The Three-dimensional Structure of Recombinant Leech-derived Tryptase Inhibitor in Complex with Tryptsin

IMPLICATIONS FOR THE STRUCTURE OF HUMAN MAST CELL TRYPTASE AND ITS INHIBITION

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The x-ray crystal structure of recombinant leech-derived tryptase inhibitor (rLDTI) has been solved to a resolution of 1.9 Å in complex with porcine trypsin. The nonclassical Kazal-type inhibitor exhibits the same overall architecture as that observed in solution and in rhodniin. The complex reveals structural aspects of the mast cell proteinase tryptase. The conformation of the binding region of rLDTI suggests that tryptase has a restricted active site cleft. The basic amino terminus of rLDTI, apparently flexible from previous NMR measurements, approaches the 148-loop of tryptase. This loop has an acidic equivalent in tryptase, suggesting that the basic amino terminus could make favorable electrostatic interactions with the tryptase molecule. A series of rLDTI variants constructed to probe this hypothesis confirmed that the amino-terminal Lys-Lys sequence plays a role in inhibition of human lung tryptase but not of trypsin or chymotrypsin. The location of such an acidic surface patch is in accordance with the known low molecular weight inhibitors of trypase.

Tryptase is the major protein component of mast cell granules (1). In contrast to many other trypsin-like serine proteinases, tryptase is present in the granules in an active form. Human mast cell tryptase is catalytically active as a tetramer, stabilized by heparin proteoglycans from the mast cells that are stored and secreted together with the protease. Human tryptase is unique inasmuch as no endogenous inhibitors have yet been detected for this enzyme, although rat tryptase has been shown to be inhibited by trypstatin (2), a fragment of the same inhibitor (22) but exhibiting a distinctive disulfide pattern. The x-ray crystal structure of recombinant leech-derived tryptase inhibitor (LDTI), a protein-type inhibitor of human mast cell tryptase from the medicinal leech Hirudo medicinalis, (18) and have expressed it in Saccharomyces cerevisiae (19). LDTI inhibits tryptase with a K_i of 1.4 nM and, in addition, trypsin and chymotrypsin in the nanomolar range. Together with the plasmin inhibitor bdelin B-3 from H. medicinalis (20) and the thrombin inhibitor rhodniin from Rhodnius prolixus (21), LDIT belongs to a small group of nonclassical Kazal-type inhibitors related to, for example, the Japanese quail ovomucoid inhibitor (22) but exhibiting a distinctive disulfide pattern.

The abbreviations used are: LDTI, leech-derived tryptase inhibitor; rLDTI, recombinant leech-derived tryptase inhibitor; HPLC, high performance liquid chromatography; TDPCA, (1, 1', 3')-terphenyl-3,'3'-dicarboximidium; DX9065a, (+/-2-14C/[3S]-3-amino-3(pyridyl)-oxy[phenyl]-3-(7-amidino-2-naphthyl) propionic acid; BACH, 2,7-bis-(4-amidinobenzylidene)-cycloheptan-1-one; BABIM, bis-(5-amidino-2-benzimidazolyl)methane; MOPS, 4-morpholinepropanesulfonic acid.

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Tryptase Inhibitor Structure

TABLE I
Sequences of Kazal-type inhibitors, aligned according to cysteines

| Inhibitor | Cysteines | Secondary structure |
|-----------|-----------|---------------------|
| LDTI      | * bbb     | bb aaaaaa bbbb     |
| BDB3      | KDCA      |                  |
| Rhodniin  | A          |                  |
| JPQ       | LLN       |                  |

(BDB3, bdellin B3; RHOD1 and RHOD2, first and second domains of rhodniin, respectively; JPQ, third ovmucoid domain from the Japanese quail. The asterisk denotes the position of the P, residue; secondary structure is denoted by a (α-helix) and b (β-sheet). Residues mutated in this work are in boldface type.

We have recently solved the structure of rLDTI in complex with porcine trypsin, solved to a resolution of 1.9Å. The compact molecule binds to the active site of trypsin in a canonical (i.e. substrate-like) manner. Its distinctive disulfide pattern results in a disk-shaped molecule that suggests a restricted active site cleft of trypsin. The deletion of one turn of α-helix allows the amino-terminal residues to follow a path along the surface of the proteinase novel for Kazal-type inhibitors. Based on the structure, we have used site-directed mutagenesis to establish a specific role of the amino-terminal basic residues of LDTI in its interaction with human lung trypsome. The implications of these results for the structure and inhibition of trypsin are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals used were of the highest quality available commercially from the following sources: Sigma, Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Biogen (Hamburg, Germany), Roth (Karlsruhe, Germany), Braun (Melsungen, Germany), Dianova (Hamburg, Germany), and Promega (Madison, WI).

Restriction endonucleases and DNA-modifying enzymes were purchased from the following sources: Boehringer Mannheim GmbH (Mannheim, Germany), New England Biolabs (Beverly, MA), and Pharmacia-Biotech (Freiburg, Germany).

Bacto-trypsin, Bacto-peptone, Bacto-yeast nitrogen base, Bacto-yeast extract, and Bacto-agar were from Difco (Augsburg, Germany).

rLDTI was isolated according to the procedure in Ref. 19, and human lung trypsome was isolated as described in Ref. 18.

Bovine pancreatic trypsin; human trypsin; and the substrates Tos-Gly-Pro-Arg-pNA (tryptase), Bz-Ile-Glu-Gly-Arg-pNA (trypsin), and Gly-Pro-Arg-pNA (tryptase) are discussed. The implications of these results for the structure and inhibition of trypsin are discussed.

**Protein Purification and Protein Chemical Characterization**—Protein isolation and purification was performed as described (18, 19) with the following modifications in cation exchange chromatography buffers: 20 mM NaH2PO4, pH 7.3, for rLDTI-var1 and 20 mM NaH2PO4, pH 7.0, for rLDTI-var2 and rLDTI-var3.

SDS-polyacrylamide gel electrophoresis of proteins was performed with 15–25% polyacrylamide gels (37). The gels were self-prepared and run in either a conventional apparatus or the PhastSystem™ (Pharmacia). Isoelectric focussing was performed with the PhastSystem™ using the calibration kit, pH 3.5–9.3, from Pharmacia.

Selected fractions from the cation exchange chromatography, usually 2–5 μmol of protein, were analyzed by reversed phase HPLC as detailed previously (18). Partial N-terminal sequence analysis was performed with the gas phase sequencer 473A (Applied Biosystems GmbH, Weitbrach, Germany) following the instructions of the manufacturer.

The protein concentration was determined using the Pierce BCA* protein assay with bovine serum albumin as standard protein (38) or by measuring the absorbance at 280 nm and using theoretical values for aromatic residues and cysteines according to Ref. 39, with a global average temperature factor of 21 Å2.

Cassette Mutagenesis and Expression of the rLDTI Variants—Standard techniques of molecular cloning were performed according to Ref. 35, with DNA sequences according to methods according to Ref. 36. The deletion variants rLDTI-var1 and rLDTI-var2 and the substitution variant rLDTI-var3 were constructed by cassette mutagenesis using the cloning vector pRM 5.1.5 (19). Briefly, after cleavage of vector DNA with XbaI/BglII, the small fragment coding for the fusion linker and the N terminus of rLDTI was substituted by appropriate hybridized oligonucleotides (cassettes). The oligonucleotide sequences for the XbaI/BglII linker, the small fragment coding for the fusion linker, and the N terminus of rLDTI were amplified by PCR using the primers: A-CTA GAT AAA AGA GTT TGC and B-CTA GAT AAA AGA GTT TGC with the primer set according to Ref. 31. The resulting cloning vectors (pRM 16.1, rLDTI-var1, rLDTI-var2, rLDTI-var3, rLDTI-var4, and rLDTI-var5) are described in the accompanying paper (36).

**Protein Expression and Purification**—Expression of rLDTI and the mutated rLDTI genes were isolated by XbaI/HindIII cleavage and ligated into yeast shuttle vector pVT102/Ua. The resulting expression vectors pRM 20.2.1, pRM 21.2.1, and pRM 22.2.1 were used to transform E. coli K12/pRM 22.2.1 and E. coli K12/pRM 22.2.1 into S. cerevisiae and transformed yeast was grown in synthetic media lacking Trp, Leu, His, and Ade and transformed in parallel with the parental strain (23) and that of rhodniin in complex with thrombin (24).

**Trypsin Inhibitor**—The starting point of the structure of rLDTI in solution (23) and that of rhodniin in complex with thrombin (24).

In this paper, we describe the crystal structure of rLDTI in complex with porcine trypsin, solved to a resolution of 1.9Å. The compact molecule binds to the active site of trypsin in a canonical (i.e. substrate-like) manner. Its distinctive disulfide pattern results in a disk-shaped molecule that suggests a restricted active site cleft of trypsin. The deletion of one turn of α-helix allows the amino-terminal residues to follow a path along the surface of the proteinase novel for Kazal-type inhibitors. Based on the structure, we have used site-directed mutagenesis to establish a specific role of the amino-terminal basic residues of LDTI in its interaction with human lung trypsome. The implications of these results for the structure and inhibition of trypsin are discussed.
tive LDTI variants were determined by titration with trypsin, assuming an equimolar interaction between each inhibitor and the enzyme. Bovine pancreatic trypsin was standardized by active site titration using p-nitrophenyl p'-guanidinobenzoate (43).

Equilibrium dissociation constants ($K_i$) were determined essentially as suggested by Bieth (44). Briefly, a constant concentration of bovine pancreatic trypsin, chymotrypsin, or trypsinase isolated from human lung tissue (in the presence of 50 μg/ml heparin to stabilize the protease) was incubated with increasing concentrations of each inhibitor; the time necessary to reach equilibration of the enzyme-inhibitor complex was determined in preliminary experiments. Subsequently, the residual enzyme activities were measured by following the hydrolysis of suitable substrates. Apparent $K_i$ values were calculated by fitting the steady state velocities to the equation for tight binding inhibitors (45) using nonlinear regression analysis as follows.

$$ V/V_0^{	ext{app}} = 1 - (E_t + I_t + K_{i,	ext{app}})^{-1} - (E_t + I_t + K_{i,	ext{app}})^{-2}E_t, \quad (\text{Eq. 1}) $$

where $V_t$ and $V_0$ are the velocities in the presence and absence of an inhibitor, and $E_t$ and $I_t$ the total concentrations of enzyme and inhibitor, respectively. Since the LDTI variants bind to only two of the four catalytic subunits of the trypsin tetramer in the range of concentrations used, Equation 1 was modified by subtracting $V_t$ (i.e. the remaining velocity at high concentrations of inhibitor) from $V_t$ and $V_0$ (18).

Equilibrium dissociation constants ($K_i$) for the complexes of the LDTI variants with chymotrypsin were obtained from the $K_{i,	ext{app}}$ values after correction for competition between the inhibitors and the substrate; even at high substrate concentrations competition was not detectable during the measurements of residual enzyme activities of trypstatase and trypsin.

$K_i$ values for the inhibition of trypstatase and factor Xa by the low molecular weight inhibitors TPDCA and DX9065a (Fig. 4) were calculated according to Dixon (46) from data measured at pH 7.6 and 25 °C using the chromogenic substrate Tos-Gly-Pro-Arg-pNA.

RESULTS
(105,589),(897,608)

Crystal Structure of LDTI in Complex with Porcine Trypsin—Apart from a few surface-located side chains, the trypsin moiety is almost completely defined by electron density. With the exception of the side chain of Tyr^217, which swings out to accommodate the amino-terminal residues of LDTI (Fig. 1; see below), no significant conformational changes are seen in the trypsin component compared with other porcine trypsin structures (29, 47–49). The LDTI moiety is well defined in the vicinity of the proteinase, but it is characterized by elevated temperature factors and disrupted density further away from trypsin. In particular, amino acid residues Lys^3–Lys^5, Gly^7–Arg^19, Ser^23, Ser^36, and the C-terminal residues Pro^41–Asn^46 are defined by either weak or no electron density. In contrast, the NMR solution structure (23) is well defined for residues Cys^4–Cys^49, while the terminal peptides Lys^1–Val^9 and Pro^41–Asn^46 are mobile.

LDTI secondary structure consists of a short central α-helix (Ser^24–Asn^30) and a small antiparallel β-sheet (residues Val^37–Gly^15, Thr^20–Tyr^21, and Ile^34–Glu^37) characteristic for the classical Kazal-type inhibitors (Fig. 2). Due to a large deletion, however, the helix is one turn shorter than that in Japanese quail ovomucoid third domain (22) or either domain of rhodniin (24) (Fig. 2). The third β-strand is in part poorly defined, suggesting a degree of flexibility in the crystal. Residues P^3–P^3’ (Cys^6–Lys^11) exhibit the highly conserved “canonical” or substrate-like conformation of the small serine proteinase inhibitors (50). As a result of its “nonclassical” disulfide pattern, the active site wedge of LDTI is rather narrow, as was also observed for rhodniin (24). In contrast to either of the two domains of rhodniin, however, the first disulfide bridge (Cys^4–Cys^29) possesses a right handed spiral conformation that causes the amino terminus to exit the active site cleft to the “south,” rather than to the “west” (Fig. 1). Such a conformation of the disulfide is sterically hindered in rhodniin and Japanese quail ovomucoid third domain, since it would result in a clash of the amino-terminal residues with those of the longer α-helix.

Although not well defined, there is sufficient density to show that the two amino-terminal lysine residues reach out toward the trypsin loop, containing residue 148 (referred to as the “148-loop”). Structure-based sequence alignment of mast cell tryptases with trypsin (16) reveals this loop to be acidic in the tryptases (see Fig. 3), making it conceivable that the basic amino terminus of LDTI makes an electrostatic contribution to its interaction with trypstatase. To test this hypothesis, a series of N-terminally truncated and charge-deleted variants were constructed using cassette mutagenesis.
Expression of Variants—The yeast expression system gave yields of the variants of the order of 12 mg/liter, whose purity and identity were analyzed using SDS-polyacrylamide gel electrophoresis, isoelectric focusing, and reverse phase HPLC. Correct processing of the α mating type leader fusion protein was verified by partial N-terminal sequencing in each case. Mass spectroscopy of the variants yielded major masses for LDTI-var1 of 4609.3 Da (calculated mass, 4609.35 Da), for LDTI-var2 of 4480.8 Da (calculated mass, 4481.18 Da), and two masses in a 1:1 ratio for LDTI-var3 of 4736.9/4719.4 Da (calculated mass, 4737.45 Da). The lower mass peak observed for LDTI-var3 was attributed to partial formation of pyroglutamate. Variants rLDTI-var1 and rLDTI-var2 both showed an additional minor mass with a reduced value of 114.0 Da compared with the major peak, interpreted as the truncation of the C-terminal Asn46 by endogenous yeast proteinases.

Inhibition of Tryptase by the Variants—Equilibrium dissociation constants were measured for each variant with trypsin, tryptase and chymotrypsin (Table II). Sequential deletion of the first and second N-terminal lysines (variants 1 and 2, respectively) had a marked effect on anti-tryptase activity but only a negligible effect on the interaction with trypsin or chymotrypsin. This was also the case for the substitution of these residues by the uncharged polar residue glutamine (variant 3), substantiating the positive charge requirement. A series of variants designed to allow thrombin inhibition (see accompanying paper (36)) showed decreased tryptase inhibition. Mutation of four residues in the reactive site (rLDTI-var8) resulted in a modest decrease in tryptase inhibition, as did variant rLDTI-var2, C-terminally elongated by an acidic peptide. Tryptase inhibition was effectively abolished in the remaining C-terminal variants rLDTI-var1, rLDTI-var4, and rLDTI-var8. Some of these effects were also mirrored in the inhibition of trypsin and chymotrypsin.

Having established the existence of a negatively charged patch on the tryptase surface to the south of the active site cleft, it was decided to test the inhibition of tryptase by bisamidine inhibitors (Fig. 4). TPDCA inhibits tryptase with a $K_i$ of 0.85 μM, whereas no inhibition could be measured for DX9065a.

**DISCUSSION**

The heparin-associated tetrameric tryptase has so far defied attempts at crystallization. In this paper, we present structural and mutagenesis data for rLDTI, a high affinity inhibitor of human tryptase. Using the structure of LDTI as a template, we are able to address aspects of the tryptase structure. Compared with the “classical” Kazal-type inhibitors (e.g. the Japanese quail ovomucoid inhibitor (22)), rLDTI has a narrow disk-like proteinase-binding region. This is a result of its characteristic disulfide pattern, which it shares with rhodniin and bdellin B-3. The structure of the rhodniin-thrombin complex (24) revealed this narrow binding region to be essential for the fit to thrombin’s restricted active site cleft, bounded to the “north” by its characteristic 60-insertion loop (51). According to the structural alignment proposed by Johnson and Barton (16), tryptase does not possess such a 60-insertion.
loop. The active site cleft should, however, be occluded to the west by a pronounced 9-residue insertion with respect to trypsin residues 173–176 (Fig. 3) immediately after the intermediate helix. The southerly exit of LDTI’s amino-terminal residues from trypsin’s active site cleft suggests that LDTI is favorably adapted to avoid this restriction in tryptase. The southerly route is made possible by the conformation of disulfide Cys4–Cys29, in turn facilitated by the deletion of the last turn in the central α-helix with respect to the ovomucoid inhibitors or rhodnin (Fig. 2). This disulfide may be in equilibrium with other conformers in the free inhibitor; a specific conformation was not observed in the NMR structure due to a lack of nuclear Overhauser effects constraining the amino terminus (23). An indication that the right handed spiral conformation might be well populated in the free inhibitor, and hence of functional significance, comes from the fact that the side chain of trypsin Tyr217 swings out to accommodate the amino-terminal residues (Fig. 1). In all other published structures of porcine trypsin (29, 47–49), this side chain makes favorable stacking interactions with the aromatic side chain of Tyr172.

The southerly route of the amino-terminal residues would lead to a juxtaposition of the basic Lys1-Lys2 amino terminus with the acidic 148-loop of human tryptases (Fig. 3). Progressive deletion of these residues results in a clear deterioration in

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**Table II**

**Equilibrium dissociation constants $K_i$ for the complexes of the LDTI variants with human lung tryptase and bovine pancreatic trypsin and chymotrypsin**

| Residues | $K_i$ (nM) |
|----------|-----------|
| 1-2 8-12 40- | Tryptase | Trypsin | Chymotrypsin |
| rLDTI | 1.2 | 1.8 | 18 |
| rLDTI-var1 | 3.9 | 1.2 | 23 |
| rLDTI-var2 | 19 | 1.8 | 21 |
| rLDTI-var3 | 43 | 1.2 | 17 |
| rLDTI-var4 | 21 | 1.4 | 70 |
| rLDTI-var5 | 5.2 | 2.4 | >100 |
| rLDTI-var6 | >100 | 3.3 | >100 |
| rLDTI-var7 | >100 | 4.4 | |

$^a$ $K_i$ for the interaction of the LDTI variants with two of the four active sites of the tryptase tetramer.

$^b$ No inhibition.

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**Fig. 4. Chemical formulae and inhibition constants for synthetic tryptase and factor Xa inhibitors.** $K_i$ values (for human enzyme) are taken from (a) Stürzebecher et al. (14), (b) Caughey et al. (13), (c) this work, (d) Stürzebecher et al. (55), (e) Tidwell et al. (56), (f) von der Saal et al. (52), and (g) Hara et al. (1994) (57). n.i., no inhibition.

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Overhauser effects constraining the amino terminus (23). An indication that the right handed spiral conformation might be well populated in the free inhibitor, and hence of functional significance, comes from the fact that the side chain of trypsin Tyr217 swings out to accommodate the amino-terminal residues (Fig. 1). In all other published structures of porcine trypsin (29, 47–49), this side chain makes favorable stacking interactions with the aromatic side chain of Tyr172.

The southerly route of the amino-terminal residues would lead to a juxtaposition of the basic Lys1-Lys2 amino terminus with the acidic 148-loop of human tryptases (Fig. 3). Progressive deletion of these residues results in a clear deterioration in
inhibitory potency of LDTI for tryptase (Table II), as does their mutation to the neutral Gln¹-Gln² variant. That these mutations had hardly any effect on the inhibition of trypsin and chymotrypsin points strongly to a specific role of electrostatic exosite interactions in the inhibition of trypstat by LDTI.

Such an electrostatic interaction is consistent with the known low molecular weight inhibitors of tryptase (13–15). The most potent inhibitors consist of two aromatic amidino functionalities linked via a suitable spacer (Fig. 4). Clearly, one amidino function would occupy the primary specificity pocket of tryptase. It is quite conceivable that the second amidino moiety could make electrostatic interactions with the acidic 148-loop, the restriction of the active site to the west and an acidic exosite interaction in the inhibition of tryptase by LDTI.

Furthermore, it is quite conceivable that the second amidino moiety could make electrostatic interactions with the acidic 148-loop, the restriction of the active site to the west and an acidic exosite interaction in the inhibition of tryptase by LDTI.

This loop may restrict the active site cleft (Fig. 3). Such a conclusion should be considered circumspectly in view of the altered properties of this variant with respect to trypsin and chymotrypsin. In contrast to the C-terminal acidic extension variants rLDTI-var1, -var4, and -var6, rLDTI-var2 shows only a mild diminution of anti-trypstat activity, comparable with that of the N-terminal deletion variant rLDTI-var2. The reason for this cannot be explained by our model of the tryptase monomer; it may reflect interactions with other subunits of the tetrameric tryptase.

Similarly, the model presented here does not explain the observed 50% maximal inhibition achieved by LDTI for the cleavage of small substrates by trypstat; presumably, the binding of LDTI to one monomer of trypstat precludes access of a further inhibitor molecule to the neighboring active site in the tetramer.

Although our data do not allow conclusions about the quaternary organization of trypstat or the role played by heparin to be drawn, the model presented here serves as a first step in understanding the structural features of this intriguing enzyme and its inhibition.

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