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Structures of thrombin retro-inhibited with SEL2711 and SEL2770 as they relate to factor Xa binding

Igor Mochalkin and A. Tulinsky*

Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, USA

Correspondence e-mail: tulinsky@pilot.msu.edu

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PDB References: thrombin–hirugen–SEL2711 complex, 7kme; thrombin–hirugen–SEL2770 complex, 8kme.

Most thrombin active-site inhibitors form a short antiparallel β-strand with residues Ser214–Gly216. However, the Selectide Corp. inhibitors SEL2711 and SEL2770 bind to thrombin in a retro fashion, making a parallel β-strand with Ser214–Gly216 similar to other retro-binding inhibitors. The crystallographic structures of thrombin–hirugen complexed with SEL2711 and SEL2770, which are isostructural with the binary thrombin–hirugen complex, have been determined and refined in the 9.0–2.1 Å resolution range to final R values of 16.5 and 16.7%, respectively. The structures of the SEL2711 and SEL2770 complexes contain 131 and 104 water molecules, respectively, both of which correspond to occupancies of greater than 0.5. The L-4-amidinophenylalanyl residues of SEL2711 and SEL2770 are fixed at the S1 specificity site, utilizing favorable ionic and hydrogen-bonding interactions between the N atoms of the amidino group and the side-chain O atoms of Asp189. The Glu192 residue of thrombin adopts an extended conformation, which allows the L-cyclohexylglycyl residue in the P2 retro-binding position of the inhibitors to occupy a similar site to the P3 aspartate in thrombin platelet-receptor peptides bound to thrombin. The N-terminal acetyl group of both inhibitors is located in the S2 subsite, while the L-3-pyridyl-(3-methyl)-alanyl of SEL2711 and the L-(*N*,*N*-dimethyl)lysine of SEL2770 occupy the S3 D-Phe subsite of D-PheProArg chloromethyl ketone (PPACK) in the thrombin–PPACK complex. The two C-terminal residues of SEL2711 (leucine and proline) point into the solvent and have no electron density in the thrombin complex. Those of SEL2770 are also positioned into the solvent, but surprisingly produce weak electron density with high B values ($\langle B \rangle$ = 50 Å^2). Since the Selectide inhibitors are about 10^4 times more specific for factor Xa, modeling retro-binding to the latter suggests that the selectivity can be a consequence of interactions of the inhibitors in the S3–S4 binding subsites of factor Xa.

1. Introduction

Under normal physiological conditions, the blood of higher organisms is maintained in a fluid state. Upon vascular injury, the hemostatic system is primed to stop the loss of blood by sealing off the site of the injury and beginning the process of tissue repair. Thrombin, a multifunctional serine protease, plays a central role in the blood-coagulation cascade by performing a number of functions, exhibiting both coagulant and anticoagulant activities. As a coagulant, thrombin activates platelets, cleaves fibrinogen to form fibrin and converts coagulation factors V, VII, VIII, XI and XIII to their active forms (Colman *et al.*, 1994). Bound to thrombomodulin,

thrombin subsequently activates protein C, which acts as an anticoagulant by converting factors Va and VIIIa to inactive forms in the presence of the cofactor protein S (Esmon, 1993).

Another prominent serine protease of the coagulation cascade is factor Xa, generated through limited proteolysis of factor X by either the tissue-factor–factor VIIa complex of the extrinsic pathway (Osterud & Rapaport, 1977; Silverberg *et al.*, 1977) or the X-ase complex, consisting of factors IXa and VIIIa, Ca^{2+} ion and phospholipid, of the intrinsic pathway (Stern *et al.*, 1985). Among all the obligatory feedback mechanisms of the blood-coagulation cascade, factor Xa activates the tissue-factor–factor VII complex and physiologically converts prothrombin to thrombin through the prothrombinase complex of factors Xa and Va, Ca^{2+} ion and phospholipid (Mann *et al.*, 1990). From a purely isolated standpoint based on the hierarchy of the stepwise coagulation process and the proteolytic functions of thrombin and factor Xa, both enzymes are attractive targets for inhibition based on drug design.

Thrombin owes its unique role in coagulation to its having three independent binding sites – the active site, the fibrinogen-recognition exosite and the heparin-binding exosite – or four sites, if the Na^+ ion binding site is counted (Tulinsky, 1996). The highly electropositive fibrinogen-recognition site assists in the divalent association of the natural substrate fibrinogen and the potent natural inhibitor hirudin to thrombin. Another highly electropositive site binds heparin, a prominent antithrombotic agent and cofactor which produces a 50000-fold acceleration of the rate of inhibition of the active site of thrombin by antithrombin III (Olson & Bjork, 1992). An allosteric change from a kinetically slow to fast form accompanying binding of an Na^+ ion affects the energetics of the active site of thrombin (Wells & Di Cera, 1992); fibrinogen binds to the fast form with high affinity and is cleaved with higher specificity (Mathur *et al.*, 1993), while the slow form activates the anticoagulant protein C more specifically (Dang *et al.*, 1995). The Na^+ binding site has been identified and characterized crystallographically with the aid of diffusion exchange of the Na^+ ion with an Rb^+ ion (Zhang & Tulinsky, 1997).

As members of the trypsin-like serine-protease family, the active sites of thrombin and factor Xa possess the catalytic triad His57–Asp102–Ser195 (chymotrypsinogen numbering; Bode *et al.*, 1992). Two insertion loops, one at position 60 and the other at position 149, partially occlude the active site of thrombin and are responsible for some of the restricted specificity of thrombin compared with trypsin (Locht *et al.*, 1997). Thrombin active-site inhibitors divide into two principal groups according to their binding orientation (antiparallel or parallel β-strand) with respect to residues Ser214–Gly216. The antiparallel group, also operative with factor Xa, consists of a variety of inhibitors which mimic natural substrates at the P1–P3 positions, the archetypes being D-phenylalanyl-prolyl-arginyl chloromethylketone (PPACK) for thrombin (Bode *et al.*, 1992) and glutamyl-glycyl-arginyl chloromethylketone for factor Xa, along with a large number of other small peptide inhibitors (Balasubramanian, 1995). This particular group also includes argatroban ((2*R*,4*R*)-4-methyl-1{N^{α}-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl}-2-piperidine carboxylic acid) and NAPAP [N^{α}-(2-naphthyl-sulfonyl-glycyl)-D-*p*-amidinophenylalanyl-piperidine] (Brandstetter *et al.*, 1992), which form an antiparallel β-strand even though their binding mode is different at the S1′–S3 subsites (Mathews & Tulinsky, 1995). The second group of thrombin inhibitors consists of hirudin-like active-site-binding inhibitors (Maraganore *et al.*, 1990; Rydel *et al.*, 1991), which show a retro-binding mode of association with thrombin by forming a parallel β-strand with Ser214–Gly216, such as the synthetic peptide BMS-183507 (Fig. 1*a*; Tabernero *et al.*, 1995), the natural product Nazumamide A (Fig. 1*b*; Nienaber & Amparo, 1996) and a RPPGF breakdown fragment of bradykinin (unpublished results of this laboratory).

A class of tri- to octapeptide competitive inhibitors of factor Xa has recently been identified from the screening of a large combinatorial library (Ostrem *et al.*, 1998). The consensus sequence is Tyr–Ile–Arg–*X*, where *X* is a hydrophobic amino acid and isoleucine is replacable with leucine. The minimal sequence is the first three residues and may be replaced by homologous non-natural amino acids. Some of these have been shown to inhibit hydrolysis of chromogenic substrates of factor Xa, but bind to the enzyme differently to substrate so that Ser195 of the catalytic triad does not attack the carbonyl group at the P1 position (Ostrem *et al.*, 1998). Selectide Corp.[1] inhibitors SEL2711 and SEL2770 (Fig. 1*c*) are members of this class of combinatorially designed potent active-site inhibitors of factor Xa which are also highly selective against thrombin and other blood proteases (Table 1), exhibiting dose-dependent efficacy following both intravenous and oral administration in a rat arteriovenous shunt thrombosis model. A reverse binding mode, similar to N-terminal hirudin active-site binding to thrombin, was suggested to explain the selectivity of this class of inhibitors (Ostrem *et al.*, 1998). However, from an NMR study of two of the inhibitors, Ac-Tyr–Ile–Arg–Ile–Pro-NH_2 and Ac-(4-amino-Phe)–(cyclohexyl-Gly)–Arg-NH_2 (peptides A and B, respectively), bound to the active site of factor Xa (Fraternali *et al.*, 1998), the authors showed that 'the inhibitors assume a compact, very well defined conformation, embedded into the substrate binding site not in the same way as a substrate'. It was also not a retro-binding mode, but had the arginine inserted in the S1 specificity site.

Diffusion of the SEL2711 and SEL2770 inhibitors into crystals of factor Xa proved unsuccessful, probably because the active site is blocked by a substrate-like intermolecular interaction (Padmanabhan *et al.*, 1993), and co-crystallization attempts with the inhibitors did not produce crystals. This led us to soak the inhibitors into thrombin–hirugen (sulfated Tyr63–*N*-acetyl-hirudin 53–64) crystals in an effort to possibly establish and corroborate the different binding mode in thrombin and then model it as it might occur in factor Xa. We report here the crystal structures of thrombin complexes of SEL2711 and SEL2770 as they may relate to those of factor Xa. The refined structures of the thrombin-inhibited

[1] Selectide Corporation, a subsidiary of Hoechst Marion Roussel.

complexes show that the inhibitors bind in a parallel β-strand manner with Ser214–Gly216, which is markedly different from the binding of peptides A and B determined by NMR (Fraternali *et al.*, 1998). A proposed retro-binding mode of SEL2711 and SEL2770 to factor Xa could account for its much higher specificity for factor Xa compared to thrombin.

Table 1
Specificity of Selectide inhibitors (K_i values in μM).

	SEL2711	SEL2770
Factor Xa	0.003	0.002
Thrombin	40	8
Protein C	10	12
Plasmin	130	60
TF/VII†	>200	>200
Trypsin	112	n/a

† Tissue factor–factor VIIa complex.

2. Experimental

2.1. Crystallization

An approximately tenfold molar excess of hirugen was added to a frozen sample of human α-thrombin solution (1.0 mg ml^{-1} in 0.75 *M* NaCl) at 277 K to form a 1:1 thrombin–hirugen complex and to prevent autocleavage of the enzyme. The solution of the complex was concentrated to about 3.5 mg ml^{-1} using a Centricon 10 concentrator with a molecular-weight cutoff of 10 kDa in a refrigerated centrifuge at 277 K. The thrombin–hirugen complex was crystallized using the hanging-drop crystallization method in a 4 µl drop, consisting of 2 µl of the protein sample and 2 µl of a well solution. The well solution contained 28% PEG (polyethylene glycol) 8000 in 0.1 *M* sodium phosphate buffer pH 7.3. A repetitive macroseeding technique was applied to enlarge the crystals. X-ray diffraction quality crystals with dimensions 0.25 × 0.20 × 0.20 mm were transferred into a protein-free storing drop containing 32% PEG 8000 in 0.1 *M* sodium phosphate buffer pH 7.3. The SEL2711 and SEL2770 inhibitors were soaked into thrombin–hirugen crystals separately. A 1 µl aliquot of a 20 m*M* solution containing the inhibitor was added to the storing drop and 1 µl of the resulting solution was removed from the drop at intervals of 10–12 h for 5 d until the final concentration of inhibitor in the drop was about 15 m*M*.

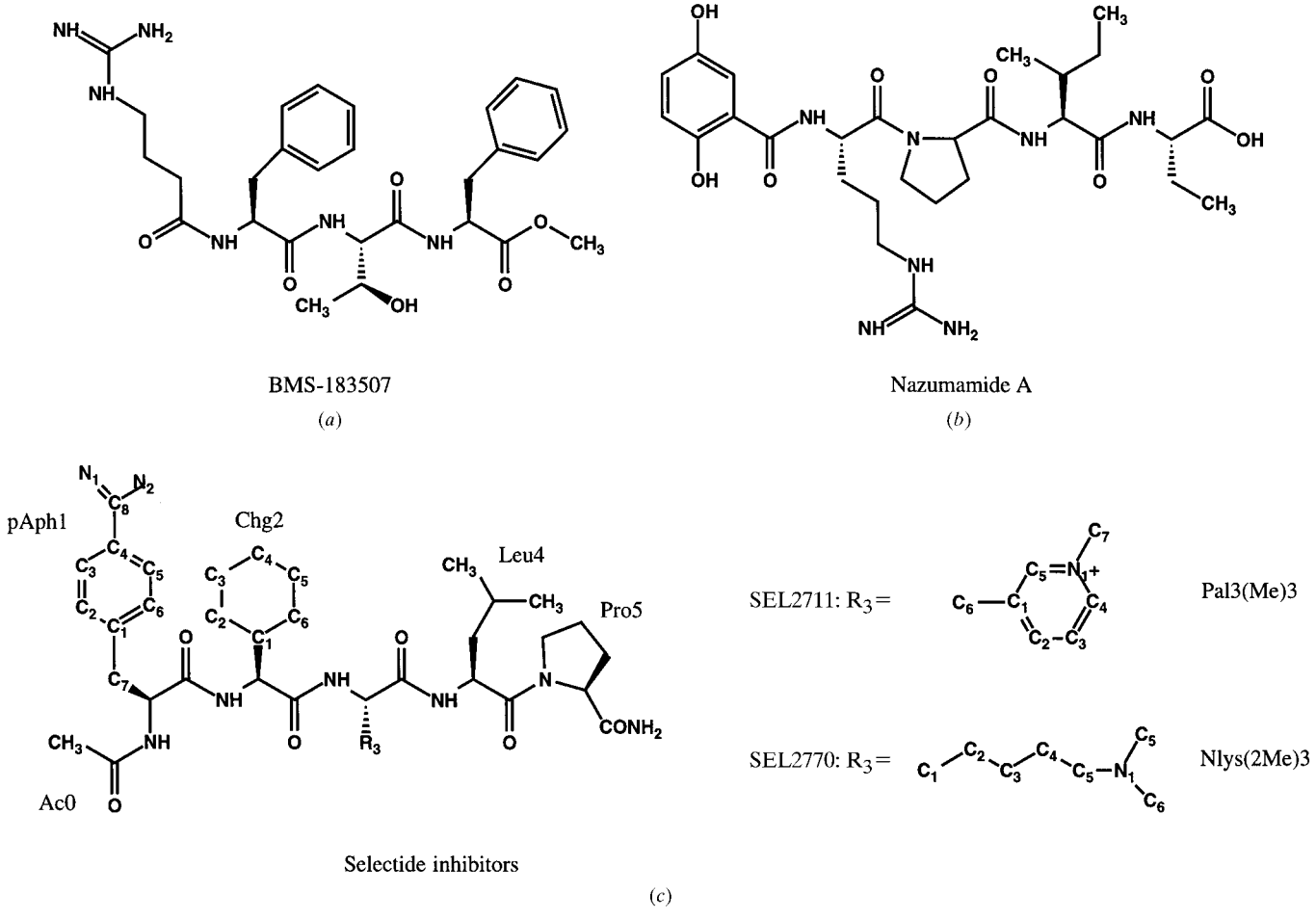

Figure 1
Active-site retro-binding inhibitors of thrombin including SEL2711 and SEL2770.

Table 2
Crystal data and intensity data-collection statistics.

	SEL2711	SEL2770
Space group†	*C*2	*C*2
Unit-cell parameters		
a (Å)	71.05	71.66
b (Å)	71.82	72.00
c (Å)	73.45	73.45
β (°)	101.2	101.2
Resolution (Å)	2.1	2.1
Observations [$I/\sigma(I) > 1.0$]	28464	24962
$I/\sigma(I)$ (outermost range) (2.3–2.1 Å)	3.2	2.3
Independent reflections [$I/\sigma(I) > 1.0$]	17688	13507
Redundancy	1.6	1.8
Completeness (%)	78	60
Outermost range (%)	52	37
$R_{\rm merge}$ (%)	6.9	4.9
Outermost range (%)	14.8	14.3

† Four ternary complexes per unit cell, one per asymmetric unit.

Table 3
Refinement summary of deviations from ideality.

Restraints	Target	SEL2711	SEL2770
Distances (Å)			
Bond distance	0.020	0.011	0.014
Angle distance	0.030	0.037	0.036
Planar 1–4 distance	0.050	0.047	0.046
Planes	0.040	0.034	0.030
Chiral volume (Å^3)	0.15	0.17	0.17
Non-bonded contacts (Å)			
Single torsion	0.60	0.22	0.24
Multiple torsion	0.60	0.25	0.28
Possible hydrogen bond	0.60	0.25	0.28
Isotropic thermal factors (Å^2)			
Main-chain bond	1.0	1.0	0.9
Main-chain angle	1.5	1.7	1.5
Side-chain bond	2.0	2.5	2.1
Side-chain angle	3.0	3.6	3.0

2.2. Intensity data collection

X-ray diffraction data of the ternary SEL2711– and SEL2770–thrombin–hirugen complexes were collected with an R-AXIS II imaging-plate detector. The radiation generated from a Rigaku RU-200 rotating-anode generator operating at 5 kW power with a fine-focus filament (0.3 × 3.0 mm) was monochromated (Cu $K\alpha$) and intensified by focusing with Yale mirrors (Molecular Structure Corp.). The crystal-to-detector distance was 10.0 cm and the detector-swing angle was 0°. Both crystals scattered X-rays to 2.1 Å resolution, although the diffraction pattern of the SEL2770 complex was considerably weaker (Table 2). Autoindexing and processing of the measured intensity data were carried out with the Rigaku *R-AXIS* software package (Higashi, 1990). The intensity data-collection statistics are summarized in Table 2.

2.3. Structure determination

The crystal structure of the SEL2711 complex was determined using thrombin coordinates from the isostructural binary thrombin–hirugen complex (PDB code 1HAH; Vijayalakshmi *et al.*, 1994). The initial coordinates were optimized by rigid-body refinement using the *X-PLOR* program package (Brünger, 1992) to an *R* value of 30.4% (resolution range 7.0–2.5 Å), followed by three cycles of coordinate and overall *B* refinement with a starting *B* value of 30.0 Å^2 using the program *PROFFT* (Hendrickson, 1985; Finzel, 1987), after which the *R* factor was 27.4% . After six cycles of individual temperature-factor refinement, the *R* value converged at 21.6%.

The crystal structure of the SEL2770 complex was solved with the coordinates of thrombin from the refined thrombin–SEL2711 structure as starting coordinates. After three cycles of overall and six cycles of individual *B* refinement, the *R* value was 20.0% (resolution range 7.0–2.5 Å). Both complexes at this stage showed electron density corresponding to hirugen and the active-site inhibitors (which were omitted from calculations) in $2F_o - F_c$ electron-density maps and $F_o - F_c$ difference density maps. In each case, the thrombin model and bound inhibitor were subjected to additional cycles of *PROFFT* refinement. Thereafter, the data were expanded to the resolution range 9.0–2.5 Å and subsequently to 2.1 Å resolution, two Na^+ ions were located and solvent water molecules were progressively found and added by examining difference density maps. The final structure of thrombin complexed with SEL2711 contained 131 molecules of water with occupancies (Ω) > 0.5, *R* = 16.5% (resolution range 9.0–2.1 Å) and $\langle B \rangle$ = 26.8 Å^2. The final SEL2770 structure contained 104 water molecules with $\Omega > 0.5$ refined to *R* = 16.7% (resolution range 9.0–2.1 Å) and $\langle B \rangle$ = 34.8 Å^2. Both complexes also contain two Na^+ ions, one intramolecular and one intermolecular, essentially identical to those previously described (Zhang & Tulinsky, 1997). The final refinement statistics of the complexes are presented in Table 3.

3. Results

3.1. Thrombin

The electron density is well defined for most of the residues of thrombin in both complexes, with the exception of the termini of the A chain (residues 1H–1B, 14K–15) and the autolysis loop (residues 148–149E), which are typically disordered in other isomorphous thrombin structures. The two complexes show no large conformational differences from each other or from other thrombin structures. The r.m.s.d. (root-mean-square deviation) of CA positions between the two structures is 0.25 Å for 253 atom pairs. Comparing the thrombin structures of the ternary complexes with that of the binary thrombin–hirugen complex (PDB code 1HAH) gives r.m.s.d.s of 0.21 and 0.30 Å for the SEL2711 and SEL2770 complexes, respectively. Ramachandran plots of the SEL2711 and SEL2770 complexes show that 196 (81.7%) and 189 (78.8%) residues of the total 240 non-glycine and non-proline amino acids of thrombin, respectively, are in the most favored regions, while 44 (18.3%) and 51 (21.2%) residues, respectively, occupy additionally allowed areas. Two Na^+ ion binding sites, one (intramolecular) responsible for the slow-to-fast

Table 4
Intermolecular and intramolecular interactions of SEL2711 and SEL2770 inhibitors in the active site of the thrombin.

Numbering and nomenclature as used in text.

		SEL2711	SEL2770	Type
Intermolecular				
pAph1 O	Gly216 N	2.91	2.82	H-bond
pAph1 N1	Asp189 OD2	2.89	3.29	H-bond/ion pair
pAph1 N2	Asp189 OD1	3.07	2.95	H-bond/ion pair
pAph1 N1	Ow438/428	3.07	3.04	H-bond
pAph1 N2	Gly219 O	3.28	2.62	H-bond
pAph1 N2	Ala190 O	2.87	3.16	H-bond
Phe227 O	Ow438/428	3.57	3.16	H-bond
Pal3(Me)3 N	Gly216 O	2.69	—	H-bond
Pal3(Me)3 N1	Ow455	3.32	—	Ion/dipole
Nlys(2Me)3 N	Gly216 O	—	3.07	H-bond
Nlys(2Me)3 N1	Tyr60A OH	—	3.60	Ion/dipole
Leu4 O	Ow679	—	2.85	H-bond
Intramolecular				
Ac0 O	Nlys(2Me)3 N1	—	3.43	Ion/dipole
Chg2 O	Nlys(2Me)3 N1	—	3.46	Ion/dipole

kinetic transition (Wells & DiCera, 1992), and the other intermolecular, have been identified in both structures. The Na^+ ions adopt distorted octahedral arrangements coordinated by solvent water molecules and the carbonyl O atoms of Arg221A and Lys224 for the intramolecular ion and from Lys169, Thr172 of one molecule and Phe204A of a symmetry-related molecule for the intermolecular site and are the same as those described previously (Zhang & Tulinsky, 1997). The temperature factors and the occupancies of the Na^+ ions in the SEL2711 complex are $\Omega = 1.00$, $B = 23.1$ Å^2 and $\Omega = 0.84$, $B = 22.9$ Å^2 for the intermolecular ion and the intramolecular ion, respectively, and the temperature factors and occupancies in the SEL 2770 complex are $\Omega = 0.79$, $B = 27.6$ Å^2 and $\Omega = 0.82$, $B = 27.2$ Å^2 for the intermolecular ion and the intramolecular ion, respectively.

3.2. Selectide inhibitors

Both SEL2711 and SEL2770 bind to thrombin in a retro fashion forming a parallel β-strand with residues Ser214–Gly216. The electron density of SEL2711 is well defined, except that there is no electron density corresponding to the C-terminal leucine and proline residues (Fig. 2*a*). The $2F_o - F_c$ electron-density map of SEL2770 has a break at the amide between the L-(*N*,*N*-dimethyl)lysine [Nlys(2Me)3] group[2] and the leucine residue, and although the density corresponding to the C-terminal dipeptide is weaker than the remainder of the inhibitor, it is sufficient to trace the conformations of the leucine and proline (Fig. 2*b*).

As in most crystal structures of thrombin complexed with active-site inhibitors containing an arginine-like residue, the specificity site of the enzyme is occupied by the arginine-like L-4-amidino-phenylalanyl (pAph1) residue in both complexes and utilizes doubly hydrogen-bonded ionic interactions between the amidino group and the side chain of Asp189 (Fig. 3, Table 4). In addition to this hydrogen-bonded salt bridge, one of the N atoms of the amidino group also makes contact with Gly219 O and Ala190 O, while the other N atom forms a hydrogen bond to a water molecule which bridges to Phe227 O (Table 4). The Glu192 residue of thrombin adopts an extended conformation into the solvent region, instead of the more common bent conformation covering the S1 binding site (Vijayalakshmi *et al.*, 1994), and provides space for the L-cyclohexylglycyl (Chg2) residues of SEL2711 and SEL2770 to occupy the proximity of the L-enantiomorphic S3 site of thrombin in a similar way to the P3 aspartate residue of a thrombin–thrombin platelet-receptor peptide complex (Mathews & Tulinsky, 1995). The Chg2 residues of both peptides are in the lower energy chair conformation; however, they differ in orientation around the CA–CB bond by about 90°. The N-terminal acetyl group of the inhibitors is located in the hydrophobic S2 site of thrombin, while the L-3-pyridyl(3-methyl)alanyl [Pal3(Me)3] residue[3] of SEL2711 and Nlys(2Me)3 of SEL2770 are in the aryl D-enantiomorphic S3 site; the pyridyl orientation of the former is approximately orthogonal to the plane of D-phenylalanyl of PPACK in the thrombin–PPACK complex (Bode *et al.*, 1992). In the case of SEL2770, the site is occupied by Nlys(2Me)3 in a partially extended conformation (Fig. 3). The two C-terminal residues, leucine and proline, point into the solvent region, having no interaction with the thrombin molecule and appear flexibly disordered in the SEL2711 complex, although they are reasonably fixed in the SEL2770 complex, high B values notwithstanding (Figs. 2 and 3). This is somewhat contradictory, since crystals of the SEL2711 complex produced a better diffraction pattern (Table 2). Cleavage of the peptide in a substrate-binding mode prior to retro-binding is a possibility, but is highly unlikely with a Pal3(Me)3 group at the P1 specificity position (see also below).

4. Discussion

Because of the structural similarity of SEL2711 and SEL2770, the binding modes of both are similar. While PPACK and many other substrate-like inhibitors show an antiparallel β-strand association with Ser214–Gly216 of thrombin, SEL2711 and SEL2770 form a parallel β-strand with these residues, making hydrogen bonds with the N and carbonyl O atoms of Gly216 (Table 4). The antiparallel β-strand association of PPACK in peptidic thrombin inhibition is more abundant because its hydrogen-bonding pattern is energeti-

[2] Nomenclature and numbering used here is the same as that in Ostrem *et al.* (1998).

[3] For convenience, a slightly different numbering was used here (Fig. 1).

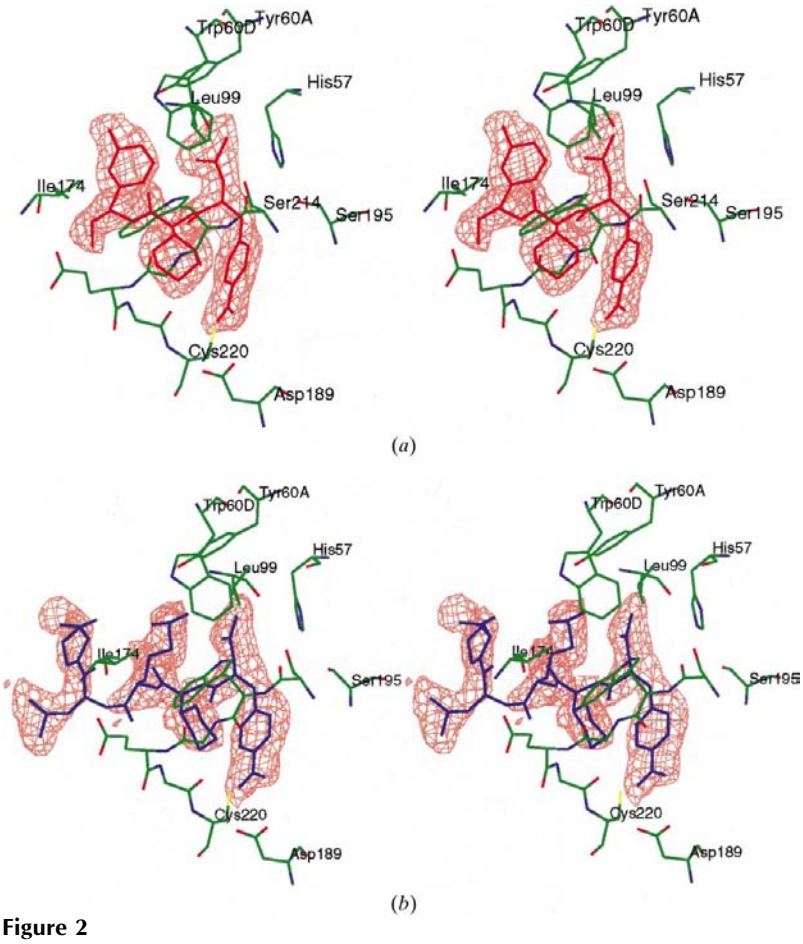

Figure 2
Stereoview of the final $2F_o - F_c$ inhibitor electron density of the thrombin complexes. (*a*) SEL2711; (*b*) SEL2770. Contoured at 1σ level; SEL2711 shown in red; SEL2770 in blue. Thrombin residues are in atom colors: carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow.

cally preferable compared with that in the parallel β-strand alignment of SEL2711 and SEL2770. The parallel β-strand arrangement, however, displays a high degree of fidelity in its positioning in the active site when compared with other known retro-binding thrombin inhibitors.

The largest structural difference between the binding of the two similar inhibitors with thrombin appears at the S3 site of the enzyme, where the more flexible Nlys(2Me)3 group of SEL2770 is able to adopt a favorable conformation in order to interact internally with itself and thrombin (Fig. 3). Superposition of the two inhibited structures shows that the quaternary N atoms of Nlys(2Me)3 and Pal3(Me)3 are 3.3 Å apart. However, in the case of the former, free bond rotations place the positively charged N atom in an electronegative environment consisting of the carbonyl O atoms of the acetyl group and Chg2 O of SEL2770, as well as Tyr60A OH of thrombin (Table 4). If the Nlys(2Me)3 group were fully extended, it could approach within 3.0 Å of the carbonyl O atom of Glu97A. Thus, the ion-dipole interactions of SEL2770 appear to be more stabilizing than this possible hydrogen bond. With much less rotational freedom, the positively charged N atom of Pal3(Me)3 cannot orient in a direction toward the same internal electronegative environment utilized by Nlys(2Me)3 in order to achieve a comparable interaction.[4]

The Chg2 residues of both inhibitors occupy the L-enantiomorphic S3 region of thrombin, similar to the aspartate of Leu–Asp–Pro–Arg in the thrombin–thrombin platelet-receptor peptide complex (Mathews & Tulinsky, 1995). The L-S3 site is surface exposed and of considerable size (Fig. 3); the Chg2 residues do not superpose on the aspartate position of the former complex, but are located adjacent to it, and even appear to be in approximately orthogonal orientations (Fig. 3; χ_1 = 173 and 71° for SEL2711 and SEL2770, respectively). Both the Chg2 groups of SEL2711 and SEL2770 and the aspartate group of thrombin platelet-receptor peptide point into solvent space. The aspartate, however, makes a water-mediated hydrogen bond with Arg221A to stabilize its orientation (Ni *et al.*, 1992; Mathews & Tulinsky, 1995), while the Chg2 groups cover the S1 site and appear to interact with the phenyl pAph1 group in the specificity site and the disulfide bridge of Cys191–220. Thus, the Chg2 groups assume the usual role of Glu192 (bent conformation covering S1) and in the process make close contacts with Cys191 S and Cys220 S, providing a more hydrophobic environment for the latter by expelling some water molecules from the region.

The different backbone associations with thrombin notwithstanding, PPACK and the Selectide inhibitors display similar binding within the S1 specificity pocket. In both structures, two N atoms of the guanidino group (PPACK) and the amidino group (SEL2711 and SEL2770) form a doubly hydrogen-bonded salt bridge with the carboxylate of Asp189. In addition, one of the N atoms also interacts with Gly219 O and the other makes a water-mediated bridge to Phe227 O. Two more water molecules, one bridging from Asp189 OD2 to Tyr228 OH and the other bound to Asp189 OD1, both also present in the apo-thrombin structure, remain in the specificity pocket on binding of the inhibitors. In the thrombin–PPACK structure, an additional water molecule in the specificity pocket mediates an interaction between the arginyl NE atom and Glu192 OE1, while in SEL2711 and SEL2770 this interaction is lost because Glu192 is in the thrombin–hirugen conformation (extended; Vijayalakshmi *et al.*, 1994). The bulkiness of the incoming aromatic ring of pAph1 or that of Chg2 appears to disrupt the usual conformation of Glu192 (bent over the S1 site) which accompanies arginyl binding at the specificity site. Residue Glu192 adopts a fully extended open conformation and does not have any

[4] Pal3(Me)3 N1–Ac0 O1, 5.0 Å; Pal3(Me)3 N1–Chg2 O, 5.1 Å; Pal3(Me)3 N1–Tyr60A OH, 4.7 Å; compare with corresponding Nlys(2Me)3 N1 distances in Table 4.

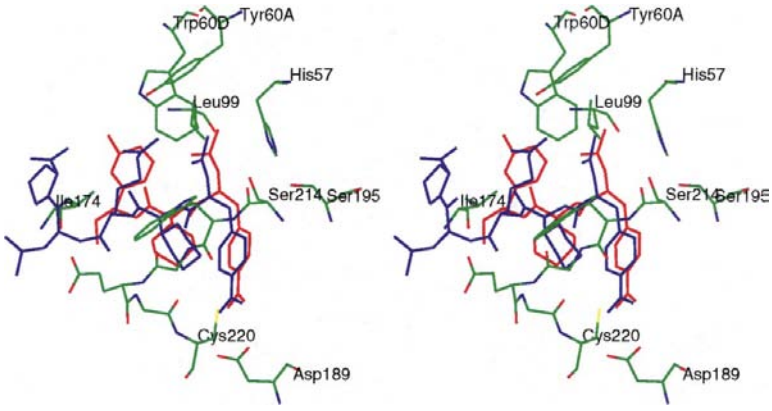

Figure 3
Stereoview of superposed Selectide inhibitors in the active site of thrombin. SEL2711 and SEL2770 are shown in red and blue, respectively; thrombin residues of the SEL2711 complex are in atom colors and numbered.

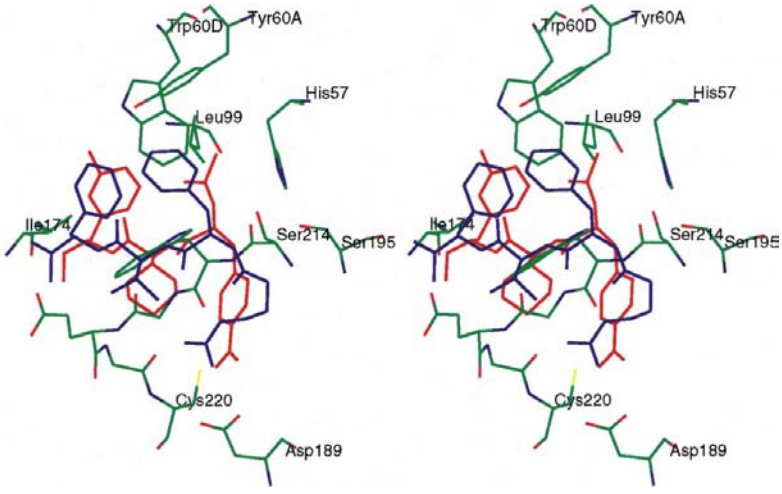

Figure 4
Stereoview of superposed SEL2711 and BMS-183507 bound to thrombin. SEL2711 and BMS-183507 are shown in red and blue, respectively; thrombin residues of the SEL2711 complex are in atom colors and numbered.

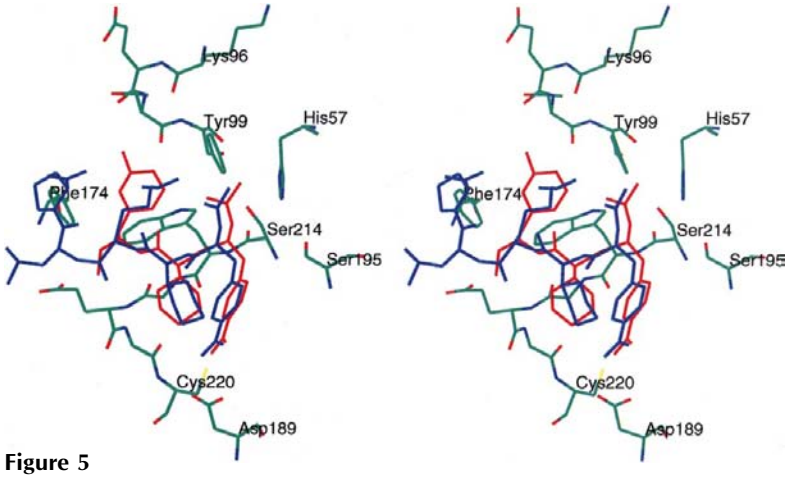

Figure 5
Stereoview of SEL2711 and SEL2770 superposed on the active site of factor Xa in a retro-binding mode. SEL2711 in red, SEL2770 in blue, factor Xa in atom colors and numbered. Since the pyrrolidine of SEL2770 collides with Phe174, a conformational change must occur in one or the other, most likely the former.

specific contacts, being positioned on the surface of thrombin, which allows the P2 Chg2 group of the inhibitors to occupy the same site as the P3 aspartate of the thrombin–thrombin platelet-receptor peptide complex.

Superposition of the Selectide inhibitors and the thrombin retro-binding BMS-183507 inhibitor (PDB code T5161) reveals a practically identical parallel β-strand association of the backbone of the inhibitors with the Ser214–Gly216 residues of thrombin (Fig. 4). Like PPACK, BMS-183507 forms three hydrogen bonds with Ser214 O, Gly216 N and Gly216 O of thrombin, while the Selectide inhibitors form only two (Table 4). The additional hydrogen bond of BMS-183507 is accomplished by the different positioning of the terminal amide group. The arginyl-like group of the BMS inhibitor has one C atom less than an arginyl side chain, so its entrance to the specificity site leads to a bowed conformation, which positions the terminal amide within hydrogen-bonding distance of Ser214 O (Fig. 4). The N-terminal arginyl-like moiety and phenyl group of BMS-183507 bind in the specificity pocket and S2 site, respectively, whereas the S1 site is occupied by the Aph1 group of the Selectide inhibitors while the N-terminal acetyl group is in the S2 position (Fig. 4). In addition, the BMS and Selectide inhibitors display different binding modes within the S1 site. Only one of the N atoms of the guanidine of BMS-183507 makes contact with a carboxylate O atom of Asp189, while the other hydrogen bonds to Gly219 O as in the Selectide inhibitors (Fig. 4; Table 4).

Since the structures of thrombin and factor Xa are similar (r.m.s.d. = 1.2 Å, 198 C^{α}-atom pairs), especially in the active-site regions, it is quite possible that the retro-binding mode of the Selectide inhibitors with thrombin may also be operative with factor Xa. This was explored by transposing the thrombin-bound Selectide inhibitors onto the active site of factor Xa (Fig. 5). The principal sequence differences in the resides surrounding the S1–S3 binding subsites of the two enzymes are Leu99, Ile174 and Glu192 of thrombin, which are replaced by Tyr99, Phe174 and Gln192 in factor Xa. An even larger difference is the absence in factor Xa of the 60-insertion loop of thrombin. The Tyr60A–Trp60D segment of the loop projects out over the S2 and D-enantiomorphic S3 binding sites in thrombin, restricting their access and thus contributing to specificity.

Theoretical considerations have led to the proposal of cation-π-electron-mediated interactions (Dougherty & Stauffer, 1990) in the S4 binding site of factor Xa (Lin & Johnson, 1995), where the π-faces of the three aromatic residues

Tyr99, Phe174 and Trp215 are sufficiently rich in π electrons that they not only form a hydrophobic pocket but also act as a cation-recognition site.[5] Thus, the charge interaction is basically an attraction of a positive charge to the π-electrons of the aromatic rings, alternatively called an ion-quadrupole attraction (Stauffer *et al.*, 1990; Schwabacher *et al.*, 1993). The cation-π-electron-binding hypothesis was modified somewhat with the structure determination of the DX-9065a (Daiichi compound) inhibited structure of factor Xa (Brandstetter *et al.*, 1996), which showed that the above aryl-binding site was further assisted by the carbonyl O atoms of Lys96–Glu97 and the side chain of the latter to form a so-called cation hole which expands the S4 binding-site region. It is noteworthy that the main chain of factor Xa between residues 94–98 is positioned differently in thrombin owing to an insertion in the vicinity of Glu97A of the latter. In the absence of the insertion, Lys96–Glu97 of factor Xa make closer contacts with groups binding at the D-S3 site. It is also noteworthy that the Arg93–Arg101 stretch has been implicated in thrombin–heparin binding (Tulinsky, 1996) by electrostatic potential-energy calculations (Karshikov *et al.*, 1992) and by mutagenesis (Gan *et al.*, 1994; Ye *et al.*, 1994; Sheehan *et al.*, 1994). Thus, under appropriate circumstances, the two sites (S4 and heparin) could interact with each other.

If the Selectide inhibitors bind to factor Xa in a similar retro manner as with thrombin, the positively charged Pal3(Me)3 and Nlys(2Me)3 groups occupy the aryl site formed by Tyr99, Phe174 and Trp215 (Fig. 5). In the case of SEL2711, the Pal3(Me)3 group, modeled as it binds to thrombin, makes edge-to-face interactions with Tyr99 and Phe174 (Fig. 5). The latter aromatic ring systems are both parallel to the pyrrolidine ring of the inhibitor in the DX-9065a–factor Xa complex (Brandstetter *et al.*, 1996). The different orientations could reflect the difference between an aromatic and aliphatic group interacting with the aryl site of factor Xa or could reflect a more accurate determination of orientation of the Pal3(Me)3 group (2.1 Å resolution) compared with the pyrrolidine (3.0 Å resolution). If no conformational changes occur in factor Xa on binding, the positively charged N atoms of the two Selectide inhibitors are too far away (>4.5 Å) to hydrogen bond with the carbonyl O atoms of Lys96–Glu97 of the S4 cation hole. The three residues of the aryl site, however, can interact with the delocalized positive charge of the Pal3(Me)3 ring in a similar way to that described for the Daiichi compound (Brandstetter *et al.*, 1996) to approximate a cation-π-electron-mediated ion-quadrupole attraction (Lin & Johnson, 1995). Since the binding constants of the two Selectide inhibitors are practically the same for factor Xa (Table 1), the Nlys(2Me)3 group most likely achieves a similar cation π-electron-mediated interaction as the Pal3(Me)3 group of SEL2711.

Conversely, if the Selectide inhibitors bind to factor Xa in a substrate-like binding mode, the amidino N atoms of the pAph1 groups of both inhibitors could form hydrogen bonds with Lys96 O and Glu97 O of the S4 cation hole; however, the binding of the Pal3(Me)3 and Nlys(2Me)3 groups in the S1 specificity site is clearly inferior to that of the doubly hydrogen-bonded salt bridge of pAph1 in retro binding. Thus, a retro-binding mode in factor Xa would appear to be more preferable. If the inhibitors bind in a retro manner in both thrombin and factor Xa, the difference of a factor of 1.3×10^4 (SEL2711) and 4×10^3 (SEL2770) in selectivity (Table 1) would arise from the contribution of the Pal3(Me)3 and Nlys(2Me)3 groups binding at the S4–S3 cation-aryl site of factor Xa. This only corresponds to 5–6 kcal mol^{-1} (1 kcal mol^{-1} = 4.18 kJ mol^{-1}), and from all appearances the site can either operate through cation-π-electron-mediated interactions (Lin & Johnson, 1995) or, with a longer inhibitor group to reach Lys96–Glu97, hydrogen bonding could also occur in the cation hole (Brandstetter *et al.*, 1996).

Two other inhibitors developed by Selectide Corp. (peptides A and B mentioned earlier), have been shown using transferred nuclear Overhauser effect NMR spectroscopy (Fraternali *et al.*, 1998) to have a very compact conformation, displaying a severe hydrophobic collapse of the P2–P3 groups in the substrate-binding site of factor Xa, but not resembling the usual substrate-binding mode. The arginine of the peptides enters the specificity S1 site differently to substrate, in an extended but curved conformation, surprisingly forming a doubly hydrogen-bonded salt bridge with Asp189, while the P2 and P3 residues (Ile–Tyr for peptide A, Chg–4-amino-Phe for peptide B), which must be the source of the selectivity for factor Xa, interact together above the S1 site with the S2 site and Tyr99. These bound structures are very different from the retro-binding mode modeled here for SEL2711 and SEL2770, where selectivity for factor Xa appears to arise from a cationic interaction with the S3–S4 sites. The only significant factor which would seem to underlie the binding difference thus appears to center around the S1 specificity site. It has already been indicated that the Pal3(Me)3 and Nlys(2Me)3 groups of SEL2711 and SEL2770 are inferior P1 residues compared with the pAph1 group, and this is the most likely to be the reason why these inhibitors do not bind like peptides A and B. This is also generally in agreement with the weaker binding constants of peptides A and B for factor Xa, which are only 1.6 and 0.3 μM,[6] respectively, and essentially result from side-chain–side-chain hydrogen-bond interactions between inhibitor and enzyme. Thus, two or more different active-site binding modes (including substrate binding) may be operative in factor Xa depending on the exact structure of the inhibitor, the selectivity of which is derived from interactions with the S4 region in the retro-binding case.

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[5] This region also corresponds to the D-enantiomorphic S3 site, which is occupied by D-Phe in the thrombin–PPACK complex. The P4, L-leucine residue of the thrombin–LDPR complex of thrombin platelet-receptor peptide also occupies this site in thrombin (Mathews & Tulinsky, 1995).

[6] However, this is important for the fast exchange of inhibitor required in the NMR work.

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