Molecular Design and Characterization of an α-Thrombin Inhibitor Containing a Novel P1 Moiety

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ABSTRACT: An inhibitor of α-thrombin was designed on the basis of the X-ray crystal structures of thrombin and trypsin. The design strategy employed the geometric and electrostatic differences between the specificity pockets of the two enzymes. These differences arise due to the replacement of Ser 190 in trypsin by Ala 190 in thrombin. The new inhibitor contained a tryptophan side chain instead of the arginine side chain that is present in the prototypical thrombin inhibitors. This inhibitor had a K_i value of 0.25 μM, displayed more than 400-fold specificity for thrombin over trypsin, and doubled the rat plasma APTT at a concentration of 44.9 μM. The X-ray crystal structure of the inhibitor/α-thrombin complex was determined. This represents the first reported three-dimensional structure of a thrombin/inhibitor complex where the specificity pocket of the enzyme is occupied by a chemical moiety other than a guanidino or an amidino group. As was predicted by the molecular model, the tryptophan side chain docks into the specificity pocket of the enzyme. This finding is in contrast with the indole binding region of thrombin reported earlier [Berliner, L. J., & Shen, Y. Y. L. (1977) *Biochemistry 16,* 4622−4626]. The lower binding affinity of the new inhibitor for trypsin, compared to that for thrombin, appears to be due to (i) the extra energy required to deform the smaller specificity pocket of trypsin to accommodate the bulky indole group and (ii) the favorable electrostatic interactions of the indole group with the more hydrophobic specificity pocket of thrombin. The neutral indole group may be of pharmacological significance because the severe hypotension and respiratory distress observed following the administration of some thrombin inhibitors have been linked to the positively charged guanidino or amidino functionalities.

The vascular system, in the healthy state, is under the strict homeostatic control of the blood coagulation system for the containment and the smooth flow of blood. The soluble component of the coagulation system, known as the coagulation cascade, is comprised of a series of soluble proteases and their regulatory cofactors. α-Thrombin is the last enzyme of this cascade that converts fibrinogen to fibrin I and fibrin II by limited proteolysis (Bailey et al., 1951; Lorand, 1951, 1952; Lorand & Middlebrook, 1952). α-Thrombin is generated by the proteolytic cleavage of prothrombin by the prothrombinase complex which includes factor V, a phospholipid surface, and factor Xa, the serine protease immediately preceding thrombin in the coagulation cascade. In the presence of Ca^{2+}, α-thrombin is also involved in the activation of factor XIII to generate factor XIIIa (Lorand, 1961; Lorand & Konishi, 1964). Factor XIIIa stabilizes the blood clot by cross-linking fibrin through N^{ϵ}-(γ-glutamyl)lysine transamide bridges (Bruner-Lorand et al., 1966; Roberts et al., 1973; Mockros et al., 1974; Shen & Lorand, 1983). The polymerized fibrin forms the general scaffold for the growth of the thrombus. The overall participation of thrombin in hemostasis and thrombosis is complex and involves more than its enzymatic function. However, the enzymatic activity of thrombin is known to be crucial not only for the arrest of blood flow but also for the ensuing growth of the thrombus. Therefore, the inhibition of the enzymatic activity of α-thrombin is considered a viable mechanistic approach for the development of chemical entities for treating hemostasis and thrombosis (Fenton et al., 1991).

α-Thrombin is a prolate ellipsoidal glycoprotein molecule made up of a short A chain (36 residues) and a long B chain (259 residues) cross-linked by one disulfide bond (Bode et al., 1992). The enzyme is a serine protease with Arg-Xxx or Lys-Xxx specificity with preference for arginine. The molecular basis for the enhanced affinity for arginine has been elegantly elucidated by Bode et al. (1992). A vast majority of the active site-directed synthetic inhibitors to date contain a guanidino or an amidino group to mimic the interaction of the natural substrate with the enzyme [for reviews, see Claeson (1994) and Powers and Kam (1992)]. The charged guanidino or amidino groups form strong ionic interactions with the carboxylate group of Asp 189[1] in the specificity pocket of the enzyme (Bode et al., 1992). The syntheses of these arginine-based inhibitors are fraught with difficulties due to the need for protection and deprotection of these groups during the course of syntheses. Furthermore, this class of inhibitors generally, with a few exceptions, lacks specificity for thrombin over many other trypsin-like enzymes prevalent in the vascular system. This is because Asp 189 is highly conserved among trypsin-like proteases present in the blood coagulation system.

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[1] The numbering of amino acid residues of α-thrombin follows the chymotrypsinogen system introduced by Bode et al. (1989).

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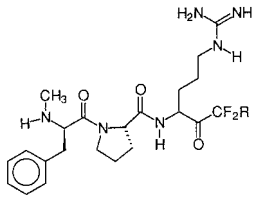
It is desirable to generate novel thrombin inhibitors that do not contain guanidino or amidino functionalities. The severe hypotension and respiratory depression observed in animals following the administration of some thrombin inhibitors have been postulated to be caused by the positively charged arginine residues present in these inhibitors (Kaiser & Hauptmann, 1992). Our first objective was to design an inhibitor with an uncharged group in P1[2] with good potency and enhanced specificity for thrombin. The difference in the electrostatics of such a ligand in comparison to that of the guanidino- or amidino-based inhibitors may have significant pharmacokinetc implications. For example, such an inhibitor may partition better into the milieu of the thrombus and inhibit the clot-bound thrombin more efficiently than free thrombin in plasma. The advantage of such an inhibitor would be the capability to prevent thrombus growth without severely compromising hemostasis. This effect, if achieved, may prove useful in ameliorating the bleeding complications associated with thrombin inhibition. Such an inhibitor may have particular use in the treatment of venous thrombi. Heparin does not effectively inhibit the propagation of venous thrombi, presumably due to the inaccessibility of clot-bound thrombin to the large heparin/antithrombin III complex (Claeson, 1994). Low-molecular weight inhibitors are known to inhibit clot-bound thrombin (Hansen & Harker, 1988; Weitz et al., 1990).

Here we report the design, synthesis, the enzyme-bound structure, and biological properties of an α-thrombin inhibitor containing an indole group that docks into the S1 specificity pocket of the enzyme. This new inhibitor, without a charge interaction in the S1 specificity pocket, could serve as a starting point for the design of nonpeptidic small molecule inhibitors of thrombin with novel pharmacokinetic properties.

EXPERIMENTAL PROCEDURES

Molecular Modeling. Molecular modeling was carried out using the SYBYL software package. The rationale for the design of Peptide II is described in the following section. The molecular geometry of Peptide II in the P3 and P2 regions was built in exact correspondence to the geometry of P3 and P2 of Peptide IA in its enzyme-bound state. The carbonyl-activating group attached to the P1 residue is difluorobutyl for Peptide IA and pentafluoroethyl for Peptide II (Figure 1). The carbonyl group was adjusted for covalent bonding geometry with O^{γ} of Ser 195, and the bond was introduced. The atoms were modified to form the hemiketal structure. One of the fluorines of the α-difluoromethylene group was adjusted to be within hydrogen bonding distance to the NE2 nitrogen of His 57 in accordance with the crystal structure of the Peptide IA/thrombin complex. The P1 group was adjusted as follows. (i) The plane of the indole ring was aligned parallel to the backbone of the Gly 216−Gly 219 region of the enzyme. (ii) The phenyl ring of the indole group was adjusted for optimal orthogonal interaction with the phenol ring of Tyr 228. (iii) The indole group was oriented for the best possible electrostatic interactions between its H5 and H6 protons and the carboxylate group of Asp 189. The hemiketal form of the inhibitor thus



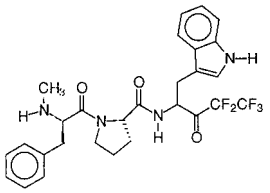


FIGURE 1: Chemical structures of Peptide IA, Peptide IB, and Peptide II.

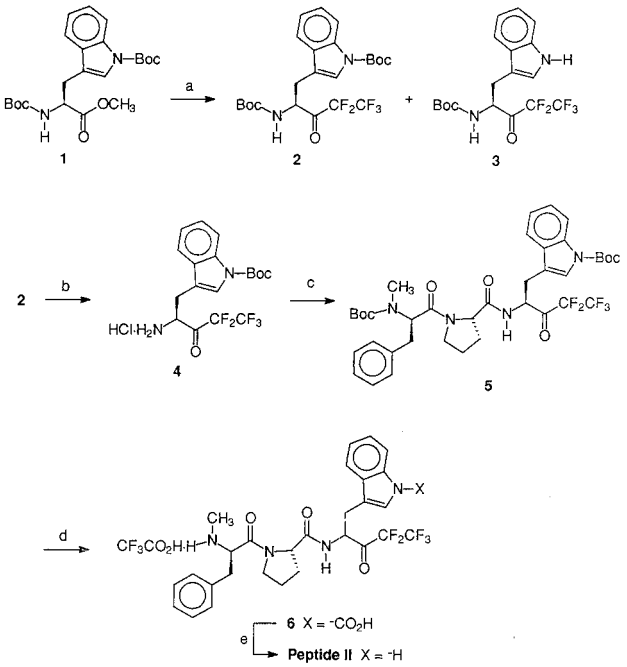


FIGURE 2: Reaction scheme for the chemical synthesis of Peptide II: (a) CF_3CF_2Li, Et_2O; (b) 2.7 N HCl in dioxane; (c) isobutyl chloroformate, *N*-methylmorpholine, *N*-Boc-*N*-methyl-D-Phe-L-Pro-OH, EtOAc; (d) CF_3CO_2H; and (e) $CHCl_3$.

generated was minimized in the field of the receptor by 2000 iterations of conjugate gradients algorithm using Kollman charges.

Synthesis of the Inhibitor. The chemical reaction scheme used for the synthesis of Peptide II is shown in Figure 2. The identities and integrities of the final and the intermediate products were ascertained by nuclear magnetic resonance (NMR),[3] mass spectral, high-resolution mass spectral, and elemental and thin layer chromatographic analyses. NMR spectra were recorded at 75 MHz for ^{13}C, 282 MHz for ^{19}F, and 300 MHz for ^{1}H. MS and HRMS spectra were recorded at 70 eV using computerized peak matching with perfluo-

[2] The nomenclature of the inhibitor residues (P1, P1′, P2, and P3) and the corresponding enzyme pockets (S1, S1′, S2, and S3) follows that of Schechter and Berger (1967).

[3] Abbreviations: APTT, activated partial thromboplastin time; HRMS, high-resolution mass spectroscopy; hirudin[53−65], *N*-acetylhirudin 53−65 with sulfato-Tyr[63]; K_i, thermodynamic inhibition constant; MS, mass spectroscopy; NMR, nuclear magnetic resonance; PPACK, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone; rmsd, root mean square deviation; TLC, thin layer chromatography.

rokerosene as reference. TLC analyses were performed with Merck-DC-F_{254} or Analtech GHLF silica gel plates with visualization by alkaline permanganate and ultraviolet irradiation. Flash chromatography (Still et al., 1978) was performed with Merck silica gel 60 (0.040−0.063 mm). CF_3CF_2Li was generated as described by Gassman and Reily (1987) using CF_3CF_2I obtained from the Richmond Chemical Co. Tryptophan derivatives were deprotected by the general procedure of Franzen et al. (1984).

*Synthesis of **2** and **3**.* A 6.85 mL volume of condensed CF_3CF_2I was added to a stirred solution of **1** (Franzen et al., 1984) in anhydrous Et_2O maintained at −60 °C under a nitrogen atmosphere. A 37.6 mL volume of 1.5 M MeLi·LiBr in Et_2O was added, maintaining the reaction temperature between −52 and −62 °C. The reaction mixture was stirred for 1 h at −55 to −60 °C. The reaction was quenched by the addition of 4.31 mL of 2-propanol, maintaining the temperature below −55 °C. The reaction mixture was allowed to warm to −35 °C and was then poured into 100 mL of 10% aqueous $KHSO_4$. The layers were separated, and the organic layer was washed with 50 mL of 10% aqueous $KHSO_4$, and 25 mL of brine and dried over $MgSO_4$ in a vacuum. Filtration and concentration gave crude products. Flash chromatography (7.5 × 19 cm silica gel column) while eluting with a gradient (15−25%) of EtOAc in hexane gave 5.43 g of **2** as a yellow foam and 1.50 g of **3** as a pale yellow solid.

*Synthesis of **4**.* A 5.0 mL volume of a solution of 4.0 N hydrogen chloride in dioxane was slowly added to a stirred solution of 1.04 g of **2** in 2.4 mL of dioxane under a nitrogen atmosphere. After 1 h, the resultant suspension was suction-filtered and the collected solid was washed three times with 10 ml portions of Et_2O. The off-white solid was dried under high vacuum over KOH pellets to provide 195 mg of **4**, as a mixture of ketone and hydrate in a 2:3 ratio. Compound **4** is susceptible to dimerization and was therefore immediately used in the following step. Dilution of the combined filtrates from above and refrigeration provided an additional 571 mg of **4**.

*Synthesis of **5**.* A 45 μL volume of *N*-methylmorpholine followed by 53 μL of isobutyl chloroformate were added to a stirred solution of 0.15 g of *N*-Boc-*N*-methyl-D-Phe-L-Pro-OH (Veber et al., 1994) in 3 mL of EtOAc maintained at −18 °C under a nitrogen atmosphere. After 15 min, an additional 45 μL of *N*-methylmorpholine, followed by 0.18 g of **4**, was added. The reaction mixture was stirred at −15 to −20 °C for 4 h, diluted with 50 mL of EtOAc, and washed twice with 25 mL portions of 10% aqueous citric acid, twice with 15 mL portions of aqueous $NaHCO_3$, and once with 25 mL of brine. Drying over $MgSO_4$, followed by filtration and concentration, gave the crude product. Flash chromatography, using a 2.5 × 12 cm silica gel column and a 45:55 (v:v) mixture of EtOAc/hexane, gave 0.16 g of **5** as a ketone/hydrate mixture.

*Synthesis of **6**.* A 2 mL volume of CF_3CO_2H was added to 150 mg of **5** under a nitrogen atmosphere, and the solution was stirred for 3 min. The solvent was removed and the residue dissolved in 3 mL of Et_2O, and 20 mL of hexane was added slowly with swirling. Suction−filtration, under a nitrogen atmosphere, provided 117 mg of **6** as an off-white solid. Compound **6** was susceptible to decarboxylation.

Synthesis of Peptide II. An 82 mg quantity of **6** was dissolved in $CHCl_3$ and stirred for 48 h. The solvent was removed, and the residue was redissolved in CH_2Cl_2/hexane and concentrated to give Peptide II (76 mg as a 1:1 mixture of DLL and DLD diastereomers) as a light yellow solid.

X-ray Crystallography. Human α-thrombin was a kind gift from Dr. John Fenton, II (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201-0509), and *N*-acetylhirudin 53−65 with sulfato-Tyr 63 (hirudin$^{53-65}$) was purchased from Baccchem (CH4416, Bubendorf, Switzerland). A ternary crystalline complex of thrombin, hirudin$^{53-65}$, and Peptide II was prepared by the general procedure of Skrzypczak-Jankun et al. (1991). An approximately 10-fold molar excess of hirudin$^{53-65}$ was added to a frozen 1 mL sample of human α-thrombin, which was subsequently diluted to 2 mL with 0.1 M phosphate buffer (pH 7.3) containing 1 mM NaN_3 and was allowed to thaw on ice. A 0.16 mL volume of 20.4 mM Peptide II in 0.1 M phosphate buffer (pH 7.3) with 0.5 mM NaN_3, 0.375 M NaCl, and 10% (v:v) DMF was added to 0.5 mL of the thrombin/hirudin$^{53-65}$ solution, and the mixture was allowed to react on ice for 15 min. The complex was concentrated six times using a centricon 10 microconcentrator (M_r cutoff = 10 000; Amicon, Beverly, MD) in a refrigerated centrifuge. The ternary complex was crystallized using the hanging drop method. Before equilibration, the drop contained 1.62 mg/mL protein, 0.375 M NaCl, 0.09 M phosphate buffer (pH 7.3), 0.5 mM NaN_3, 4.9 mM Peptide II, and 15% (w:v) polyethylene glycol 8000. The well solution contained 30% (w:v) polyethylene glycol 8000 in 0.08 M phosphate (pH 7.3) with 0.5 mM NaN_3. Crystals up to 0.4 × 0.3 × 0.1 mm^3 were obtained in 1 month.

X-ray diffraction data were collected with a Raxis-IIC image plate detector using graphite-monochromated CuKα radiation from a Siemens 18 kW rotating anode operating at 50 kV and 90 mA. Data were processed using the XDS package by Kabsch (1988).

The crystals were of the same space group (*C*2) as the crystals of the thrombin/hirugen complex (Skrzypczak-Jankun et al., 1991), and the cell dimensions were only slightly different: a = 70.55 Å, b = 72.05 Å, c = 73.0 Å, and β = 101.0° in contrast to a = 70.70 Å, b = 72.46 Å, c = 72.9 Å, and β = 100.93° for the thrombin/hirugen complex. The crystal detector distance was 9.0 cm. A total of 61 748 observations yielded 23 326 unique reflections with an *R*-sym of 4.9%. The data set is 95.8% complete to 2.0 Å.

The structure of the ternary complex of thrombin/hirugen$^{53-65}$/Peptide IA (H. A. Schreuder, unpublished results) was used as a starting model for structure refinement. The starting *R*-factor for the initial model was, without the inhibitor, 29.4% for all 17 899 observed reflections between 8 and 2.1 Å. The resulting $F_o - F_c$ omit map showed clear and unambiguous electron density for the inhibitor. The inhibitor was fitted to the electron density maps. The model was refined by six cycles of map inspection and rebuilding using the program FRODO (Jones, 1985) followed by energy minimization and *B*-factor refinement using the program X-plor (Brünger, 1992). The hirudin$^{53-65}$ peptide did not show full occupancy in previous thrombin/hirudin$^{53-65}$ complexes. Therefore, we refined a group occupancy for this peptide that resulted in an occupancy of 0.65. The final model for the thrombin/Peptide II complex contains 197 water molecules, and one Na ion and has an *R*-factor of 0.205 for 20 706 reflections greater than zero between 8.0 and 2.0

Å. Comparison of the final model and the thrombin/PPACK complex (Bode et al., 1992; PDB code 1PPB) showed that the two models were similar with root mean square deviations (rmsds) of 0.6 Å for the 278 equivalent C^{α} atoms and an rmsd of 0.2 Å for 226 C^{α} atoms deviating by less than 3Å, even though the two models are completely independent.

Measurements of Thermodynamic Inhibition Constants (K_i). The K_i values of the new inhibitor were assessed for human α-thrombin and porcine pancreatic trypsin in chromogenic assays using Sar-Pro-Arg-*p*-nitroanilide and *N*-benzoyl-L-Arg-*p*-nitroanilide, respectively, as substrates. The thrombin assay mixture contained 0.033 unit of human α-thrombin (Sigma Chemical Co., St. Louis, MO), 0.2 mM substrate, and the inhibitor in a final volume of 3 mL buffered at pH 7.4 with 0.1 mM Tris-HCl. The trypsin assay mixture contained 10 ng of the enzyme (Sigma Chemical Co.), 50 mM $CaCl_2$, 0.5 mM substrate, and the inhibitor in a final volume of 0.1 mL buffered at pH 7.8 with 50 mM Tris-HCl. Thrombin assays were carried out at 30 °C, and the trypsin assay was carried out at ambient temperature for 15 min (Tuppy et al., 1962). Both assay mixtures contained 6.7% dimethyl sulfoxide carried over from the stock solutions of substrates and inhibitors. In both cases, rates of substrate cleavage were monitored by following absorbance changes at 405 nm.

Measurement of Anticoagulation Activity. Measurements of activated partial thromboplastin time (APTT) were carried out using the reagents and methods of Dade Diagnostics, Inc. (Aguada, Puerto Rico). All clotting times were measured semiautomatically using an MLA-Electra 700 apparatus (MLA Inc., Pleasantville, NY). The inhibitor concentration required for doubling the clotting time was calculated by simple linear regression.

RESULTS AND DISCUSSION

The crystal structure of an enzyme/ligand complex is a valuable basis for the design of novel inhibitors. However, the possible lack of correspondence between the crystal structure and the dynamic structures of the interacting molecules in solution, at the time scale of biochemical reactions, is a major concern in ligand design. In order to address this issue, we compared the crystallographically determined structures of several α-thrombin/inhibitor complexes. The geometries of the enzyme active site among these complexes are very similar. The difference in thrombin conformation between thrombin/hirudin and thrombin/PPACK complexes is limited to the short autolysis loop spanning residues Lys 145−Gly 150 (Rydel et al., 1990). This is impressive in view of the fact that hirudin is a protein containing 65 amino acids whereas PPACK is a modified tripeptide. The subject matter of this study is the specificity pocket of α-thrombin. The geometry of this pocket is essentially invariant in the crystal structures of these complexes whether or not the pocket is occupied (Bode et al., 1989; Rydel et al., 1990; Skrzypczak-Jankun et al., 1991). Therefore, we considered tenable the use of the crystal structures of thrombin/inhibitor complexes for the design of novel inhibitors.

The molecular structures of the peptides discussed in this study are shown in Figure 1. The molecular design of Peptide II was based on the crystal structure of Peptide IA complexed with α-thrombin and hirudin[53−65] (H. A. Schreuder, and C. Tardif, unpublished results). Peptide IA differs from the well-known thrombin inhibitor, PPACK, only in the chemical structure of its P1′ group, i.e. an α-difluorobutyl ketone group in the former and a chloromethyl ketone group in the latter. The two peptides interact with the enzyme identically except in the region where the differences reflect the differences in their chemical compositions. Both inhibitors form a hemiacetal structure with Ser 195 of the enzyme. The P3 fragment of the Peptides (D-Phe) is docked into the aryl binding pocket (S3 pocket) largely defined by Trp 215, Leu 99, Ile 174, Tyr 60A, and Trp 60D. The amino nitrogen and carbonyl oxygen of the P3 residue are favorably positioned for strong hydrogen bonding to the backbone heteroatoms of Gly 216. The P2 fragment (Pro) is inserted into the hydrophobic pocket (S2 pocket) defined by the Leu 59−Asn 62 insertion loop of the enzyme. The side chain of the P1 group (Arg) is inserted into the specificity pocket (S1 pocket) and forms the archetypal salt linkages with Asp 189 of the enzyme.

Table 1: Measured Inhibition Constants (Nanomolar) and APTT (Micromolar) of the Inhibitors

compound	$K_{i(thrombin)}$	$K_{i(trypsin)}$	$K_{i(thrombin)}/K_{i(trypsin)}$	APTT[a]
Peptide IA	2	1	2	1.6
Peptide IB[b]	80	23	3.5	16
Peptide II	247	>100000	<0.0025	44.9

[a] See Experimental Procedures. [b] The difference in chemical structure between Peptide IA and Peptide IB is only in the actvating group attached to the carbonyl carbon atom of the P1 residues, an α,α-difluorobutyl group in the former and a pentafluoroethyl group in the latter.

Peptide IA has similar affinities for trypsin and thrombin (see Table 1). Our initial goal was to replace the guanidino group of the P1 fragment of Peptide IA with an uncharged group that would retain the inhibitor potency and enhance thrombin specificity. Therefore, our target was the specificity pocket (S1 pocket) of the enzyme. The S1 pocket of α-thrombin is known to be very similar to that of trypsin-like enzymes. The pocket is enclosed on one side by residues Gly 216−Gly 219 in a twisted β-strand arrangement in relation to the arginine side chain of the inhibitor. The pocket is closed off at the bottom by Asp 189 which is involved in salt linkages to the guanidino group of the P1 arginine residue. This latter interaction provides a significant fraction of the binding energy for the inhibitor. The pocket is closed on the other side by residues 190−193, forming an irregular strand. Gln 193 folds over the P1 side chain and closes the pocket in the front. The back part of the pocket is enclosed by the side chains of Tyr 228, Leu 160, Val 138, and Ala 190.

An important geometric and electrostatic difference between trypsin-like enzymes in general and thrombin is the replacement of Ser 190 in the former with Ala 190 in the latter. This residue change renders the thrombin S1 pocket somewhat bigger and more hydrophobic (Bode et al., 1992). An inspection of the side chain moiety docked in the S1 pocket also reveals the possibility of filling the space within that cavity better with a suitable ligand. The extra bulk in the new putative P1 moiety should approach Tyr 228 and Ala 190 for optimal fit. In consideration of these desired spatial and electrostatic features of the S1 pocket, we designed Peptide II as a potential thrombin inhibitor.

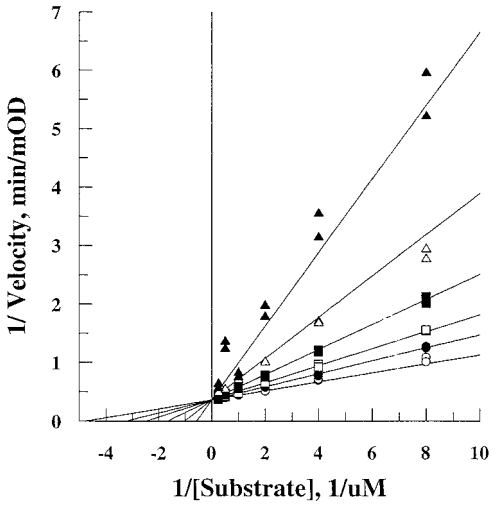


FIGURE 3: Double-reciprocal plot for the inhibition of α-thrombin by Peptide II. Inhibitor concentrations used in the experiment were as follows: (○) 0.0 μM, (●) 0.125 μM, (□) 0.25 μM, (■) 0.5 μM, (△) 1.0 μM, and (▲) 2.0 μM.

The tryptophan side chain of Peptide II is clearly bulkier and significantly more hydrophobic than the arginine side chain. In the proposed binding mode of this molecule, the electronic quadrupole of the phenyl ring of the indole group was close to the carboxylate group of Asp 189 of the enzyme for electrostatic interactions. The phenyl ring of the P1 indole group was intended to interact orthogonally and edgewise with the phenol ring of Tyr 228 (Perutz et al., 1986; Burley & Petsko, 1988). The carbonyl carbon atom of the pentafluoroethyl ketone was intended to form the tetrahedral hemiacetal complex with Ser 195 as is known to occur in serine protease inhibitors containing activated ketones.

The target molecule, Peptide II, was synthesized by the reaction scheme presented in Figure 1 as a diastereomeric mixture (Peptide II is a 50:50 mixture of *RSS* and *RSR* configurations). The *in vitro* anticoagulant activity of the inhibitor was evaluated by measurement of APTT (see Experimental Procedures). Peptide II doubled the rat plasma APTT at a concentration of 44.9 μM (Table 1). The K_i values of Peptide II[4] for α-thrombin and trypsin were determined in chromogenic assays using Sar-Pro-Arg-*p*-nitroanilide and benzoyl-L-arginine-*p*-nitroanilide, respectively, as substrates. Peptide II inhibits thrombin competitively with a K_i value of 0.25 μM (Table 1 and Figure 3). The measured K_i value for trypsin was greater than 100 μM. By optimization of the P1 residue alone, an improvement of more than 800-fold was thus obtained in the selectivity for thrombin over trypsin, as measured by $K_{i(thrombin)}/K_{i(trypsin)}$, for Peptide II in comparison to the parent peptide (Peptide IA). A more relevant comparison, to assess the impact of the P1 group substitution, is between Peptide II and Peptide IB since these peptides share the same carbonyl-activating group. In this case, the improvement in selectivity is more than 1400-fold. Table 1 shows that substitution of the arginine side chain by the tryptophan side chain increases the K_i value by a factor of 3.1. The corresponding loss in anticoagulant activity (APTT) was less than 3.

Peptide II retains the structural features of the prototypical D-Phe-Pro-Arg type inhibitors in the P1′, P2, and P3 moieties. By analogy to the known structures, the indole moiety of Peptide II was designed to be docked into the S1 pocket of α-thrombin. The competitive pattern of inhibition strengthens the validity for this mode of binding. However, alternate binding modes could not be ruled out *a priori*. In the prototype inhibitors, the guanidino or amidino moiety of the P1 residue is known to provide a significant fraction of the total binding energy. Since this strong interaction was not present in Peptide II, insertion of the new P1 group into the S1 pocket was not guaranteed. Furthermore, the charged prototype inhibitors are likely to approach the active site in a preferred orientation due to its interaction with the electrical dipole on the thrombin surface extending from the exosite to the specificity pocket. Alternate binding modes have been observed in elastase for two inhibitors that did not contain the activated carbonyl groups (Clore et al., 1986; Takahashi et al., 1988). The determination of the exact orientation of the new group was also crucial for further modifications to enhance the potency and selectivity of the molecule. Local deformation of the enzyme cavity was also a concern since the oriented volume of the new P1 group could potentially be too large for the specificity pocket and such deformations in protein structure cannot be reliably predicted by molecular modeling. Therefore, the crystal structure of the Peptide II/thrombin complex was determined.

Figure 4 shows the crystal structure of Peptide II in the enzyme active site. The crystal structure is very similar to the molecular model, described above, on which the inhibitor was based. The P3, P2, and P1′ groups assume the predicted patterns of interaction with the enzyme. The phenyl ring of D-Phe occupies the S3 pocket in a position orthogonal to Trp 214 of the enzyme. The backbone amino proton and the carbonyl oxygen of D-Phe are hydrogen bonded to the backbone of Gly 216. The pyrrolidine ring of Pro is inserted into the hydrophobic S2 pocket under the Leu 59−Asn 62 insertion loop of the enzyme. The ketone carbon of the pentafluoroethyl group forms a hemiketal with O^{γ} of the catalytic Ser 195 as indicated by the contiguous electron density in the difference Fourier map (Figure 5). The electron density for the indole methylene group was also unambiguous in the S1 pocket. The plane of the indole ring is parallel to the backbone of residues 215−217. The nitrogen atom of the indole ring is within hydrogen bonding distance of one of the carboxylate oxygens of Glu 192. It is noteworthy that Glu 192, as in other thrombin structures, has high thermal mobility as is reflected in the relatively high *B*-factors. The phenyl ring of the indole moiety of the inhibitor interacts with the phenyl ring of Tyr 228 with an approach distance of 5 Å and an interplanar angle of 120°. In the predicted model, this interplanar tilt was 92°. We do not consider this to be a major discrepancy because the crystal structure is a time-averaged structure and motions of this magnitude are known to occur in the interior of globular proteins (Brooks et al., 1988).

Berliner and Shen (1977) reported physical evidence for an indole binding site near the active site of thrombin by proflavin dye displacement experiments, spin-labeled electron spin resonance studies, and TOS-Arg-OMe esterase rate measurements on human α-thrombin. They deduced that this apolar binding site is extraneous to the "basic side chain binding site" or the S1 specificity pocket. To the best of our approximation, the indole binding site proposed by

[4] The hemiacetal structure formed between the inhibitor and the enzyme is reversible through reaction with bulk water. Thus, the K_i value is a valid parameter for evaluating the inhibitor potency.

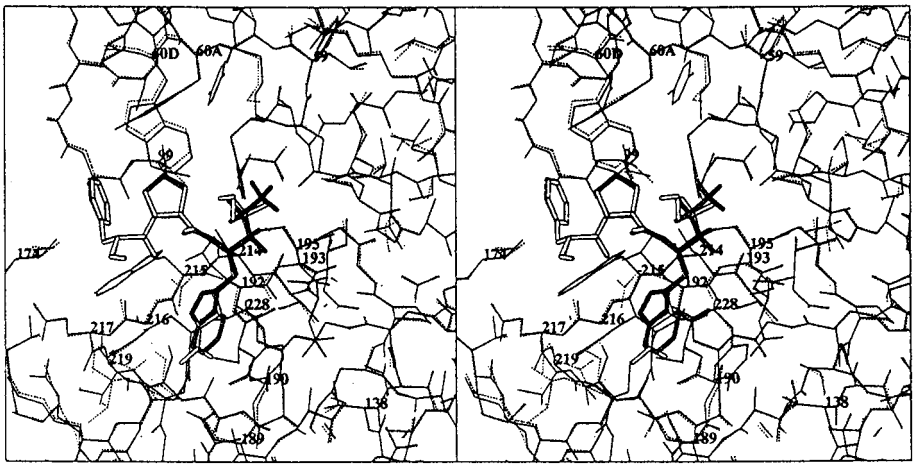


FIGURE 4: Stereoview of the active site of the thrombin/hirudin$^{53-65}$/Peptide IA and the thrombin/hirudin$^{53-65}$/Peptide II ternary complexes. The thrombin molecule is drawn in thin continuous lines for the Peptide II complex and in thin broken lines for the Peptide IA complex. Peptide II is drawn in thick dark lines. The major differences between the two complexes are the orientations of the peptide bond plane between Gly 219 and Cys 220 and of the side chain of Gln 192.

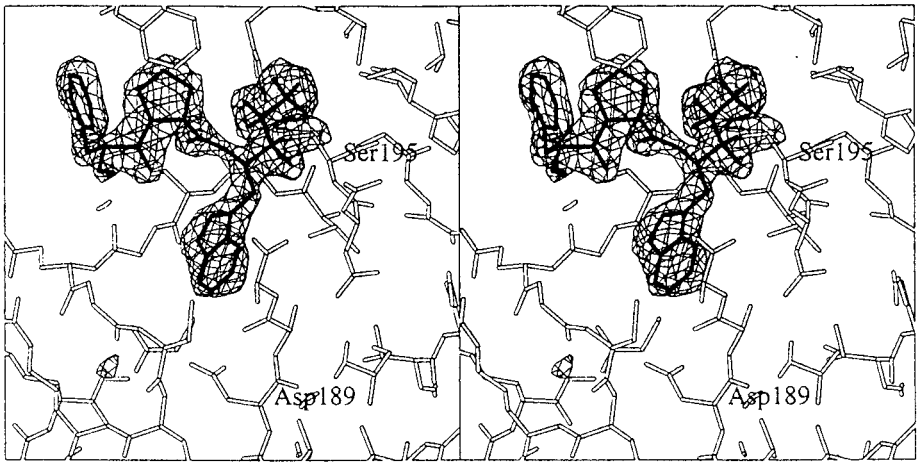


FIGURE 5: Stereoview of the F_o-F_c omit map contoured at 3.5Å. The C^β and C^γ atoms of Ser 195 are not included in the depiction. The electron density of the inhibitor is well-defined, and the covalent bond to Ser 195 shows strong and continuous density.

Berliner and Shen is in the region corresponding to the S2−S1′ pocket. It is possible to dock an indole moiety in this region that would afford several favorable interactions with the enzyme. In one such orientation, the indole ring could have favorable edge-on interactions with the aromatic rings of Trp 60D, His 57, and Tyr 60A and a strong electrostatic interaction between the π-electrons of the ligand and the charge on the amino group of Lys 60F. However, the data we present here give unequivocal proof that an indole moiety does bind in the specificity pocket of the enzyme. In the case of the present ligand, accessory binding sites in P3, P2, and P1′ may guide the indole moiety into the specificity pocket.

The increased hydrophobicity and size of the S1 pocket of thrombin compared to that of trypsin was the basis for the design of Peptide II. In all known trypsin structures, the Ser 190 side chain has a χ_1 value in the neighborhood of +60°, and as such, the side chain of this residue is pointed toward the P1 fragment of the inhibitor. This orientation of the Ser 190 side chain could cause steric hindrance when the P1 fragment is bulky as in Peptide II. This steric hindrance can only be removed by local deformation of the enzyme cavity since the strong interaction energies, particularly the covalent bond to Ser 195, would largely dictate the orientation of the inhibitor. We believe that the extra energy required to induce the fit of the inhibitor in the smaller active site of trypsin and its more optimal electrostatic interactions for the thrombin specificity pocket are responsible for the approximately 400-fold selectivity of this compound for thrombin over trypsin. Even in the thrombin active site, two significant changes are obvious between the thrombin/hirudin$^{53-65}$/Peptide IA and the thrombin/hirudin$^{53-65}$/Peptide II complexes. When compared to that of the former complex, the plane of the peptide bond between Gly 219 and Cys 220 and the side chain of Gln 192 had to be reoriented to accommodate the bulkier indole ring in the latter complex (see Figure 4).

In conclusion, we have described the design, synthesis, biological properties, and the enzyme-bound structure of a novel active site-directed inhibitor of α-thrombin. To our knowledge, this is the first reported three-dimensional structure of a thrombin/inhibitor complex where the inhibitor does not contain a charged P1 moiety. The new compound's activity was reduced only 3-fold compared to that of the parent compound, Peptide IB, even though the well-known salt linkage to Asp 189 is not present between the new ligand and the enzyme. In comparison to Peptide IB, the new ligand is approximately 1400-fold more selective for thrombin over trypsin solely due to the newly incorporated moiety in P1. Clearly, higher potency and selectivity could be achieved

through further modifications of the peptide in regions other than P1. The binding mode of the new ligand to the enzyme resembled the mode of interactions that were the basis of its design. Thus, we believe that the crystal structures of the available thrombin/inhibitor complexes are predictive of the bioactive conformations of the enzyme and could be successfully used for the design of thrombin inhibitors with novel pharmacological properties.

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