-thrombin, while the $K_i$ value for bovine trypsin was identical to that of wild-type rLDTI.

The amino acid substitutions at the reactive site of rLDTI (rLDTI-var\textsuperscript{u3}) and the additional insertion of a glycine spacer in rLDTI-var\textsuperscript{u2} to give rLDTI-var\textsuperscript{u4} resulted in a remarkable improvement in affinity for human $\alpha$-thrombin, leaving that for bovine trypsin unchanged but strongly reducing that for bovine

FIG. 2. Comparison of the fit of LDTI (top) and rhodniin (bottom) into the active site cleft of thrombin. Residues of LDTI that would clash with residues of the 60-loop of thrombin are shaded. For simplicity, main chains are drawn as a smooth rope. This figure was prepared using MOLSCRIPT (42).
chymotrypsin in both cases.

The highest affinity toward α-thrombin was achieved by combining the mutations of rLDTI-var3 and rLDTI-var4 to produce rLDTI-var65, which resulted in an over 18,000-fold increase in affinity for thrombin compared with the wild-type form. This increase in affinity was paralleled by an increased selectivity for thrombin versus the other serine proteinases tested.

The degree of anticoagulatory activity displayed by the variants in clotting assays (Table I) correlates with the $K_i$ values determined for thrombin inhibition. Despite the 1000-fold lower $K_i$ value measured for the complex of thrombin with rLDTI-var65 compared with that with hirudin, very little difference is seen between their anticoagulatory activities.

**DISCUSSION**

The results presented here show that serine proteinase inhibitors can be suitably modified for a specified target enzyme using a structure-based approach. The failure of rLDTI variants var01 and var02 to yield the anticipated inhibition of thrombin, however, highlights the difficulties of such an approach when insufficient structural information is available at the outset.

The design of rLDTI-var03 involved identifying LDTI residues colliding with thrombin residues of the 60-insertion loop and replacing them with the corresponding residues of rhodniin. Closer inspection of the proposed interaction suggests that the major obstruction for binding to thrombin comes from the side chain of Ile9, which would clash with the side chain of Lys8 in hirudin. Closer inspection of the proposed interaction suggests that the major obstruction for binding to thrombin comes from the side chain of Ile9, which would clash with the side chain of Lys8 in hirudin. The design of rLDTI-var03 involved identifying LDTI residues colliding with thrombin residues of the 60-insertion loop and replacing them with the corresponding residues of rhodniin. Closer inspection of the proposed interaction suggests that the major obstruction for binding to thrombin comes from the side chain of Ile9, which would clash with the side chain of Lys8 in hirudin. Closer inspection of the proposed interaction suggests that the major obstruction for binding to thrombin comes from the side chain of Ile9, which would clash with the side chain of Lys8 in hirudin.

Figure 3. Modeled complex of rLDTI-var03/rLDTI-var05 with thrombin. The Kazal-type domain occupies the active site (top center), with the acidic hirudin tail binding to the basic fibrinogen recognition exosite to the right. This figure was prepared using SETOR (45).

**Table I**

| $K_i$ for thrombin inhibition | Concentration required for doubling of | | |
|-----------------------------|---------------------------------------|-------------------------------|
|                            | Thrombin time | Activated partial thromboplastin time | Prothrombin time |
| rLDTI                       | >300 nM       | >10 µM                          | ND             |
| rLDTI-var01                 | >300 nM       | >10 µM                          | ND             |
| rLDTI-var02                 | >300 nM       | >10 µM                          | ND             |
| rLDTI-var63                 | 9.4 nM        | 0.24 3.7                        | ND             |
| rLDTI-var64                 | 14.9 nM       | 0.032 0.43                      | 0.76           |
| rLDTI-var65                 | 16.0 pm       | 0.013 0.09                      | 0.20           |
| Hirudin                     | 27.0 fm       | 0.011 0.11                      | 0.23           |

* ND, not determined.

The values reported above correspond to a greater than 1000-fold increase in affinity; the additional binding at the exosite (rLDTI-var65) corresponds to a greater than 100-fold increase in affinity.

The occupancy of the fibrinogen recognition exosite can facilitate binding at the active site, reminiscent of “allosteric linkage” (31). The action of rLDTI-var4 resembles that of the serpin heparin cofactor II. Unusual among inhibitors of serine proteinases with trypsin-like specificity, this serpin possesses a Leu residue at P1 rather than the preferred Arg (32, 33). Accommodation of this unfavorable residue requires the addition of heparin, whose action is 2-fold; 1) it links the heparin binding sites of thrombin and heparin cofactor II, and 2) it simultaneously exposes the acidic N-terminal peptide of heparin cofactor II, making it available for binding to the fibrinogen recognition exosite (34–36). This complex mechanism ensures that of all the hemostatic proteinases, only thrombin is inhibited. By analogy, it should be possible to suitably modify the reactive site of rLDTI-var4 to increase the selectivity for thrombin.

Although thrombin binds the archetypal serine proteinase inhibitor BPTI with a $K_i$ value greater than micromolar (3), a mutant with the single mutation Glu192 → Gln inhibits thrombin with an affinity in the nanomolar range (37, 38). We have recently solved the structure of thrombin E192Q in complex with BPTI (39), which shows dramatic rearrangements of the surface loops surrounding the active site including a remodeling of the fibrinogen recognition exosite. The results presented here corroborate our conjecture that access to thrombin’s active site cleft can be increased upon energetically favorable exosite binding, which might be necessary for thrombin to perform some of its diverse functions. It is conceivable that progres-

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2 A. S. Tanaka, C. A. Sampaio, M. T. Stubbs, H. Fritz, and E. A. Auerswald, unpublished results.
sively tighter binding at the exosite(s) could widen the active site cleft to varying degrees, although there exists as yet no direct structural evidence for this. Interactions at the active site and at the fibrinogen recognition exosite are additive, as shown by the inhibition data obtained for rLDTI-varG5.

The best inhibitor designed by our approach, variant rLDTI-varG5, could be useful as a potential novel anticoagulant. The favorable combination of both active site and fibrinogen recognition exosite binding yields a recombinant equivalent of the synthetic divalent inhibitors epitomized by hirulog (40) and hirutonin exosite binding yields a recombinant equivalent of the favorable combination of both active site and fibrinogen recognition exosite binding yields a recombinant equivalent of the synthetic divalent inhibitors epitomized by hirulog (40) and hirutonin (41). Despite the 1000-fold lower affinity for thrombin compared with hirudin, the anticoagulatory activity of rLDTI-varG5 matches that of hirudin in plasma-based clotting assays. The suitability of this variant for possible therapeutic or in vivo applications will depend on several aspects, however: its potential antigenicity, its bioavailability, and its effectiveness in preventing bleeding. These items must be addressed thoroughly before a therapeutic application can be envisaged.

In conclusion, we have been able to construct three potent inhibitors of thrombin. They represent suitable models for the design of tighter, more specific or more selective coagulation inhibitors.

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