Bifunctional Peptide Boronate Inhibitors of Thrombin: Crystallographic Analysis of Inhibition Enhanced by Linkage to an Exosite 1 Binding Peptide

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ABSTRACT: The affinity of the hirudin$^{49-64}$ segment for exosite 1 of thrombin has been used previously to enhance the potency of simple competitive inhibitors [DiMaio, J., Gibbs, B., Munn, D., Lefebvre, J., Ni, F., Konishi, Y. (1990) *J. Biol. Chem. 265*, 21698−21703., and Maraganore, J. M., Bourdon, P., Jablonski, J., Ramachandran, K. L., and Fenton, J. W., II (1990) *Biochemistry 29*, 7095−7087.]. Using a similar approach, we have enhanced the activity of two active site directed thrombin inhibitors by attaching this segment via a novel reverse oriented linker to each of two tripeptide boronate inhibitors. At P_1, compound **1** contains an arginine-like, isothiouronium, side chain, while compound **2** contains an uncharged, bromopropyl residue. Inhibition of human α-thrombin by compound **1** shows slow, tight-binding competitive kinetics (final K_i of 2.2 pM, k_1 of 3.51×10^7 M^{-1} s^{-1}, and k_{-1} of 1.81×10^{-4} s^{-1}). The addition of hirugen peptide (20 μM) competes for exosite 1 binding and restores the k_1 and k_{-1} to that of the analogous tripeptide, 0.29×10^7 M^{-1} s^{-1} and 0.13×10^{-4} s^{-1}, respectively. Compound **1** has enhanced specificity for thrombin over trypsin with K_{iTry}/K_{iThr} of ∼900 compared to the analogous tripeptide, with K_{iTry}/K_{iThr} of ∼4. Compound **2** acts as a competitive inhibitor (K_{iThr} of 0.6 nM) and is highly selective with no effect on trypsin. Crystallographic analysis of complexes of human α-thrombin with compound **1** (1.8 Å) and compound **2** (1.85 Å) shows a covalent bond between the boron of the inhibitor and Ser^{195} (bond lengths B−O of 1.55 and 1.61 Å, respectively). The isothiouronium group of compound **1** forms bidentate interactions with Asp^{189}. The P_2 and P_3 residues of the inhibitors form interactions with the S_2 and S_3 sites of thrombin similar to other D-Phe-Pro based inhibitors [Bode, W., Turk, D., and Karshikov, A. (1992) *Protein Sci. 1*, 426−471.]. The linker exits the active site cleft of thrombin forming no interactions, while the binding of Hir$^{49-64}$ segment to exosite 1 is similar to that previously described for hirudin [Rydel, T. J., Tulinsky, A., and Bode, W. (1991) *J. Mol. Biol. 221*, 583−601.]. Because of the similarity of binding at each of these sites to that of the analogous peptides added alone, this approach may be used to improve the inhibitory activity of all types of active site directed thrombin inhibitors and may also be applicable to the design of inhibitors of other proteases.

Thrombin, a serine protease with trypsin-like specificity, plays a control role in blood coagulation cleaving fibrinogen and the thrombin receptor during fibrin formation and platelet activation, respectively. Therapeutic control of thrombin is currently undertaken by two principal types of drug which act directly (heparin) or indirectly (coumarins). Since both types suffer from side effects and variable clinical efficacy, intensive research has been undertaken over the past two decades to develop safe, synthetic thrombin inhibitors.

A common feature of nearly all the low molecular weight inhibitors of thrombin so far described is a positive charge at P_1 coinciding with the primary specificity of the enzyme which preferentially cleaves Arg−Xaa bonds. Affinity and specificity for thrombin is enhanced by the inclusion of certain hydrophobic groups at P_2 to P_4, the paradigm being the sequence H-D-Phe-Pro (*1*).[1] The most highly potent of these compounds are the active site directed inhibitors which include acylating groups interacting with His^{57} or Ser^{195} within the catalytic triad, for example, H-D-Phe-Pro-boroArg-OH (*2*) which binds thrombin with a picomolar inhibition constant (K_i < 4 pM). Compounds lacking the latter groups are much less potent (e.g., H-D-Phe-Pro-Arg, $K_i = 0.58 \times 10^{-6}$ M) (*3*), and in order to improve affinity and specificity,

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[1] Abbreviations: α-aminoboronic acid or Boro(aa) is where the CO_2 of the equivalent amino acid is replaced by BO_2; Z is the benzyloxycarbonyl protecting group for the amino group; H is used to designate an unprotected amino function; TBTU is Knorr's reagent, 2H-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; the residues of the inhibitor are labeled with the suffix I, and numbered, where appropriate, consistent with the exosite 1 binding residues of hirudin (Hir$^{49-64}$); Bpg, 3-bromopropylglycine; Irg, 3-isothiouronuimpropylglycine; Thr, thrombin; Try, Trypsin; Xa, Factor X activated; Pla, Plasmin; t-PA, tissue plasminogen activator; PCa, protein C activated; UK, urokinase; Ela, elastase; Chy, chymotrypsin.

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bifunctional peptides (*4, 5*) were developed by linking such compounds via a peptide bond at the C-terminus to a sequence derived from the hirudin structure (Hir^{49-69}). Hir^{49-69} binds at the fibrinogen recognition site (exosite 1) on the thrombin surface. Optimal enhancement of potency requires an additional poly-Gly linker sequence. Extensive structure activity studies of these bifunctional inhibitors have since shown that this linker must span at least 18 Å by containing at least 11 atoms. Since the P1−P1′ amide bond is liable to proteolysis by thrombin, nonpeptide and non-scissile linkers have also been incorporated. (*6*)

Direct linking of Hir^{49-69} is not compatible with certain active site directed thrombin inhibitors since modification of such groups as aldehyde to give ketones or trifluoromethyl ketones to give difluoro derivatives or modification of halomethylene ketones, phosphonic acids, or boronic acids increases the (+I) electron donation to the group and reduces the acylating ability. Particularly when boron-based groups are considered, the hydrolytic lability of the essentially ionic boron−nitrogen bonds and the oxidative instability of boronic acids (*7*) mean that it is not feasible to form a direct link between the boronic acid "C-terminus" of a tripeptide boronate with a hirudin peptide.

In this paper, we report how we have overcome this problem by introducing Hir^{47-64} via a linker to the P_3 N-terminus of each of two tripeptide boronate inhibitors of thrombin: Z-D-Phe-Pro-boroIrg and Z-D-Phe-Pro-boroBpg. We demonstrate an increase in potency for both compounds including an increase of several orders of magnitude in specificity compared to the parent tripeptides. Since crystallographic analysis shows that the binding at the S_1-S_3 sites and at exosite 1 is unchanged compared to peptides alone, the strategy adopted for the synthesis of these bifunctional inhibitors may be applicable for the enhanced delivery of other labile acylating groups to the active site of thrombin or other serine proteases.

MATERIALS AND METHODS

Synthesis. Z-D-Phe-Pro-boroIrg and Z-D-Phe-Pro-boroBpg were synthesized as the pinanediol esters as described previously (*8*). The benzyloxycarbonyl group of Z-D-Phe-Pro-boroBpg was removed by catalytic hydrogenation over 5% palladium on charcoal at atmospheric pressure. The Hir^{49-64} sequence was assembled using standard Fmoc chemistry on Peg-PS resins and extended by two glycines. The terminal Fmoc group was removed and the neo-N-terminus acylated by glutaric anhydride on the solid phase. Compounds **1** and **2** (Figure 1) were then synthesized by coupling the tripeptide unit, H-D-Phe-Pro-boroBpg, using TBTU as described previously (*9*).

The resin containing compound **2**, with the bromopropyl side chain P_1, was reacted with ethanolic thiourea to give the isothiouronium product, compound **1**. The compounds **1** and **2** were cleaved from the resin and simultaneously deprotected by trifluoroacetic acid (TFA) and 5% (w/v) phenol as a scavenger. The TFA solutions were concentrated under reduced pressure and the compounds isolated by purification on semipreparative Rp HPLC, on a Vydac C18 3 × 30 cm column, with acetonitrile/0.1% TFA and water as mobile phase. In studies where the length of the glycine linker was varied, the preparative HPLC typically showed an 8 ± 2 min decrease in retention time between the bromo starting material and the isothiouronium product. Amino acid analysis indicated expected ratios for full incorporation of D-Phe and Pro. The molecular weight was confirmed by electrospray mass spectrometry (ES-MS).

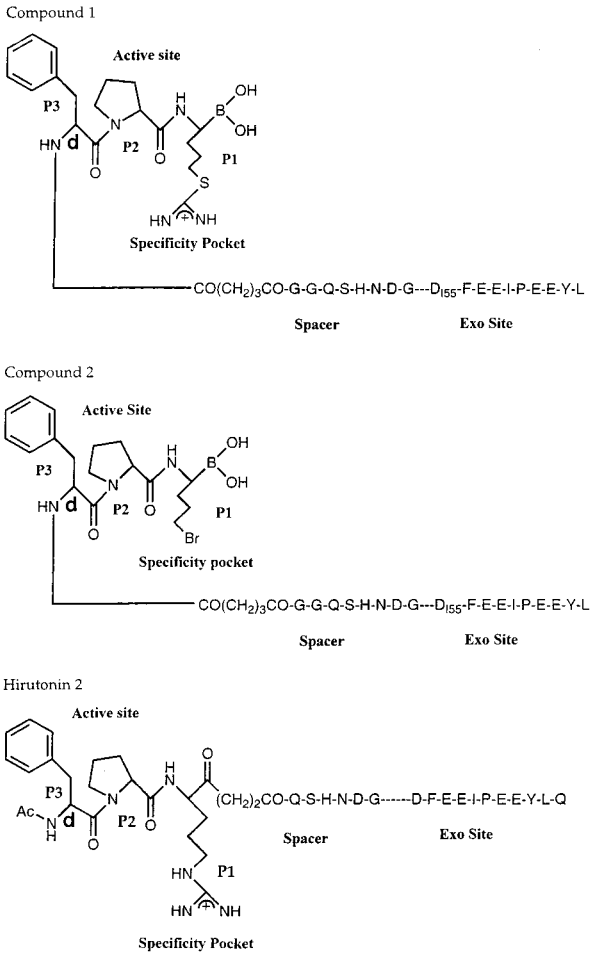

FIGURE 1: Schematic representation of the compounds **1** and **2** and Hirutonin 2. Amino acids are represented by single-letter codes for the spacer and exosite binding portions, while the active site binding unit is drawn in full. d-Enantiomers are represented by d, and numbering is based on the hirudin sequence. Drawings are annotated for the site of interaction of the inhibitor with thrombin.

Determination of K_i and Specificity. Enzyme activity (E_o, 0.5 nM) was measured from the rate of hydrolysis of substrate S2238 in 0.05 M (phosphate), 0.175 M NaCl pH 7.4 in a Molecular Devices plate reader at 25 °C.

K_i values of rapidly equilibrating inhibitors (Z-D-Phe-Pro-boroBpg and compound **2**) were determined from Dixon plots. Progress curves for slow binding inhibitors (Z-D-Phe-ProboroIrg and compound **1**) were analyzed according to the following mechanism:

$$E + I \underset{k_{-1}}{\overset{k_1}{=}} EI \qquad (1)$$

Values of absorbance (A) against time (t) were fitted by nonlinear regression using the program "Enzfitter" (*10*) according to eq 2 (*11*) where values of v_o (initial velocity in the absence of inhibitor), [S] (substrate concentration), [I] (inhibitor concentration), and K_m are known.

$$A = A_o + (v_o k_{-1} t)/k' + \{(v_o k_1[I])/(1 + [S]/K_m)k'^2\}(1 - e^{-k't}) \quad (2)$$

Values were generated for A_o (initial absorbance), k' (apparent first-order rate constant), and individual rate constants (k_1 and k_{-1}).

The value of K_i was then determined from eq 3:

$$K_i = k_{-1}/k_1 \quad (3)$$

Specificity was measured by determining K_i values for each enzyme using conditions which have been described previously (*8*), except that the substrate for FXa was S-2765 (Nα-Z-D-Arg-Gly-Arg-pNA.HCl, Chromogenix Ltd) and for TF/FVIIa was CBS 34.47 (H-D-Cha-α-Aib-Arg-pNA).

*Crystallography of Thrombin Complexed to Compound **1** or Compound **2***. The complexes of human α-thrombin with compound **1** or **2** were prepared by mixing thrombin with each compound at a 1:10 molar ratio, and the mixture was left for 1 h at room temperature. The complexes were crystallized by the hanging drop vapor diffusion method. Crystals were grown from 20% PEG 8000, 0.05 M $NaHPO_4$ buffer (pH = 7.15), and 0.05 M NaN_3. Crystals, typically of dimensions 0.30 × 0.15 × 0.15 mm^3, appeared in 3−4 weeks.

X-ray diffraction data were collected for the complex of human α-thrombin and compound **1** using an R-axis II image plate mounted on a Rigaku RU200 rotating anode generator. Data for the complex of human α-thrombin with compound **2** was collected at BW7B EMBL-Hamburg at a wavelength of 0.83 Å using a Marresearch image plate. The crystals were prepared for flash cooling by soaking in crystallization buffer made up with 25% PEG400. Data sets were collected to a maximum Bragg spacing of 1.8 Å for the complex of compound **1** and 1.85 Å for the complex of compound **2**. Diffraction data was processed with Denzo (*12*) and Scalepack (*12*). The crystals formed with both compounds belonged to space group C2, with unit cell dimensions for the complex with compound **1**, $a = 70.2$ Å, $b = 71.3$ Å, $c = 71.7$ Å, and $\beta = 100.2°$, and for the complex with compound **2**, $a = 71.2$ Å, $b = 72.0$ Å, $c = 73.0$ Å, and $\beta = 100.9°$ (Table 1). This compares to the complex of thrombin and hirugen (*13*) with crystals of *C*2 space group and unit cell dimensions of $a = 70.7$ Å, $b = 72.5$ Å, $c = 72.9$ Å, and $\beta = 100.9°$. Initial molecular replacement solutions were determined using Amore (*14*) using the structure of the ternary complex of PPACK.desamino-hirugen.thrombin (*15*) as the model for rotation and translation searches. For the complexes of compound **1** or **2** with α-thrombin, the translation function gave a correct solution with correlation values of 0.753 and 0.758 and *R*-factors of 0.34 and 0.35, respectively (Table 1).

Rigid body refinement reduced the *R*-factors for the complex of compound **1**/α-thrombin and compound **2**/α-thrombin to 0.29 and 0.30, respectively (Table 1).

Weighted difference maps (mfo-dfc) calculated after the rigid body refinement using Refmac (*14*) showed density for the inhibitor at the active site and exosite 1 of the protein. Atomic models of the inhibitors were generated using the program Spartan (version 4.1, Wave Functions Inc. Irvine CA). The coordinates for the inhibitor were placed in the electron density manually at the two binding sites using the interactive graphics program "O" and were subjected to several cycles of refinement using Refmac (*14*). Water molecules were added using ARP (*16*). The refined models had working *R*-factors of 18.6% and 17.6% and free *R*-factors of 22.7% and 22.4% for the complexes of compound **1**/α-thrombin and compound **2**/α-thrombin, respectively (Table 1). The coordinates of the complexes have been deposited in the Brookhaven Protein data bank under the file names 1A3b and 1A3e for the complexes of compound **1**/α-thrombin and compound **2**/α-thrombin, respectively.

Table 1: Summary of the Crystallographic Data for the Complexes of Compound **1**/Human α-Thrombin and Compound **2**/Human α-Thrombin

data	compound **1**/human α-thrombin	compound **2**/human α-thrombin
space group	*C*2	*C*2
unit cell	$a = 70.2$ Å, $b = 71.3$ Å, $c = 71.7$ Å, $\beta = 100.2°$	$a = 71.2$ Å, $b = 72.0$ Å, $c = 73.0$ Å, $\beta = 100.9°$
resolution range (Å)	20−1.80 (1.85−1.80)[a]	20−1.85 (1.92−1.85)
number of unique reflections	32 141	30 363
overall completeness	99.2% [98.0][a] (20−1.80 Å)	98.5% [97.4] (20−1.85 Å)
R-merge (%)	5.3 (32.5)	7 (39.0)
R-factor (working) (%)	18.6	17.6
R-factor (free) (%)	22.7	22.4
rms deviations		
bands (Å)	0.011	0.016
angles (deg)	1.3	2.245

[a] Figures in brackets refer to the highest-resolution bin data.

RESULTS

Synthesis. Compounds **1** and **2** were assembled as described above, from the hirudin exosite 1 binding sequence (Hir^{49-64}), without sulfation on tyrosine (Tyr^{I63}), with an N-terminal extension of two glycines. During preliminary studies, compound **2** was synthesized varying the size of the linker from G_2 to G_6. Optimum inhibition of thrombin occurred at G_2, and this was selected for further study (Figure 1). The flexible glutaric acid linker was selected to minimize steric interference in the binding of the inhibitor to the active site and exosite 1.

After liberation from the resin and Rp HPLC purification the composition of compounds **1** and **2** was confirmed by ES-MS and amino acid analysis, which showed the correct ratios for incorporation of the D-Phe and Pro, although the aminoboronate unit was not quantified.

*Inhibitory Properties of Compounds **1** and **2***. Compound **1** is similar to the parent peptide, Z-D-Phe-ProboroIrg, in acting as a slow, tight-binding inhibitor of thrombin, with an equivalent final K_i in the picomolar range ($K_{iThr} = 2$ pM as opposed to 4 pM, Table 2). In respect of the analogous peptide boronate (Z-D-Phe-Pro-boroBpg), compound **2** exhibited an order of magnitude increase in inhibition ($K_{iThr} = 0.78$ nM, Table 2). In both cases, the increased affinity of compounds **1** and **2** is due to binding at the exosite 1, since addition of hirugen peptide, which binds only to the exosite, competes for the interaction (Table 3). This is observed as a reduction in the apparent K_i (Table 3) for the inhibitors in the mixture, to values comparable to those for the structurally

Table 2: Inhibition Constants and Specificity K_{ienz}/K_{iThr}[a] of the Bifunctional Peptide Boronate Thrombin Inhibitors Compared to Tripeptide Inhibitors

enzyme	Z-D-Phe-Pro-boroIrg	compound **1**	Z-D-Phe-Pro-boroBpg	compound **2**
α-thrombin	0.004[b] [1][a]	0.002[b] [1]	6.62 [1]	0.78 [1]
γ-thrombin	nd[c]	nd[c]	35.8 [5.41]	0.8 [1.02]
factor Xa	5.7 [1425]	3550 [1 775 000]	ne[d] (35.3 μM)	ne[d] (4.5 μM)
trypsin	0.0164[b] [4.1]	1.81[b] [905]	2670 [403]	ne[d] (4.5 μM)
activated protein C	9.9 [2475]	180 [90 000]	6350 [959]	ne[d] (4.5 μM)
tissue factor/factor VIIa	29.7 [7425]	84 [42 000]	3700 [559]	10 700 [13 718]
chymotrypsin	1019 [254 750]	10 500 [5 250 000]	1270 [192]	5930 [6910]
kallikrein	126 [31 500]	10 200 [5 100 000]	148 000 [22 357]	ne[d] (4.5 μM)
elastase	21900 [5 475 000]	2800 [1 400 000]	430 [65]	770 [987]
plasmin	9.6 [2400]	7100 [3 550 000]	11 300 [1707]	ne[d] (4.5 μM)
urokinase	17.9 [4475]	400[b] [200 000]	ne[d] (35.3 μM)	ne[d] (4.5 μM)

[a] [ ] is value of K_{ienz}/K_{iThr}. [b] Final K_i from slow, tight-binding kinetics. [c] Not determined. [d] No effect at maximum concentration tested in brackets ( ).

Table 3: Measured Inhibition Constants (Nanomolar) for the Inhibitors in the Presence of Increasing Concentrations of Hirugen (Micromolar)[a]

	compound **1**			compound **2**
concentration of Hirugen	$k_1 \times 10^7$ (M^{-1} s^{-1})	$k_{-1} \times 10^{-4}$ (s^{-1})	K_i (app)	K_i (app)
0	3.51	1.81	0.005	0.9
0.74	1.49	1.08	0.0074	4.5
2.22	0.74	0.9	0.0122	8.0
6.67	0.29	0.54	0.0188	24.4
20	0.12 [0.29][b]	0.4 [0.13][b]	0.0353 [0.0043][b]	63.4 [6.62][b]

[a] See experimental procedures. [b] Figures in brackets refer to the final K_i for the analogous tripeptide inhibitors, Z-D-Phe-Pro-boroIrg and Z-D-Phe-Pro-boroBpg for compounds **1** and **2**, respectively, in the absence of Hirugen.

related tripeptide. Analysis of the kinetics of binding of compound **1** to thrombin shows that the rate ($k_1 = 3.51 \times 10^7$ M^{-1} s^{-1}, Table 3) of formation of the complex was enhanced compared to the parent inhibitor that binds only at the active site ($k_1 = 0.29 \times 10^7$ M^{-1} s^{-1}, Table 3). Despite the increase in k_1, the inhibitory constant of compound **1** is of the same order as the tripeptide, due to a co-incident increase in the dissociation rate for the complex ($k_{-1} = 1.81 \times 10^{-4}$ s^{-1}, compared to 0.13×10^{-4} s^{-1}, Table 3). Addition of increasing concentrations of the hirugen peptide, which competes for binding at the exosite 1, reduces k_1 to the order of that for the tripeptide (Table 3). Such an effect has previously been demonstrated for hirulog-1 (*4*).

The specificity of both compounds is enhanced as demonstrated by the fact that they show higher K_i values with other coagulation proteases than are obtained with the corresponding tripeptide (Table 2). In particular, there is a marked improvement in the selectivity of compound **2** for thrombin, which has no effect on Try, FXa, PCa, Pla, or UK when tested at concentrations over 5000-fold higher than its K_{iThr}. Lack of specificity is of significance in assessing the pharmacological properties of inhibitors; for example, an anticoagulant level of Ac-D-PhePro-boroArg also inhibits PCa ($IC_{50Pca} = 0.75$ μM) compromising both the profibrinolytic and anticoagulant effects of this enzyme (*17*, *18*). Overall, in terms of selectivity for thrombin compared to PCa, compound **1** (ratio $K_{iPca}/K_{iThr} = 9 \times 10^4$) is the most selective boroArg analogue reported to date, compared to DUP-714 (*2*), SDZ-217766 (*19*), and Z-D-Dpa-Pro-boroIrg (Scully et al., unpublished results) (ratios $K_{iPca}/K_{iThr} = 17\,500$, 52, and 1860 respectively).

Crystallography. The crystal structures for the complexes of compound **1**/human α-thrombin and compound **2**/human α-thrombin were solved at 1.8 and 1.85 Å, respectively (Table 1).

The boron functionalities of compounds **1** and **2** covalently derivatize the active site Ser^{195} of α-thrombin, with B−O bond lengths of 1.55 and 1.61 Å, respectively (Figures 2 and 3). The oxygens B−O1A are oriented toward $His^{57}N\epsilon2$ (2.54 and 2.6 Å), and B−O1B occupies the oxyanion binding

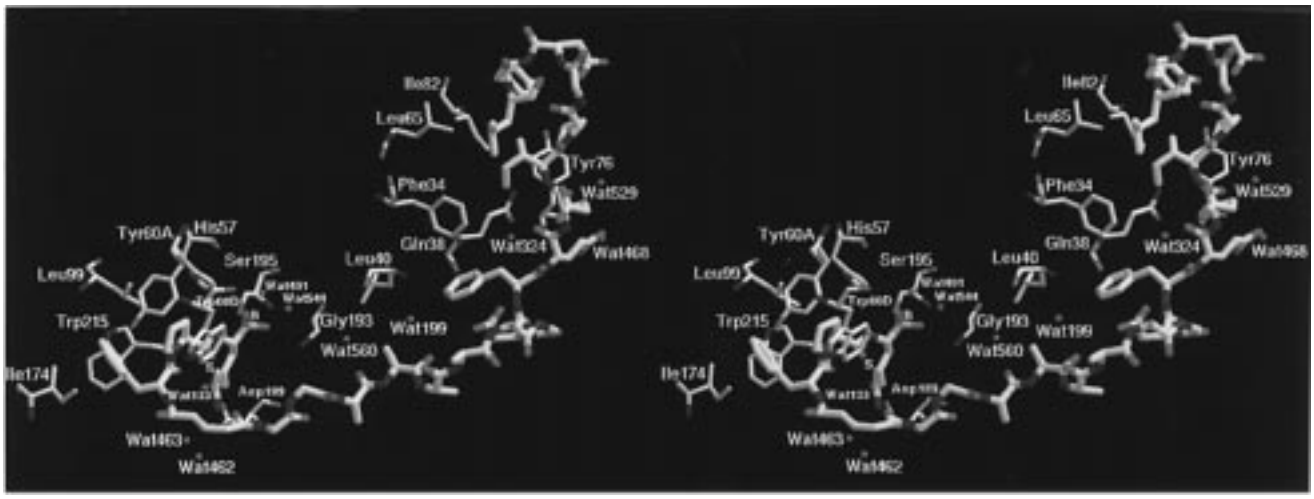

FIGURE 2: Interactions of Compound **1** (thick lines) with thrombin. Thrombin residues and water molecules that form strong interactions with the compound are numbered.

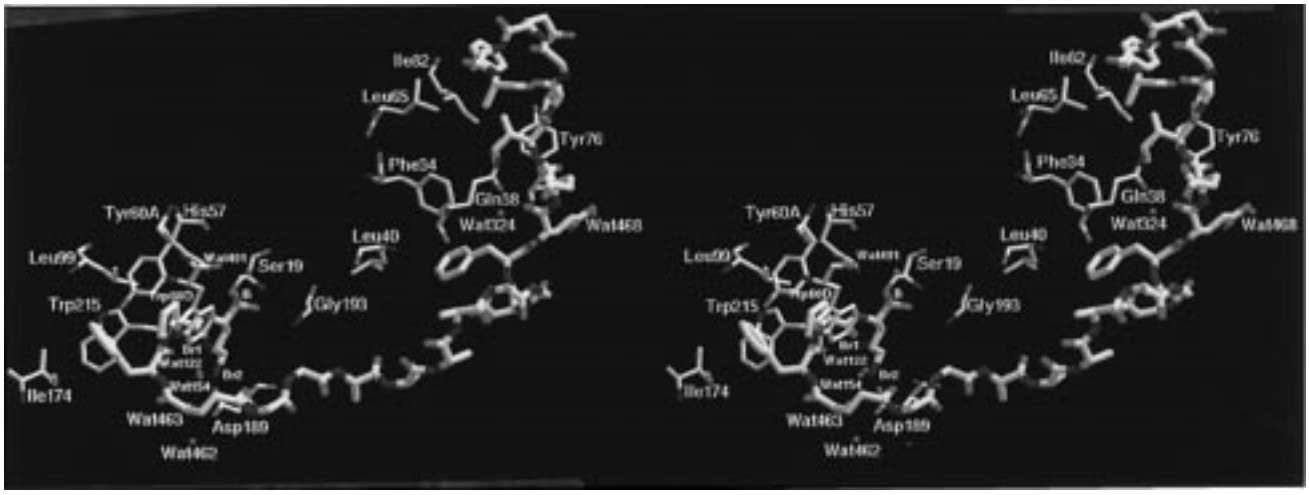

FIGURE 3: Interactions of Compound **2** (thick lines) with thrombin. Thrombin residues and water molecules that form strong interactions with the compound are numbered.

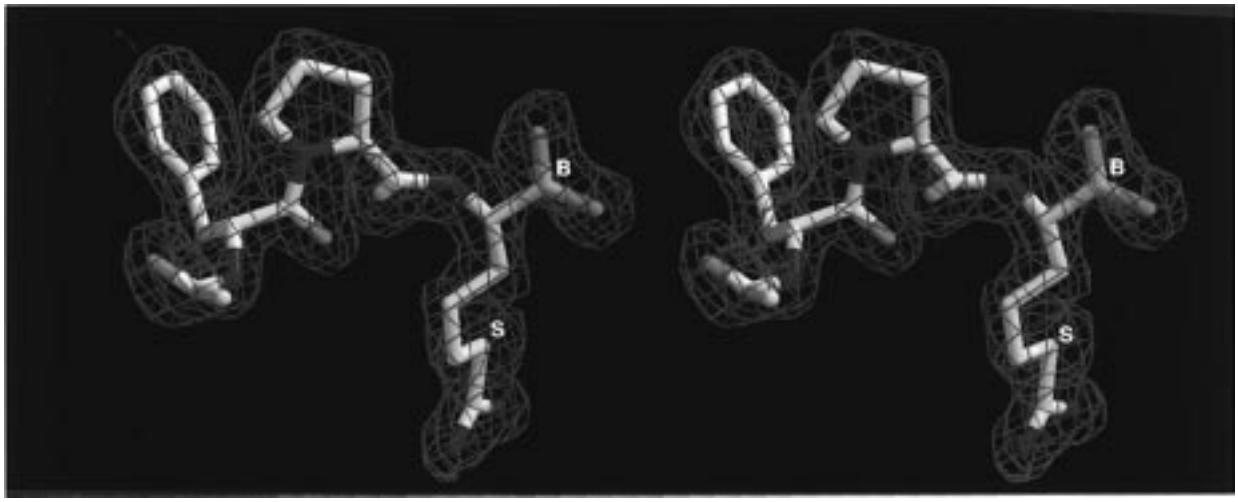

FIGURE 4: Electron density maps (2mfo-dfc) of the P_1-P_3 active site residues of the refined structure of the compound **1**/α-thrombin complex contoured at 1σ. The position of the boron atom is labeled B. Residues Ser^{195} and Asp^{189} of α-thrombin are shown.

pocket, with hydrogen bonding to the α-amino groups of Ser^{195} (3.19 and 3.07 Å) and Gly^{193} (2.96 and 3.19 Å) for complexes of compounds **1** and **2**, respectively (Figures 3 and 4, respectively). These interactions are typical of the "transition-state" analogue interactions achieved at the active site of serine proteases with peptidyl boronates (*20*−*22*). Strong interactions are achieved with the active site serine, as are seen with the pyridinium methyl ketone, P596, (*23*), which acylates $Ser^{195}O\gamma$ at 1.46 Å, unlike the noncovalent hirutonin-2, (*24*) (CO-Ser^{195} $O\gamma$ 2.8 Å). P596 and hirutonin-2 have, for comparison, distances to the α-amino groups of Ser^{195} of 2.8 and 3.4 Å, and of Gly^{193} of 2.8 and 3.1 Å, respectively.

The nitrogens of the P_1 isothiouronium side chain of compound **1** form a bidentate interaction with the Asp^{189} carboxylate (N1A-2.84 Å-Asp^{189}OD2, and N1B-2.68 Å-Asp^{189}OD1, Figure 4) that delimits the bottom of the so-called "specificity pocket". This has been seen previously for arginine-based bifunctional compounds such as hirulog-1 (*13*), hirutonin-1 and −2 (*24*), P596 (*23*), and CVS995 (*25*) and differs from the monocoordinate interaction observed by the bifunctional MQPA analogues P498 and P500 (*26*).

In contrast, for compound **2**, the bromopropyl side chain at P_1 is fully extended into the thrombin S_1 site toward the Asp^{189}. The bromine is seen in two positions in the electron density map, at, for Br1, 5.03 and 5.88 Å, and, for Br2, 4.86 and 4.62 Å from the carboxylate oxygens OD1 and OD2 of Asp^{189}, respectively (Figure 5). The bromine atom is hydrogen bonded via a water molecule, WAT^{122}, to the γ-carboxyl group of Asp^{189} (Br1-2.56 Å-WAT^{122} and Br2-2.13 Å-WAT^{122}, and the position of the WAT^{122} is WAT^{122}-2.99 Å-Asp^{189}OD1, WAT^{122}-3.36 Å-Asp^{189}OD2). Due to the dual, and almost equal, occupancy of the bromine atom (B factors of 36 and 41 $Å^2$), the WAT^{122} has a relatively high thermal factor (*B* factor) of 42 $Å^2$, which is higher than the average value of 30 $Å^2$ for the buried, protein-associated waters. Tripeptide boronates with only ornithine and lysine residues form similar water bridges to Asp^{189} (ϵNH-2.6 Å-WAT6-2.6 Å-Asp^{189}OD for BoroLys and ϵNH-2.9 Å-WAT^6-3.0 Å-Asp^{189}OD for BoroOrn), (*22*). Interestingly, like here, this geometry preserves the covalent acylation of the $Ser^{195}O\gamma$ by boron and interaction of the side chain amino with Gly^{219} (3.2 and 3.3 Å for BoroLys and BoroOrn, respectively).

Both compounds achieve the canonical interactions at P_2 and P_3 seen with PPACK (*27*). That is, at the S_2 site, hydrogen bonding interaction of the P_1 α-amino with Ser^{214} (3.0 and 3.19 Å for compounds **1** and **2**, respectively) and

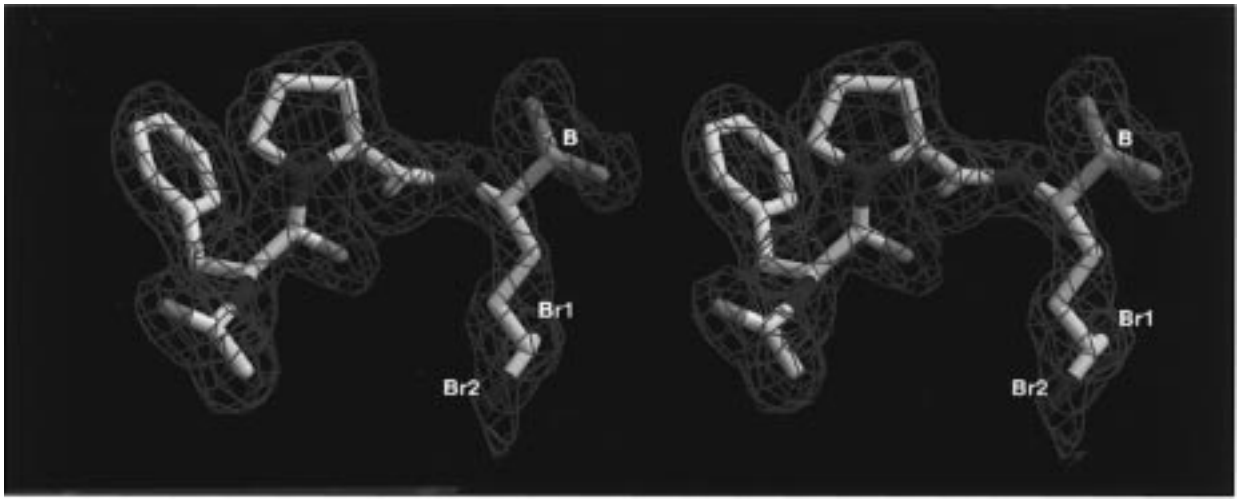

FIGURE 5: Electron density maps (2mfo-dfc) of the P_1-P_3 active site residues of the refined structure of the compound **2**/α-thrombin complex contoured at 1σ. The position of the boron atom is labeled B. The two positions observed for the bromine (Br2 and Br1) and the WAT^{122} is shown. Residues Ser^{195} and Asp^{189} of α-thrombin are shown.

docking of the P_2 Pro^{I2} in the site formed by the 60D loop are achieved. In the S_3 site, orientation of the inhibitor is achieved by antiparallel binding with Gly^{216} and an edge on aryl−aryl interaction of the P_3 Phe^{I3} phenyl residue with Trp^{215}. The D stereoconfiguration of the P_3 residues of the inhibitors orients the glutaric linker "up" out of the active site canyon, exiting at a point where the FpA chain continues to run along for a further 3 amino acids (G^{I13}-G^{I12}-E^{I11}), before forming its turn which places Phe^{8} in S_3 (*28*). These positions (S_4-S_6) are unoccupied by the inhibitors **1** and **2**.

Crystallographic analysis of the compounds in complex with thrombin show disorder of the linker (alkyl chain and $Gly^{I}Gly^{I}$) and exosite binding segments (Asn^{I52} and Leu^{I64}) for both compounds. Strong interactions are only seen with P'_6 (Gln^{I49}), P'_7 (Ser^{I50}), P'_{12} (Asp^{I55}), P'_{13} (Phe^{I56}), and P'_{14} (Glu^{I57}) residues. Phe^{I56} occupies the first of the two hydrophobic sites of the thrombin exosite 1(*29*), formed by residues Phe^{34}(CE1-3.42 Å-Phe^{I56}CE1), and weaker interactions with Leu^{40}, Arg^{73}, and Thr^{74}. Thermodynamic analyses of these interactions have shown both to contribute 1−2 kJ mol^{-1} each (*29*, *30*) to the interaction. While the intervening sections therefore may adopt several conformations, there are clearly few direct interactions with the surface residues of thrombin. A possible conformation of the five residues P'_8 (His^{I51}) to P'_{11} (Gly^{I54}) would allow them to loop out from the surface, as seen for the tetra-Gly linker of Hirulog-1 (*13*), with the carboxylate residue of Asp^{I55} within hydrogen bonding distance (∼3 Å of the backbone amino group of Asn^{I52} and Asp^{I53}, tending to stabilize the loop). The necessity to form the loop brings the Ser^{I50} α-carboxyl to within 8.7 Å from the α-carboxyl of Asp^{I55}, which are well-ordered in the density map, compared to an average expected separation for a fully extended peptide chain of ∼3.6 Å/residue. As this is thermodynamically unfavorable, the complex may be unstable as seen by the increased k_{-1}.

The remainder of the peptide Glu^{I57} to Leu^{I64} is relatively disordered, as indicated by poorly defined electron density in the map, as was observed for the hirulog-1 complex (*31*). It is possible to model it as the 3^{10} helix as seen with peptides, such as Hirugen (*13*), or as with recombinant hirudin (*32*) or Hirutonin-2 (*24*). In those cases the stabilization of the helix is considered to be provided by sulfation on the penultimate residue, Tyr^{I63}, or for the unsulfated recombinant hirudin or Hirutonin-2 by the presence of an additional C-terminal residue, Gln^{I65}.

Ile^{I59} of the inhibitors is essential for hydrophobic packing with thrombin and the inhibitor, thereby initiating the helical twist in this second hydrophobic patch (*33*). The sulfate group of Tyr^{I63} in hirudin-derived C-terminal peptides (*13*) hydrogen bonds directly to Ile^{82}αNH (2.59 Å) and, via an ordered (*B*-factor 30 $Å^2$) water (3.12 Å), to the α-carboxyl of Glu^{80} (2.71 Å), the side chain of which is salt bridged to Arg^{67}. These interactions are reproduced in the complexes of the unsulfated peptides **1** and **2** by the interaction of Tyr^{I63} with water molecules that form a bridge to thrombin main chain residues. Thus, for compound **2** the Tyr^{I63} hydroxyl is hydrogen bonded to a water (Wat^{71}, 2.84 Å). The water molecule (Wat^{71}) is close to a water (WAT^{156}, 2.45 Å), which in turn binds to Glu^{80}αCO (2.91 Å) and to the hydroxyl of Tyr^{76} (3.07 Å). For WAT^{71} the only other interaction is to bridge to Ile^{82}αNH (3.01 Å). Occupation of the site by water was also seen in the complex of P596, where Tyr is replaced by Cha (*23*).

DISCUSSION

As noted above, the design of bifunctional peptide boronates based on the principle used in the development of the hirulog series of compounds in which the S_3-S_1 binding peptide is linked via the C-terminus to a linker and thence to the exosite 1 binding peptide was not appropriate because of reduction in acylating potential and hydrolytic lability. In adopting a strategy in which the exosite 1 binding peptide is attached by reverse orientation of the linker (Figure 1) to the NH_2-terminus of a low molecular weight peptide boronate, we were aware that the resulting compound may well have a reduced rather than enhanced inhibitory potency. This was anticipated in part because the particular potency of tripeptide boronates is due to their ability to form transition-state-like adducts and new interactions of the linker might disrupt the geometry and prevent such binding. Inhibitors with the most favorable structure (Type 1) (*34*, *35*) act as slow, tight-binding inhibitors with K_i values < 100 pM. Z-D-Phe-Pro-boroIrg, the parent inhibitor of

compound **1**, is an example of a Type 1 inhibitor, and the attachment of a linker and an exosite 1-binding peptide could have interfered with the attainment of the transition-state-like adduct. This would reduce the affinity and possibly alter its properties to that of a Type 2 inhibitor (rapidly equilibrating, $K_i > 100$ pM). In fact, this proved not to be the case, and the overall affinity remained constant although k_1 and k_{-1} were each increased by an order of magnitude. The increased association rate (k_1) approaches the collisional limit. This might have been expected since the large polar area of the exosite 1 site is known to be essential for the initial docking between hirudin and thrombin. The increase in off rate (k_{-1}), however, shows the complex to be less stable. The reasons for this lack of stability are not apparent in the crystallographic analysis since binding within the specificity pocket is very similar to that of the free peptide.

Apart from the different characteristic interactions associated with the isothiouronium rather than bromopropyl groups of compounds **1** and **2** in the specificity pocket, the only differences in the active sites of the complexes are the presence of water molecules around the His^{57} and oxygen O1A of the inhibitor. In both cases a covalent adduct is formed between the boron of the inhibitor and the hydroxyl oxygen of Ser^{195}. This interaction may be the same as that previously reported in an NMR study of a trypsin/peptide boronate complex (*8*). For the complex of the slow, tight-binding inhibitor compound **1**, there are two water molecules associated with the boronic acid group. One water (WAT^{491}) is 2.69 Å from oxygen 01A and one water (WAT^{544}) is 2.45 Å from O1B; this compares to equivalent waters seen in the complex of the analogous tripeptide inhibitor Z-D-Phe-Pro-boroIrg with human α-thrombin (E. Skordalakes, unpublished data) which has a water molecule (WAT^{491}) 2.64 Å from oxygen O1A and a water (Wat^{544}) at 2.33 Å from O1B. In the complex of compound **2**, there is no water molecule equivalent to Wat^{544} although a water (equivalent to Wat^{491}) is still associated with O1A, at 2.78 Å, and is 4.36 Å from O1B.

In summary, it was found that water (WAT^{544}) is present only in the complexes that more closely resemble the transition state, where there is interaction with Asp^{189}, as for compound **1** where it is 3.4 Å from the boron. It is therefore possible to propose that WAT^{544} adopts the position of the deacylating water expected for productive catalytic turnover of the enzyme. It has recently been proposed (*36*) that a conserved water is observed that hydrogen bonds to the carbonyl of residue[41], and Wat^{544} occupies a position at 3.2 Å from Leu^{41}. Further supporting evidence for this role is that WAT^{544} is associated with the inhibitor oxygen (here O1B of compound **1**) that occupies the oxyanion pocket, an interaction possible in the natural amide bond cleavage.

The thrombin S_1 pocket is ampipathic in nature and hydrophobic at the surface becoming more solvated around Asp^{189}, and it has been possible to exploit this by forming interactions with neutral amino acid residues at P_1 (*8*, *37*−*39*). Compared to an inhibitor with an ethyl residue at P_1, Z-D-Phe-Pro-boroEtg ($K_{iThr} = 2\ \mu$M), Z-D-Phe-Pro-boroBpg is a potent ($K_{iThr} = 7$ nM) and specific inhibitor of thrombin (K_{iThr}/K_{iTry} ratio of 405). This class of peptide boronate inhibitors all act as Type 2 inhibitors presumably because of the weaker binding of the neutral P_1 compared to the bidentate binding of Arg or Irg. For this reason, we considered that the potency of the bifunctional compounds would inevitably be reduced. In fact, again, the potency was enhanced. Despite the high specificity of the parent peptide, Z-D-Phe-Pro-boroBpg, there was also some enhancement of selectivity. Moreover, the potency of the compound in prolonging the thrombin clotting time of plasma was also increased 10-fold. Some precedent for this increase in potency has recently been reported in studies where small, low-affinity (millimolar) ligands were linked, giving compounds of submicromolar affinity (*40*).

Because of the differences in structure at P1 and also in kinetic properties between compounds **1** and **2**, it was anticipated that the complexes of compounds **1** and **2** with thrombin would differ fundamentally in structural features responsible for interactions with the enzyme. On the contrary it was found instead that both complexes adopted canonical interactions with α-thrombin in the S2 and S3 positions, that the linker departed the active site in the same orientation, and, despite the disorder of the linker segment, that canonical "hirudin-like" interactions were also achieved at the exosite 1.

No difference was also observed for the detailed geometry of interaction for compounds **1** and **2** with the catalytic triad. Direct observation of the boron atom of these compounds was not possible because of the weak scattering of X-rays by boron. Its position was therefore refined from the directly observed positions of the surrounding atoms in its environment (C, 3 × O). These positions are accurate to only ± 0.12 Å, greatly limiting the accuracy of any estimated geometry for the boron. In fact, it is impossible to distinguish between a trigonal or tetrahedral arrangement about the boron. Indeed ^{11}B NMR studies have shown previously identical signals for complexes formed with Type 1 and Type 2 inhibitors (*41*).

To obtain an approximation of the geometry of the active site boron in covalent complexes with thrombin, the data obtained at a resolution of 1.6 Å of the complex between Z-D-Phe-Pro-boroMpg and human α-thrombin (E. Skordalakes, unpublished data) was used. The boronic acid group (B, O1A and O1B) was subjected to unrestrained minimization using the program Refmac (*14*), with the van der Waal's radii of the atoms set to 0.1 and no tethering or constraint for the covalent boron to $Ser^{195}O\gamma$ bond distance. After 10 cycles of refinement this gave a boron to $Ser^{195}O\gamma$ distance of 1.6 Å and angles consistent with a distortion toward tetrahedral. The small O1A−B−O1B angle (105°) may reflect coordination of O1B in the oxyanion pocket and thus partial satisfaction of the charge. The bond lengths to boron so determined are consistent with small molecule tetrahedral complexes, as well as those reported for peptidyl boronate complexes with other proteases (*20*−*22*). The geometry seen here about boron was therefore distorted between trigonal, as expected for the uncomplexed compounds, since boron is not hydrated at pH ∼7 (*42*) and tetrahedral with $Ser^{195}O\gamma$ as the apical ligand.

The crystal complexes of compounds **1** and **2**, that are formed from high concentrations in the crystallizing solution, do not therefore reflect the differences in the kinetics of inhibition observed with the, near equilibrium, concentrations in in vitro assay. It may be that the observed kinetics are composites of multiple factors involving free rotation of inhibitor bonds, dehydration, or hydration of functional

groups of receptor or ligand and allosteric changes in the protein structure and not merely gross structural changes in the receptor ligand complex.

Conclusion. Inhibition of serine proteases by peptide boronates is sensitive to structural changes which retard the approach to formation of a tetrahedral adduct. The improvement of the properties of both Type 1 and Type 2 peptide boronates toward inhibition of thrombin and successful engagement of the active site serine by the bifunctional approach described herein encourages us to suggest that this approach may also be applicable in the development of inhibitors using other acylating moieties and for other proteolytic enzymes.

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