Crystal Structure of Kunitz Domain 1 (KD1) of Tissue Factor Pathway Inhibitor-2 in Complex with Trypsin

IMPLICATIONS FOR KD1 SPECIFICITY OF INHIBITION*

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Kunitz domain 1 (KD1) of tissue factor pathway inhibitor-2 inhibits trypsin, plasmin, and factor VIIa (FVIIa)/tissue factor with $K_i$ values of 13, 3, and 1640 nM, respectively. To investigate the molecular specificity of KD1, crystals of the complex of KD1 with bovine $\beta$-trypsin were obtained that diffracted to 1.8 Å. The $P_1$ residue Arg-15 (bovine pancreatic trypsin inhibitor numbering) in KD1 interacts with Asp-189 (chymotrypsin numbering) and with the carbonyl oxygens of Gly-219 and Oγ of Ser-190. Leu-17, Leu-18, Leu-19, and Leu-34 in KD1 make van der Waals contacts with Tyr-39, Phe-41, and Tyr-151 in trypsin, forming a hydrophobic interface. Molecular modeling indicates that this complementary hydrophobic patch is composed of Phe-37, Met-39, and Phe-41 in plasmin, whereas in FVIIa/tissue factor, it is essentially absent. Arg-20, Tyr-46, and Glu-39 in KD1 interact with trypsin through ordered water molecules. In contrast, insertions in the 60-loop in plasmin and FVIIa allow Arg-20 of KD1 to directly interact with Glu-60 in plasmin and Asp-60 in FVIIa. Moreover, Tyr-46 in KD1 electrostatically interacts with Lys-60A and Arg-60D in plasmin and Lys-60A in FVIIa. Glu-39 in KD1 interacts directly with Arg-175 of the basic patch in plasmin, whereas in FVIIa, such interactions are not possible. Thus, the specificity of KD1 for plasmin is attributable to hydrophobic and direct electrostatic interactions. For trypsin, hydrophobic interactions are intact, and electrostatic interactions are weak, whereas for FVIIa, hydrophobic interactions are missing, and electrostatic interactions are partially intact. These findings provide insight into the protease selectivity of KD1.

Proteases and their inhibitors are important in the regulation of many physiologic processes such as fibrinolysis, blood coagulation, complement fixation, fertilization, angiogenesis, hippocampal plasticity, inflammatory response, and bone resorption and remodeling (1–5). The protease inhibitors are grouped into the Kunitz (6), Kazal (6), serpin (7), and mucus (8) families. Two members of the Kunitz family of inhibitors that are involved in regulating coagulation and fibrinolysis are tissue factor pathway inhibitor (TFPI)1 and TFPI-2. TFPI-2, also known as matrix serine protease inhibitor or placent protein 5, features a domain organization similar to TFPI and contains three Kunitz-type inhibitory domains in tandem with a short acidic amino terminus and very basic carboxy-terminal tail (9, 10). A variety of cells, including keratinocytes, dermal fibroblasts, smooth muscle cells, synctiotrophoblasts, synovial-blasts, and endothelial cells, synthesize and secrete TFPI-2 into the extracellular matrix (ECM) (11–15). TFPI-2 is found in three forms with $M_r$ 27,000, $M_r$ 30,000, and $M_r$ 32,000 (16). These three forms are believed to correspond to differentially glycosylated forms of TFPI-2 (16).

TFPI-2 exhibits inhibitory activity primarily toward trypsin, plasmin, and factor VIIa (FVIIa)/tissue factor (TF) through its Kunitz domain 1 (KD1) and is believed to be the major inhibitor of plasmin in the ECM (17). However, TFPI-2 has little inhibitory activity toward urokinase-type plasminogen activator, tissue-type plasminogen activator, or thrombin (18). By inhibiting plasmin, TFPI-2 decreases the activation of the matrix metalloproteinas pro-MMP-1 and pro-MMP-3 and thereby suppresses formation of the active MMP-2. As a consequence of decreased MMP-2 activation, ECM degradation and tumor growth and metastasis are reduced (19–22). Moreover, TFPI-2 expression is reported to be up-regulated in atherosclerotic coronary arteries, indicating that it may play a role in prevention of atherosclerotic plaque rupture (23). Also, TFPI-2 has recently been shown to delay retinal degeneration by stimulating proliferation of the retinal pigment epithelium, although the mechanism by which this occurs is unclear (24). Thus, understanding the protease specificity of KD1 is biologically significant.

TFPI-2 inhibits proteases via the $P_1$ arginine residue2 (Arg-
15) in KD1 (17, 25). Although KD1 is specific for inhibiting plasmin, the other two Kunitz domains in TFPI-2 have no discernable inhibitory activity and may serve to bind to nearby proteins to localize TFPI-2 in the ECM. These two Kunitz domains may also serve as spacers to correctly position KD1 to interact with plasmin. As a prelude to crystal structure determinations, mutagenesis and molecular modeling of KD1 were employed to understand the specificity of interaction between KD1 and trypsin, plasmin, or FVIIa/TF (17). We now report the crystal structure of KD1 complexed with trypsin that precisely defines the interactions between KD1 and this protease. The crystal structures of KD1, plasmin, and FVIIa/TF were then used to refine the protease-inhibitor models built previously (17). Knowledge gained from these studies may help in the development of a potent and specific TFPI-2 KD1 molecule that can selectively inhibit plasmin without targeting other proteases. Such a molecule could have a large pharmacologic impact specifically in preventing tumor metastasis, retinal degeneration, and degradation of collagen in the ECM.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of KD1**—The first Kunitz-type domain of human TFPI-2 (KD1) was overexpressed as an amino-terminal His_6-tagged fusion protein in _E. coli_ strain BL21(DE3) pLysS and pET28a expression vectors were products of Novagen Inc. (Madison, WI). Amon Ultra-15 and Ultra-4 centrifugal filter devices (5000 molecular weight cutoff) were purchased from Millipore (Bedford, MA). Q-Sepharose FF, SP-Sepharose, and His-Trap HP columns were obtained from Amersham Biosciences. NoveX® 4–20% Tris-glycine polyacrylamide gels were purchased from Invitrogen. Kanamycin and TPCk-trypsin were obtained from Sigma. Human thrombin was prepared as described previously (26). All other reagents were of the highest purity commercially available.

**Expression and Purification of KD1**—The first Kunitz-type domain of human TFPI-2 (KD1) was overexpressed as an amino-terminal His_6-tagged fusion protein in _E. coli_ strain BL21(DE3) pLysS using the T7 promoter system. The recombinant plasmid derived from pET28a, containing the full-length sequence followed by a thrombin cleavage site and the cDNA encoding the first Kunitz-domain of TFPI-2, was prepared according to standard procedures. The recombinant construct was examined for in-frame orientation and integrity by nucleic acid sequencing. The His_6-tagged KD1 fusion protein was expressed in _E. coli_ grown in rich media containing 10 mg/liter kanamycin and induced at 37 °C with 1 mM isopropyl thigalactosyranoside at mid-log-phase (A_600 = 0.4–0.7).

The induced cells were harvested and lysed using a lysis-buffer-nucleodiase mix (0.2% lysozyme, 20 μg/mL DNase I, and 20 μg/mL RNase A in 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1 mM MgCl_2, and 1 mM phenylmethylsulfonyl fluoride). Cell lysis was carried out at room temperature for 2 h, and the lysate was subjected to centrifugation (20,000 × g for 15 min). The cell pellet was then resuspended in a detergent solution (2% Igepal® CA-630 in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA) and sonicated at 50% power, and inclusion bodies were collected by centrifugation (20,000 × g for 15 min). The inclusion bodies were then washed twice with water following brief sonication and centrifugation (20,000 × g for 15 min). The highly enriched inclusion bodies were then solubilized overnight in 50 mM Tris-HCl (pH 8.0) containing 8 M urea, 0.5 M NaCl, and 10 mM 2-mercaptoethanol. The suspension was centrifuged at 47,000 × g for 30 min, the supernatant was filtered (0.2 μ filters), and the filtrate was subsequently loaded onto a nickel-charged His-Trap column. The column was washed with equilibration buffer (50 mM Tris-HCl (pH 8.0) containing 6 M urea, 0.5 M NaCl, and 10 mM 2-mercaptoethanol), followed by equilibration buffer containing 25 mM imidazole. The His_6-tagged KD1 fusion protein was eluted from the column in equilibration buffer containing 500 mM imidazole.

The His-Trap purified protein was reduced by the addition of 50 mM dithiothreitol. This solution was incubated overnight with rocker shaking at 4 °C, diluted to a concentration of ~0.5 mg/mL in 50 mM Tris-HCl (pH 9.0) containing 6 M urea and 0.02% azide, and dialyzed against 20 volumes of the same buffer at 4 °C. The refolding was then initiated by dialysis against 50 mM Tris-HCl (pH 9.0) containing 0.3 M NaCl, 2 mM urea, 2.5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.02% azide (buffer A) essentially as described by Stone et al. (27) for the refolding of human tissue factor. The dialysis was performed for 48 h at 4 °C, and the solution was subsequently dialyzed against fresh buffer A for another 48 h at 4 °C. The solution was then dialyzed extensively at 4 °C against 50 mM Tris-HCl (pH 9.0). The refolded protein solution was then filtered (0.2 μ filters) and applied to a Q-Sephrose FF column equilibrated at 4 °C with 50 mM Tris-HCl (pH 9.0). The protein was eluted from the column using a linear 0–1 M NaCl gradient, and the fractions were analyzed by SDS-PAGE. The fractions containing pure His_6-tagged KD1 were pooled and digested with human thrombin at a 1:1000 enzyme/substrate molar ratio for 2 h at 37 °C. Complete digestion of KD1 by thrombin was confirmed by SDS-PAGE analysis of transient aliquots. Thrombin-treated KD1 preparations were then applied to a His-Trap column to remove the His_6 peptides followed by SP-Sepharose chromatography equilibrated with 50 mM HESS (pH 6.0) buffer to remove traces of thrombin. The pure, His_6-tag-free KD1 preparations were then dialyzed extensively against 20 mM Tris-HCl (pH 7.5), concentrated to >10 mg/mL in Amicon Ultra-15 (5000 molecular weight cutoff) filters, and stored at ~80 °C. Each batch preparation was characterized with respect to protein concentration (A_280), purity (SDS-PAGE analysis), and inhibition kinetics as previously described (17).

**Crystallization**—For crystallographic purposes, the His_6-tag-free preparations of KD1 were found to be essential to achieve high concentrations of KD1 without precipitation. Bovine trypsin (TPCK-treated) was dissolved in 1 mM HCl. For crystallization of the KD1-trypsin complex, the solution was set up at 27 mg/mL in a 1:1.1 molar ratio using sitting drops (equal volume of protein and mother liquor) subjected to vapor diffusion at 37 °C against a reservoir containing 100 mM Heps (pH 7.5) with 40% (NH_4)_2SO_4, with crystals appearing in 2 days. The crystals were soaked in 33% glycerol and flash frozen.

**X-ray Data Collection**—The data set was collected at the Advanced Light Source (Berkeley, CA) using beam line 8.2.2 and an ADSC Q315 detector. One crystal diffraction to a resolution of 1.8 Å and belonged to the orthorhombic space group P2_12_1 with two molecules of the KD1-trypsin complex in the asymmetric unit. The data were processed using the programs DENZO and SCALA (28). The statistics for data collection are summarized in Table I.

**Structure Determination and Refinement**—Most of the calculations were performed using the CCP4 suite (29). The starting phases for refinement were obtained by molecular replacement using the program EPMR (30, 31). The starting search model for KD1 was derived from the crystallographically determined structures of KD1 (this report), FVIIa/TF (Ref. 32), and the trypsin complex (Protein Data Bank code 1TAW, Ref. 32). The rotation and translation search gave two trypsin-KD1 complexes per asymmetric unit. The model was rebuilt using the 2F – F and F – F electron density maps. Five percent of the data were kept out of refinement for cross-validation (33). Subsequently, solvent molecules were added to the model. The final refinement statistics are given in Table I. The coordinates have been deposited into the Research Collaboratory for Structural Bioinformatics Protein Data Bank with accession code 1ZRO.

**Molecular Modeling**—Three-dimensional structural information on complexes formed between KD1 and plasmin and between KD1 and FVIIa was obtained using molecular modeling strategies. The crystallographically determined structures of KD1 (this report), FVIIa/TF inhibited with a BPTI mutant (Ref. 34, Protein Data Bank code 1FAK), FVIIa/TF (Ref. 35, Protein Data Bank code 1DAN), and plasmin (Ref. 36, Protein Data Bank code 1BML) served as templates in building these models. The relative positions of the inhibitor and proteinase domains were maintained, and minor adjustments were only made in the side chains. Hydrophobic/van der Waals contacts, hydrogen bonds, and ionic interactions were observed between each proteinase/inhibitor complex. A set of these interactions were taken into consideration while evaluating each inhibitor–proteinase complex, and it was assumed that all potential hydrogen bond donors and acceptors would participate in these interactions. Bulk solvent was excluded from the proteinase-inhibitor complex, and, accordingly, it was anticipated that hydrogen bonds and ionic interactions that may play an important role in specificity could be accurately evaluated. The protocols for modeling these complexes have been described previously (37).

**RESULTS**

KD1-Trypsin Crystallization and Structure Determination—His_6-tagged KD1 was cloned and expressed in _E. coli_ strain BL21(DE3)pLysS. The fusion protein was refolded/renatured in the presence of redox exchange buffer, the His_6 tag was

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removed by thrombin proteolysis, and the His$_6$ tag-free KD1 was purified to homogeneity. The KD1 fragment was then incubated in a 1:1.1 molar ratio with bovine trypsin containing $\sim$1 mM Ca$^{2+}$, and crystals were obtained at 37 °C in 100 mM Heps (pH 7.5) and 40% ammonium sulfate after 2–3 days. The crystals diffracted to 1.8 Å and were found to belong to the space group P2$_1$2$_1$2, with unit cell dimensions of $a = 74.107, b = 77.013, c = 125.424$. There were two KD1-trypsin complexes in the asymmetric unit. An initial structure was obtained by molecular replacement using the structure of APPI-trypsin (Protein Data Bank code 1TAW, Ref. 32) as a search model and refined using CCP4/REFMAC to a final $R_{work}$ of 23.0 and $R_{free}$ of 29.5 (29). The refinement statistics and geometry of the final structure are summarized in Table I.

**TABLE I**

| Data collection and refinement statistics | KD1/Trypsin |
|-----------------------------------------|-------------|
| Space group                             | P2$_1$2$_1$2, | |
| Unit cell dimensions (Å)                | 74.107, 77.013, 125.424 |
| Measured reflections                   | 449.141 |
| Unique reflections                      | 63,596 |
| Redundancy                              | 7.1 |
| Overall completeness (%)$^a$            | 94.8 (100) |
| $R_{merge}(%)^b$                        | 7.3 (35.6) |
| $I_{ave}/I_{cryst}$                     | 29.8 (6.9) |
| Resolution (Å)                          | 10–1.8 |
| No. of atoms/residues                   | 572 |
| Protein                                 | 2 |
| Water                                   | 2 |
| Root mean square deviations             | 2.025 |
| Bond angle (°)                          | 2.108 |
| Ramachandran plot                       | Favored |
| Allowed                                 | 14.4 |
| Generously allowed                      | 0.6 |
| Disallowed                              | 0.4 |

$^a$ Numbers in parentheses represent data in the highest resolution shell, 1.802–1.847 Å.

$^b$ $R_{merge}(I) = \sum_{hkl}I_{hk} - \langle I_{hk}\rangle/I_{hk}$.

$^c$ $R_{merge} = \sum_{hkl}I_{hk} - [F_{calc}/F_{obs}]R_{free}$, $R_{free}$ was computed identically, except that 5% of the reflections were omitted as a test set.

protease inhibitory activity of KD1. This segment forms a hairpin-like structure, which is located outside the segment formed by residues 34–39 in KD1. A disulfide bridge (Cys-14-Cys-38) joins these two segments together along with several H-bonds forming a two-stranded antiparallel β-sheet. The P$_1$ residue, Arg-15 of KD1, makes the primary contact with the S$_1$ residue, Asp-189 of trypsin. The side chain NH$_2$ and NH$_2$ of Arg-15 in KD1 make H-bonds with the hydroxyl of Ser-190 and the carbonyl oxygen of Gly-219 in trypsin, as has been observed previously for the APPI-trypsin complex (32). As noted in previous structures of BPTI-protease complexes, H-bonds between the carbonyl oxygen of Pro-13 in KD1 and nitrogen of Gly-216 in trypsin and between the backbone nitrogen of Arg-15 in KD1 and carbonyl oxygen of Ser-214 in trypsin were also observed (Fig. 1B). The negative charge that develops during complex formation at the carbonyl oxygen of Arg-15 in KD1 is stabilized by H-bonds involving the backbone nitrogens of Gly-193 and Ser-195 (Fig. 1B).

In the KD1-trypsin complex, trypsin Gln-192 is well ordered in the structure. The side chain nitrogen of Gln-192 in trypsin forms an H-bond with the carbonyl oxygen of Cys-14 in KD1 as well as with a water molecule. This water molecule makes three additional H-bonds in a tetrahedral arrangement involving Ne of Arg-15 and the carbonyl oxygen of Cys-14 in KD1 and the carbonyl oxygen of Gly-219 in trypsin. Moreover, the side chain oxygen of trypsin Gln-192 is linked via well-ordered water molecules to the carbonyl oxygens of Ala-16 and Leu-34 in KD1, as well as to the side chain nitrogen of Asn-143, the hydroxyl of Tyr-151, and the carbonyl oxygens of Cys-220 and Ser-146 in trypsin (Fig. 1C). Such interactions further stabilize the KD1-trypsin complex.

**Hydrophobic Interactions That Stabilize the KD1 Structure and Its Interaction with Trypsin**—KD1 consists of a hydrophobic core, which is composed of Leu-9, Tyr-11, Tyr-21, Tyr-22, Phe-33, and Tyr-35 (Fig. 2A). These residues are part of the 10–20 segment as well as the two β-strands in KD1 (Fig. 1A), and they are defined by the high-resolution electron density map shown in Fig. 2B. The hydrophobic residues Leu-17, Leu-18, Leu-19, and Leu-34 in KD1 interact with a complementary hydrophobic patch in trypsin composed of Tyr-39, Phe-41, and Tyr-151. The details of these interactions are shown in Fig. 3 and appear to play a major role in the KD1-trypsin interaction.

**Electrostatic Interactions in the KD1/Trypsin Complex**—In addition to the primary interactions involving the S1 site and hydrophobic interactions, several electrostatic interactions are also important for the antiprotease protease interaction (37). In the case of KD1, Arg-20, Tyr-46, and Glu-39 have been implicated to be important for protease specificity (17). The interaction of Arg-20 of KD1 with trypsin is shown in Fig. 4A. Arg-20 of KD1 interacts with the side chain O of Asn-44 in KD1 as well as with the carbonyl oxygens of His-57, Tyr-59, and Ser-96 in trypsin via a chain of water molecules. The carbonyl oxygen of Leu-19 in KD1 interacts with Lys-60 in trypsin via a single water molecule, and the backbone nitrogen of Leu-19 in KD1 forms an H-bond with the side chain oxygen of Tyr-39 in trypsin.

Interactions involving Tyr-46 and Glu-39 of KD1 with trypsin are shown in Fig. 4, B and C, respectively. Tyr-46 of KD1 interacts with the carbonyl oxygens of Ala-56, His-57, and Tyr-59 in trypsin indirectly via several water molecules. Similarly, Glu-39, which is part of the acidic patch in KD1, interacts with the carbonyl oxygen of Asn-97, as well as with the side chains of Gln-175 and Lys-224 in trypsin via water molecules. Cumulatively, such electrostatic interactions, although long range, could play a role in the interaction of KD1 with trypsin.
In this report, we have defined the specific interactions between KD1 and trypsin at the interface. Our analyses indicate that in addition to the S1 site interactions, Gln-192 in trypsin also contributes to stabilization of the complex (Fig. 1C). As much as plasmin also has Gln at position 192, the residue at position 151 is Phe instead of Tyr-151 in trypsin, and at position 143, it is Glu versus Asn as in trypsin. Furthermore, because of deletions in the 140-loop in plasmin (36, 40), there is no residue corresponding to trypsin Ser-146 in plasmin. Thus, the interactions related to Gln-192 may be, at best, partial in plasmin. In FVIIa, Lys is present at position 192, which is unable to have interactions with KD1 similar to Gln in plasmin or trypsin. Interestingly, Lys-192 makes direct contact with Asp-11 in the first Kunitz domain of TFPI (37); however, residue 11 in KD1 is Tyr, which appears to preclude such interactions. Thus, residue 192 appears to have

![Fig. 1. KD1-trypsin complex. A, overall view of the KD1-trypsin complex. The two β-barrel subdomains of trypsin and the Kunitz inhibitory domain of KD1 are depicted. The α-helices are presented as ribbon cylinders, and β-strands are presented as thick arrows. Trypsin is in magenta, and KD1 is in yellow. Carbons are green, oxygens are red, and nitrogens are blue. The P1 residue, Arg-15, of KD1 is poised to interact with the S1 site residue, Asp-189, in trypsin. The active site triad (His-57, Asp-102, and Ser-195) in trypsin is also shown. The residue numbers for KD1 are shown to assist in tracing the polypeptide backbone, and the loops in trypsin that are in close proximity to KD1 are labeled. Calcium in trypsin is shown as a white sphere, and the carboxyl terminus is labeled as C. B, active site interactions between trypsin and KD1. Residues of trypsin are labeled in magenta, and those of KD1 are labeled in yellow. H-bonds are depicted with white dashed lines. NH₁ and NH₂ of Arg-15 from KD1 are shown interacting via H-bonds with the side chain of Asp-189 as well as with the side chain oxygen of Ser-190 and carbonyl oxygen of Gly-219 of trypsin. The carbonyl oxygen of Arg-15 in KD1 is depicted interacting through H-bonds with the backbone nitrogens of Gly-193 and Ser-195 in trypsin. Moreover, common to all Kunitz inhibitor-protease interactions, the carbonyl oxygen of Pro-13 and the amide nitrogen of Arg-15 in KD1 make an H-bond with the nitrogen of Gly-216 and with the carbonyl of Ser-214 (depicted by dashed arrow) in trypsin, respectively. C, interactions of Gln-192 in trypsin with KD1. The side chain nitrogen as well as oxygen of Gln-192 in trypsin forms H-bonds with KD1, as well as with other residues in trypsin, through a network of well-ordered water molecules as depicted.](image1)

![Fig. 2. Hydrophobic core in KD1. A, residues comprising the hydrophobic core in KD1. KD1 is shown as a ribbon with β-strands in yellow. Carbons are green, oxygens are red, and nitrogens are blue. The internal hydrophobic core of KD1 is composed of Leu-9, Tyr-11, Tyr-21, Tyr-23, and Tyr-35. B, electron density surrounding some of the hydrophobic core residues in KD1. The hydrophobic core residues that are depicted are Leu-9, Tyr-11, Tyr-22, and Phe-33, and water molecules in the vicinity are shown as red spheres. The electron density is a 2Fᵦ – Fᵦ map contoured at 1.2σ.](image2)

**DISCUSSION**

In this report, we have defined the specific interactions between KD1 and trypsin at the interface. Our analyses indicate that in addition to the S1 site interactions, Gln-192 in trypsin also contributes to stabilization of the complex (Fig. 1C). As much as plasmin also has Gln at position 192 (738), the residue at position 151 is Phe instead of Tyr-151 in trypsin, and at position 143 (687), it is Glu versus Asn as in trypsin. Furthermore, because of deletions in the 140-loop in plasmin (36, 40), there is no residue corresponding to trypsin Ser-146 in plasmin. Thus, the interactions related to Gln-192 may be, at best, partial in plasmin. In FVIIa, Lys is present at position 192 (341), which is unable to have interactions with KD1 similar to Gln in plasmin or trypsin. Interestingly, Lys-192 (341) in FVIIa makes direct contact with Asp-11 in the first Kunitz domain of TFPI (37); however, residue 11 in KD1 is Tyr, which appears to preclude such interactions. Thus, residue 192 appears to have
more stabilizing interactions in trypsin than in plasmin and to have the least stabilizing interactions in FVIIa.

The KD1 structure is stabilized by a number of hydrophobic residues including Leu-9, Tyr-11, Tyr-21, Tyr-22, Phe-33, and Tyr-35 (Fig. 2). Phe-33 is internal to the hydrophobic core, whereas Tyr-11 is peripheral. Thus, change of Phe-33 to Ala should have a destabilizing effect on the structure of KD1 and result in reduced affinity for all three proteases (trypsin, plasmin, and FVIIa), consistent with the previously observed mutagenesis data (17). Moreover, change of Tyr-11 to Ala should result in minimal change in affinity for all three proteases, which is also consistent with the observed mutagenesis data (17).

Hydrophobic interactions involving Leu-17, Leu-18, Leu-19, and Leu-34 appear to play a significant role in the interaction of KD1 with trypsin (Fig. 3). Such is undoubtedly expected to be the case with plasmin (Fig. 5A), but not with FVIIa/TF. Residues Tyr-39, Phe-41, and Tyr-151 in trypsin and residues Phe-37, Met-39, and Phe-41 in plasmin provide a complementary hydrophobic interface. Such a complementary interface appears to be weak in FVIIa, in which Leu-41 interacts with Leu-18 in KD1. Notably, residue 18 in Kunitz domain 1 of TFPI is Met, which points into the hydrophobic core rather than toward the interface. Moreover, residue 19 in Kunitz domain 1 of TFPI is Lys instead of Leu in KD1, which supports the hypothesis that hydrophobic interface contacts are not important to FVIIa interaction with KD1 or with the first Kunitz domain of TFPI.

Leu-17 in KD1 appears to be important for hydrophobic interactions with all three proteases. In trypsin, it is nestled against Tyr-151 and the side chain of Gln-192 as well as Leu-34 of KD1, whereas in plasmin, Leu-17 of KD1 is nestled against the side chains of Gln-192 and Glu-687 as well as Leu-34 of KD1. In the case of FVIIa, it is situated in the center of the side chains of Lys-192 and Gln-143 as well as Leu-34 in KD1. Thus, change of Leu-17 to Ala would result in disruption of these van der Waals contacts and impairment of KD1 inhibition of all three proteases. This is consistent with earlier experimental observations (17).

Electrostatic interactions also play a role in the interaction of KD1 with trypsin (Fig. 4, A–C). These interactions are mediated through water molecules in the case of trypsin and are long range. Because the strength of the Coulomb interactions is di-

![Figure 3. KD1-trypsin hydrophobic interactions.](image)

**FIG. 3.** **KD1-trypsin hydrophobic interactions.** KD1 is shown as yellow ribbons, and trypsin is shown as magenta ribbons. Residues from KD1 are labeled in yellow, and those from trypsin are labeled in magenta. Leu-17 and Leu-34 of KD1 interact with Tyr-151 of trypsin, and Leu-18 and Leu-19 of KD1 interact with Tyr-39 and Phe-41 in trypsin.

![Figure 4. Electrostatic interactions between KD1 and trypsin.](image)

**FIG. 4.** **Electrostatic interactions between KD1 and trypsin.** KD1 is shown as yellow ribbons and β-strands, and trypsin is shown as magenta ribbons and β-strands. H-bonds are shown as white dashed lines, and water molecules are shown as red spheres. A, Arg-20 of KD1 interacts with trypsin. The side chain of Arg-20 of KD1 interacts with water molecules that link to the trypsin backbone. The carbonyl oxygen of Leu-19 in KD1 forms an H-bond with the backbone nitrogen of Leu-19 in KD1. B, Tyr-46 of KD1 interacts with trypsin. The side chain of Tyr-46 of KD1 interacts with water molecules that link to the trypsin backbone. C, Glu-39 of KD1 interacts with trypsin. Glu-39, which is part of an acidic patch in KD1, interacts with water molecules.
interact with the hydroxyl group of Tyr-46 in KD1 (Fig. 5B).
Similarly, Glu-39 in KD1 is poised to make a salt bridge with Arg-175 (719) of plasmin. Arg-100 (644) and Arg-221 (767) could interact with Asp-10 as well as Glu-39 of KD1 through water molecules (Fig. 5C). Thus, electrostatic interactions are noticeably more prominent in the case of plasmin versus trypsin.

Direct electrostatic interactions involving Arg-20 and Tyr-46 of KD1 are also quite likely for FVIIa. Asp-60 (196) in FVIIa is ideally situated to make a salt bridge with Arg-20 in KD1. Moreover, because of the insertions in the 60-loop, Lys-60A (197) and/or Lys-60C (199) in FVIIa are expected to form an H-bond(s) with the side chain hydroxyl of Tyr-46 in KD1. Glu-39 in KD1, which is part of the acidic patch, does not appear to make any direct interactions with FVIIa. Thus, FVIIa falls between trypsin and plasmin in terms of electrostatic interactions with KD1.

Based upon the preceding considerations, it is expected that change of Asp-10 to Ala should only affect KD1 inhibition of plasmin with very little effect on the other proteases. In contrast, mutations at Arg-20 should affect KD1 inhibition of all three proteases. Such effects should be drastic when Arg-20 is changed to Asp, intermediate when Arg-20 is changed to Ala, and minimal when Arg-20 is changed to Lys. These conclusions are consistent with the mutagenesis data involving residues Asp-10 and Arg-20 (17).

In this study, we describe the structure of the human TFPI-2 KD1 with bovine trypsin and refine the models of the interaction of KD1 with plasmin and FVIIa. The specificity of KD1 for trypsin is mainly attributable to an extensive hydrophobic patch interface and weaker electrostatic interactions. In the case of FVIIa, the hydrophobic interactions are weak, and the electrostatic interactions are modest. Nonetheless, fibroblasts express TF as well as TFPI and TFPI-2, and the possibility exists that the FVIIa/TF complex localized on the fibroblast surface is inhibited by large concentrations of TFPI-2 released into the vicinity. If so, such a mechanism would bypass the need for factor Xa that is required by TFPI prior to inhibition of the FVIIa/TF complex (37). In the case of plasmin, both hydrophobic and electrostatic interactions are stronger than those present in either trypsin or FVIIa. Moreover, augmentation of the acidic patch by mutation of nonacidic residues in the vicinity of Glu-39 and Asp-10 in KD1 to acidic residues could further strengthen the electrostatic interactions involving the basic patch in plasmin composed of Arg residues 100 (644), 175 (719), and 221 (767). Such changes could lead to improved specificity and selectivity of KD1 for therapeutic inhibition of plasmin.

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