Protein Science (1996), *5*:221-228. Cambridge University Press. Printed in the USA.

Crystallographic determination of the structures of human α-thrombin complexed with BMS-186282 and BMS-189090

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(RECEIVED September 12, 1995; ACCEPTED November 10, 1995)

Abstract

The crystallographic structures of the ternary complexes of human α-thrombin with hirugen (a sulfated hirudin fragment) and the small-molecule active site thrombin inhibitors BMS-186282 and BMS-189090 have been determined at 2.6 and 2.8 Å. In both cases, the inhibitors, which adopt very similar bound conformations, bind in an antiparallel β-strand arrangement relative to the thrombin main chain in a manner like that reported for PPACK, **D**-Phe-Pro-Arg-CH_2Cl. They do, however, exhibit differences in the binding of the alkyl guanidine moiety in the specificity pocket. Numerous hydrophilic and hydrophobic interactions serve to stabilize the inhibitors in the binding pocket. Although PPACK forms covalent bonds to both serine and the histidine of the catalytic triad of thrombin, neither BMS-186282 nor BMS-189090 bind covalently and only BMS-186282 forms a hydrogen bond to the serine of the catalytic triad. Both inhibitors bind with high affinity (K_i = 79 nM and 3.6 nM, respectively) and are highly selective for thrombin over trypsin and other serine proteases.

Keywords: crystallography; human α-thrombin–inhibitor complexes; structure-based drug design

The serine protease thrombin occupies a central position in the blood coagulation cascade. It cleaves circulating fibrinogen to fibrin monomers, which then polymerize and are covalently linked by thrombin-activating factor XIIIa, which is also a product of thrombin activation. In addition, thrombin activates several other coagulation and plasma factors, such as factors V, VIII, protein C, and protein S. Thrombin also induces platelet aggregation and promotes the proliferation of endothelial cells, the liberation of tissue plasminogen activator, and the contraction and dilation of blood vessels. Selective inhibition of thrombin may result in efficient control of various pathophysiologic states, such as thrombosis and arteriosclerosis, and aid in the prevention of myocardial infarction (Berliner 1992; Fenton, 1986; Fenton et al., 1991).

Human α-thrombin consists of two polypeptide chains, A and B, connected through a single disulfide bond. The A chain has 36 residues (after the loss of a tridecapeptide during activation with prothrombin) and the B chain has 259 residues. The B chain is glycosylated at Asn 60G and contains the active site residues His 57, Asn 102, and Ser 195. The numbering scheme for the thrombin residues in this paper are assigned by homology with chymotrypsin as described by Bode et al. (1989). A suffix, as in

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Abbreviations: BMS-186282, [*S*-(*R**,*R**)]-4-[Aminoiminomethyl)-amino]-*N*-[[1-[3-hydroxy-2-[(2-naphthalenylsulfonyl)amino]-1-oxopropyl]-2-pyrrolidinyl]methyl]butanamide; BMS-189090, [*S*-(*R**,*R**)]-1-(Aminoiminomethyl)-*N*-[[1-[*N*-[(2-naphthalenylsulfonyl)-L-seryl]-2-pyrrolidinyl]-methyl]-3-piperidenecarboxamide; PPACK, **D**-Phe-Pro-Arg-CH_2Cl; MD-805, (2R,4R)-4-methyl-1[N^a-(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidinecarboxylic acid (also called MQPA or Argatroban); NAPAP, N^a-(2-naphthyl-sulfonyl-glycyl)-D-*para*-amidinophenylalanylpiperidine; RMSD, RMS deviation.

Asn 60G above, denotes an insertion in the thrombin sequence relative to that of chymotrypsin.

Although thrombin can be inhibited by a number of different compounds, the development of a direct-acting, specific, active site agent remains a major target of pharmaceutical research (Claeson et al., 1993). A number of small synthetic inhibitors (Balasubramanian et al., 1993; Shuman et al., 1993) have been patterned after the tripeptide sequence Gly-Val-Arg, which is found in fibrinopeptide A immediately N-terminal to the peptide bond (Arg 16–Gly 17) that is cleaved by thrombin. These pseudopeptide inhibitors form hydrogen bonds to the thrombin main chain similar to that found in an **antiparallel** β sheet. Examples include the tripeptide PPACK (Bode et al., 1989, 1992; Skrzypczak-Jankun et al., 1989) and also some of the small inhibitors based on benzamidine and arginine such as MD-805 and NAPAP (Banner & Hadváry, 1991, 1993) (Fig. 1).

Hirudin, a 65-amino acid polypeptide isolated from the medicinal leech, is currently in Phase III clinical trials (Markwardt, 1991; Vandenbos et al., 1993). Hirudin forms a noncovalent, high-affinity ($K_i = 25$ fm) complex with thrombin in which the N-terminus blocks the active site of the enzyme, whereas the C-terminus binds to a site distant from the site of the proteolysis (Rydel et al., 1990, 1991). This "exosite" is associated with regulation of the thrombin activity by fibrinogen. Hirudin fragments (such as the deca- and dodecapeptides from the C-terminus) also inhibit the enzyme (Topol et al., 1993). These polypeptides, like hirudin itself, also form a pair of hydrogen bonds to Gly 216, but the β-strand arrangement is **parallel**. A novel series of tripeptide analogues, typified by BMS-183507, which were initially modeled after the Phe-Pro-Arg peptides but were found to bind to thrombin in the parallel or "retro" fashion observed for hirudin, have also been reported (Iwanowicz et al., 1994; Tabernero et al., 1995).

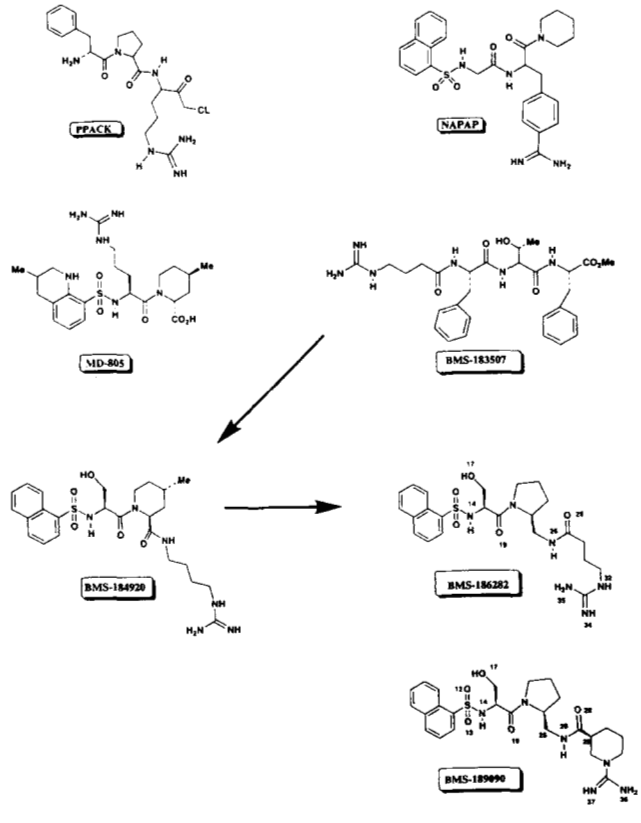

Fig. 1. Structures of PPACK and NAPAP and the development of BMS-186282 and BMS-189090 from MD-805 and BMS-183507.

Our recent efforts in this area have centered on identification of a novel inhibitor that incorporates the critical pharmacophores of known inhibitors such as MD-805, **D**-Phe-Pro-Arg analogues, and the BMS-183507 inhibitors (Kimball et al., 1994). SAR studies in this series led to BMS-184920 as an early lead compound. Replacement of the piperidine ring with a pyrrolidine ring and reversal of the amide bond connecting the pyrrolidine ring and the guanidine function led to BMS-186282, with a sevenfold improvement in activity. Optimization of the side-chain length and replacement of the alkyl side chain in BMS-186282 with a conformationally constrained ring system led to BMS-189090, one of the most potent and selective thrombin inhibitors reported (Fig. 1; Table 1).

The crystallographic structures of the ternary complexes of α-thrombin and hirugen in complex with BMS-186282 and BMS-189090 have been determined in an effort to explain the structure–activity relationships of these compounds, including the 20-fold difference in activity between the two, and to facilitate the design of new direct acting inhibitors.

Results and discussion

We distinguish between two general classes of thrombin active site inhibitors by their modes of binding. Inhibitors based on PPACK, MD-805, and NAPAP have interactions in each of the primary specificity pockets: hydrophobic groups are inserted into both the **P** (proximal or S_2 apolar hydrophobic pocket) and **D** (distal or aryl binding) pockets, and a guanidine group forms strong hydrogen bonds to the side chain of Asp 189 in the specificity pocket. The backbone of the inhibitor lies alongside that of the extended thrombin segment Ser 214–Gly 216 and forms a pair of hydrogen bonds to Gly 216 in a manner typical of an **antiparallel** β-strand. The oxyanion hole is empty. This antiparallel motif is observed commonly in the binding of substrates to other serine proteases and is also observed in the structure of a human fibrinogen fragment bound to bovine thrombin (Martin et al., 1992).

In contrast, the three N-terminal residues of the natural substrate hirudin (Grütter et al., 1990; Rydel et al., 1990, 1991) bind to the thrombin active site in a different fashion. Although they also form a pair of hydrogen bonds to Gly 216, the alignment of the backbone relative to the Ser 214–Glu 217 segment is **par-**

Table 1. *Enzyme selectivity of BMS-186282 and BMS-189090 K_i values for selected serine proteases (relative to thrombin)*

	Thrombin	Trypsin	Plasmin	tPA	Factor Xa
186282	78.9 nM (1)	0.972 μM (12)	106 μM (1,300)	>157 μM (>2,000)	63.7 μM (800)
189090	3.64 nM (1)	47.0 μM (13,000)	>165 μM (>45,000)	>157 μM (>43,000)	4.7 μM (1,300)

allel and the pair of hydrogen bonds forms a **parallel** β-strand to the thrombin main chain. Hydrophobic groups exist in both the **P** and **D** pockets, but the basic side chain that binds in the Asp 189 specificity pocket is missing.

The binding of the small molecule inhibitor BMS-183507 (Tabernero et al., 1995) exhibits some of the features of each class. In this complex, the Phe 1-*allo* Thr 2-Phe 3 portion of the inhibitor binds like the first three N-terminal residues of hirudin with the inhibitor backbone forming a parallel β-strand to the main-chain segment Ser 214–Gly 219. The Phe 1 and Phe 3 side chains bind in the **P** and **D** pockets, respectively, whereas the *allo*-Thr 2 side chain lies above Gly 219. The alkyl guanidine group binds in the specificity pocket forming two hydrogen bonds; one of the terminal nitrogen atoms is hydrogen bonded to a carboxylate oxygen of Asp 189 and the second terminal nitrogen forms a hydrogen bond to the carbonyl of Gly 219. Also, the secondary nitrogen of the guanidine group is involved in an intramolecular hydrogen bond with the carbonyl oxygen of Phe 1, resulting in a bent conformation of the alkyl guanidine. There are no specific interactions between the residues of the catalytic triad and the inhibitor, and a water molecule is hydrogen bonded to the side chain of Ser 195.

The crystal structures of the ternary complexes described here demonstrate that both BMS-186282 and BMS-189090 fall into the class of inhibitors that bind in an **antiparallel** fashion and share a number features common to those reported previously for the complexes with PPACK, MD-805, and NAPAP. The bound

Table 2. *Hydrogen bond contacts for BMS-186282 and BMS-189090*

Atom	Atom	Residue	Distance (Å)
BMS-186282			
N14	O	Gly 216	2.8
O19	N	Gly 216	3.2
O17	Oε1	Glu 192	3.2
O12	H_2O	602	3.2
N26	O	Ser 214	3.2
N26	Nε2	His 57	3.6
O28	Oγ	Ser 195	3.3
N34	H_2O	601	2.5
N35	O	Gly 219	2.7
N35	Oδ1	Asp 189	3.1
BMS-189090			
N14	O	Gly 216	2.7
O19	N	Gly 216	3.1
O12	H_2O	602	3.6
O17	H_2O	602	3.1
O17	Oγ	Ser 11	3.5
N26	O	Ser 214	2.7
N26	Nε2	His 57	3.1
N36	O	Gly 219	2.8
N36	H_2O	601	3.3
N36	O	Ala 190	3.0
N37	O	Gly 219	2.8
N37	O17	BMS189090	3.1

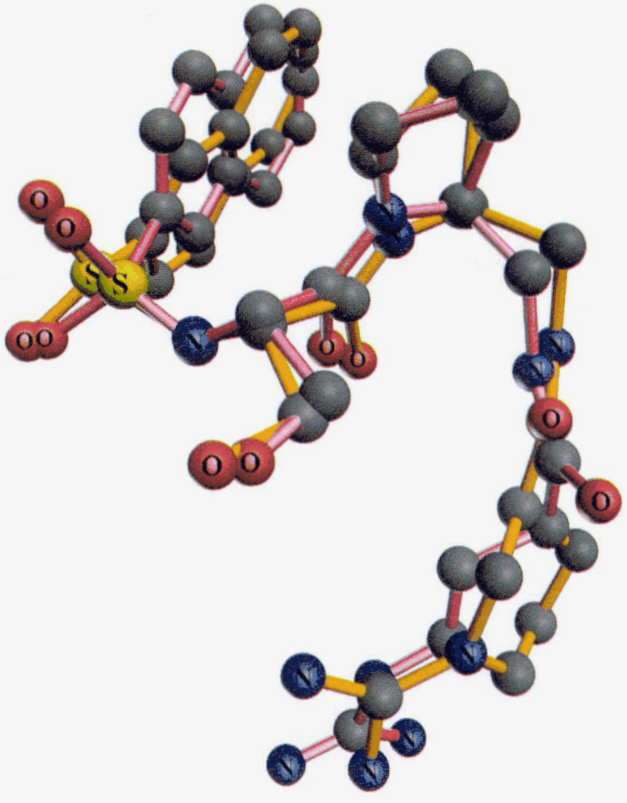

Fig. 2. Superimposition of the bound conformations of BMS-186282 and BMS-189090.

conformations of BMS-186282 and BMS-189090 are remarkably similar to each other (Figs. 2, 3 and Kinemage 1; Table 2):

1. The BMS inhibitors bind with the peptide bond facing the catalytic triad and the sulfonamide oxygens exposed to the opening of the active site cleft. Numerous close hydrogen bonding and hydrophobic contacts to the protein serve to stabilize the inhibitor in the active site.

2. For both of the BMS inhibitors, the backbone lies next to the extended thrombin segment Ser 214–Glu 217 and forms a pair of hydrogen bonds to Gly 216 in a manner typical of antiparallel β-strands. Both inhibitors form a hydrogen bond to the carbonyl oxygen of Ser 214.

3. The naphthalene group of the inhibitor lies in the **D** pocket formed by the side chains of Trp 215, Ile 174, and Glu 97A–Leu 99.

4. The proline groups of the inhibitors bind in the **P**-pocket, making contacts with Tyr 60A and Trp 60D.

5. The alkyl guanidine forms hydrogen bonds with the carbonyl oxygen of Gly 219. In BMS-186282, only N35 is involved in this hydrogen bond, but in BMS-189090, there is a bidentate hydrogen bonding arrangement wherein both of the alkyl guanidine nitrogens form hydrogen bonds to the carbonyl oxygen.

Although the binding of the two inhibitors to the active site residues of α-thrombin is very similar, there are some significant differences in their conformations:

1. In the BMS-189090 complex, the hydroxyl, O17, is hydrogen bonded to one of the waters of hydration, which is in turn hydrogen bonded to one of the sulfonamide oxygens and to the nitrogen of Gly 219. This hydroxyl group also forms a hydro-

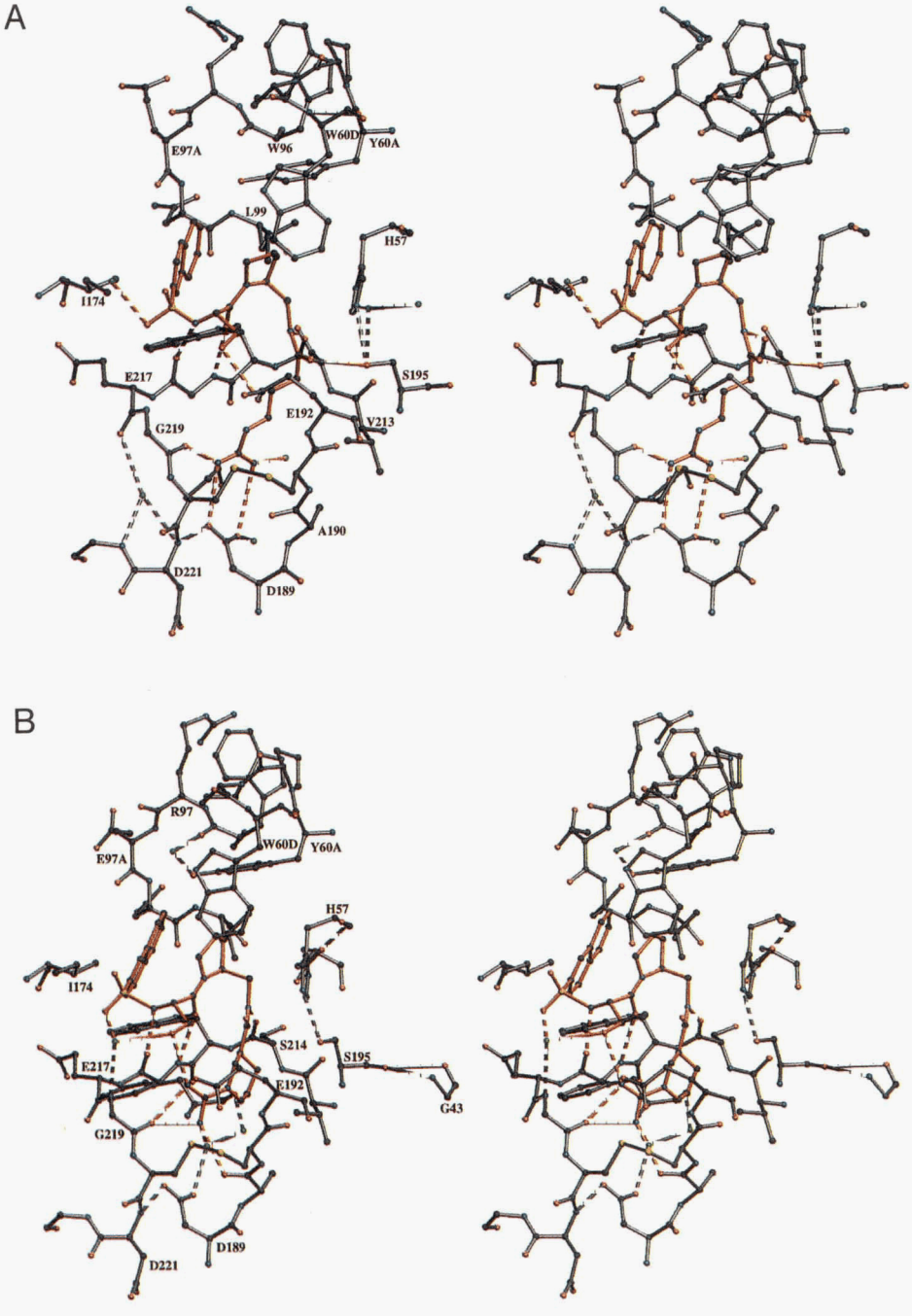

Fig. 3. Final three-dimensional structure of α-thrombin complexed with (**A**) BMS-186282 and (**B**) BMS-189090, showing the bound conformation of the inhibitor, the active site residues, and solvent molecules in the active site.

gen bond to Ser 11 of a symmetry related molecule. In the BMS-186282 complex, this hydroxyl group is hydrogen bonded to one of the side-chain oxygens of Glu 192, whereas the sulfonamide oxygen is hydrogen bonded to a water of hydration. In the BMS-189090 complex, Glu 192 is turned away and forms a hydrogen bond to the side-chain atoms of Thr 147.

2. The cyclic side chain in BMS-189090 changes the orientation of the carbonyl oxygen O28. In the BMS-186282 complex, O28 is hydrogen bonded to Oγ of Ser 195, but in the BMS-189090 complex, this oxygen does not participate in any hydrogen bonding interactions.

3. The cyclic side chain in BMS-189090 results in approximately a 2-Å shortening in length of the alkyl guanidine moiety relative to BMS-186282. As a consequence, the two inhibitors exhibit differences in the details of their binding to the side-chain atoms of the specificity pocket defined by residues Asp 189–Ser 195. In the BMS-186282 complex, one of the guanidine nitrogens, N34, is hydrogen bonded to one of the waters of

hydration, whereas the other nitrogen, N35, forms hydrogen bonds to the carbonyl oxygen of Gly 219 and to one of the side chain oxygens of Asp 189. There are no hydrogen bonds to N32. In BMS-189090, **both** of the nitrogens form hydrogen bonds to the carbonyl oxygen of Gly 219. In addition, N36 forms hydrogen bonds to one of the waters in the active site and the carbonyl oxygen of Ala 190, whereas N37 is hydrogen bonded to the hydroxyl O17.

In the previously reported structures of α-thrombin complexed with PPACK (Bode et al., 1992), MD-805, NAPAP (Banner & Hadváry, 1991), and a series of inhibitors related to Ro 46-6240 (Hilpert et al., 1994), the alkyl guanidine group of the inhibitors formed at least one hydrogen bond with the side-chain oxygens of Asp 189. A recent report (Weber et al., 1995) of the amidine, lysine, homolysine, and ornithine analogues of Ac-(**D**)Phe-Pro-boroArg-OH described the hydrogen bonding arrangements of these basic side chains with the side-chain atoms of Asp 189.

In the BMS-186282 complex, the side-chain oxygens of Asp 189 form hydrogen bonds to a water molecule, the amide nitrogen of Asp 221 and one of the guanidine nitrogen atoms, but in the BMS-189090 complex (as was the case for the lysine and ornithine analogues of Ac-(**D**)Phe-Pro-boroArg-OH), there are no hydrogen bonds between the inhibitor and the side-chain atoms of Asp 189. In the BMS-189090 complex, the side-chain atoms of Asp 189 form hydrogen bonds to a water of hydration, the amide nitrogen of Ala 190 and the amide nitrogen of Asp 221.

The thrombin residues occupy approximately the same positions in the active sites of both of these ternary complexes. The most obvious differences are the displacements of the side chains of Trp 60D and Glu 192. In the complex with BMS-186282, Glu 192 forms a hydrogen bond with the hydroxyl O17, but in the BMS-189090 complex, the side-chain atoms of Glu 192 are swung away and the hydroxyl forms a hydrogen bond with a water molecule. In the BMS-189090 complex, the space left in the specificity pocket by the shortening of the inhibitor side chain is filled by a water molecule, which is positioned about where N34 is in the BMS-186282 complex. This is the water molecule that is hydrogen bonded to Asp 189.

The other major difference involves the interactions of the inhibitors with the residues of the catalytic triad. In the thrombin-PPACK complex, there are covalent bonds formed to both Ser 195 and His 57. In BMS-186282, the carboxyl oxygen O28 is hydrogen bonded to Oγ of Ser 195, which is in turn hydrogen bonded to Nε2 of His 57 (3.2Å). The N26-Nε2 (His 57) distance is 3.6 Å. In the BMS-189090 complex, the N26-Nε2 (His 57) distance is 3.1 Å, and the inhibitor does not form a hydrogen bond to the side chain of Ser 195.

The crystal structures of the complexes of BMS-186282 and BMS-189090 with human α-thrombin support the concept that effective inhibitors form numerous close contacts (both hydrogen bonded and hydrophobic) with the protein. The antiparallel sheet arrangement between the inhibitors and the thrombin backbone segment Ser 214–Gly 219 is virtually identical. Both inhibitors form hydrogen bonds to the histidine of the catalytic triad, and in the complex with BMS-186282, the carbonyl oxygen of the inhibitor forms a hydrogen bond to the hydroxyl group of the catalytic serine 195. Although the alkyl guanidine groups of both BMS-186282 and BMS-189090 form hydrogen bonds in the specificity pocket, there does not seem to be a requirement that the hydrogen bonds be with the side-chain atoms of Asp-189, as demonstrated in the complex with BMS-189090. In fact, other interactions within the pocket more than compensate for the lack of direct interactions with Asp 189 and, by means that are not entirely clear, make BMS-189090 a 20-fold stronger inhibitor than BMS-186282.

Materials and methods

Enzyme assays

Enzymatic activity of human thrombin (Sigma) was measured in a buffer containing 0.145 M NaCl, 0.005 M KCl, 1 mg/mL polyethylene glycol (PEG-8000), 0.030 M HEPES, pH 7.4, and 0.03 U/mL final thrombin concentration using a microtiter plate based assay (Balasubramanian et al., 1993). The enzyme was incubated at room temperature with the inhibitor for 3 min prior to starting the reaction with 10 μM S-2238 (**D**-Phe-Pip-Arg-pNA, $K_m = 2.54$ μM). Time-dependent optical density change was followed at 405 nm using a microplate reader (Molecular Devices UV_{max}) at room temperature. The IC_{50} values obtained were the average of at least four determinations performed in duplicate.

The K_i values were determined from the IC_{50} values (Cheng & Prusoff, 1973) using experimentally-determined K_m values.

Bovine pancreatic trypsin (Sigma) was assayed as for thrombin except that the buffer was 2 mM $CaCl_2$, 50 mM Tris/Cl, pH 8.0, and the reaction was started with 100 μM Chromozym-TRY (Carboxybenzoxy-Val-Gly-Arg-pNA, $K_m = 46$ μM). Human plasmin (KabiVitrum) was measured in 50 mM Tris/Cl, pH 7.8, and the reaction was started with 100 μM S-2251 (D-Val-Leu-Lys-pNA, $K_m = 98$ μM). Recombinant tissue plasminogen activator (Genetech) and human Factor Xa (KabiVitrum) were assayed in the same buffer as thrombin and the reactions were started with 100 μM spectrozyme t-PA (methylsulfonyl-D-cyclohexyltyrosyl-Gly-Arg-pNA, $K_m = 90$ μM) and 100 μM S-2222 (phenyl-Ile-Glu-Gly-Arg-pNA, $K_m = 87$ μM), respectively.

Crystallization and data collection

Human α-thrombin obtained in buffer (50 mM sodium citrate, 0.2 M NaCl, 0.1% PEG 8000, pH 6.5) from Enzyme Research Laboratories, Inc. (South Bend, Indiana) was used to make the inhibitor complex without further purification. Hirugen, which is a sulfated hirudin fragment (residues 54–65), was obtained from Bachem Biosciences, Inc. (Philadelphia, Pennsylvania). This peptide, with the sequence (H-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3H)-Leu-Gln-OH), was added to bind noncovalently to the fibrinogen-binding exosite of the thrombin molecule in order to inhibit autocatalytic cleavage of the Arg 77-Asn 78 bond of α-thrombin during crystallization. The hirugen was dissolved to 1.25 mg/mL in 0.1M HEPES buffer pH 7.3 and stored in frozen aliquots at −20 °C to be used in subsequent experiments.

BMS-186282 and BMS-189090 were provided by the thrombin active-site project team in Discovery Chemistry of the Bristol-Myers Squibb Pharmaceutical Research Institute in the form of partially hydrated lyophilized trifluoracetic acid salts, which were dissolved in 0.1 M HEPES, pH 7.3, for use as a 4.0 mg/mL stock solution. Aliquots of the inhibitor stock solutions were also stored at −20 °C for later use. Both the hirugen and the inhibitor were added directly to the thawing thrombin to give ap-

proximately 10 times molar excess of both compounds. The reaction was incubated on ice with intermittent mixing for at least 1 h before concentrating the sample. Additional HEPES buffer was added (approximately 1:1, v/v) to complete the exchange of the shipping buffer of the thrombin during concentration in an Amicon centricon microconcentrator (10,000 MW cutoff). More hirugen and inhibitor were added after the initial concentration to ensure excess of the compounds. Concentration of the protein complex mixture was suspended at approximately 3–6 mg/mL; final buffer is approximately 50–75 mM HEPES, pH 7.3, 30 mM NaCl, with the active site inhibitor in a least fourfold excess, and hirugen in at least twofold excess.

Crystallization of the thrombin inhibitor complexes was carried out at room temperature by the hanging drop vapor diffusion method (McPherson, 1982) in Linbro tissue culture plates (ICN Biomedicals, Aurora, Ohio). Crystals were obtained from solutions containing the protein complex, mixed with an equal volume of the reservoir solution containing 18–20% (w/v) PEG 8000 (EM Science, Gibbstown, New Jersey), 40 mM sodium acetate, pH 3.0, 10 mM magnesium chloride or lithium chloride. This crystallization procedure produced small, rod-shaped crystals within a few weeks. However, macroseeding was necessary to obtain crystals of a size adequate for the diffraction experiment. Crystals with dimensions up to 0.25 mm grew within three weeks after seeding.

Crystals were mounted in thin-walled glass capillaries for data collection. X-ray diffraction data were measured at room temperature with a Siemens X-1000 area detector system and mirror-monochromatic Cu Kα radiation generated by a Rigaku RU2000 X-ray generator operating at 3 kW. Frames were processed with the XDS program (Kabsch, 1988a, 1988b). Details of the data collection and reduction are given in Table 3.

Table 3. *Some details of data collection and reduction*

	BMS-186282	BMS-189090
Swing angle (2θ) (°)	15	15
Detector distance (mm)	100	140
Frame width (°)	0.20	0.25
No. of frames	634	360
No. seconds/frame	120	180
R_{sym}, all data ($I > 0\sigma$)	10.6	6.6
R_{sym}, all data ($I > 3\sigma$)	6.7	5.2
Completeness (all data) (%)	75.6	74.5
Maximum resolution observed	2.6	2.8
No. reflections measured	24,598	12,793
No. unique reflections	10,811	9,687
Space group	$P2_12_12$	$P2_12_12$
a (Å)	108.17	167.78
b (Å)	81.22	49.04
c (Å)	46.00	43.79
V_m (Å^3/Da)	2.32	2.07
No. of reflections in refinement	9,341	8,053
Crystallographic R factor	17.7	16.2
RMSD in bond distance	0.013	0.011
RMSD in bond angle	1.75	1.68
RMSD in improper angle	1.58	1.43

R_{sym} is defined as: $\left(\Sigma\left(ABS(I(h,i) - I(h))\right)\right)/\left(\Sigma(I(h,i))\right)$.

The crystals containing BMS-186282 are isomorphous with crystals containing BMS-183507, LY288570 (Chirgadze et al., 1992), and the "OR1" form of PPACK (Banner & Hadváry, 1993). However, crystals containing BMS-189090 or its diastereomer (at C29) mixture, BMS-188311, grew in a form that has not been reported previously.

Structure solution and refinement

The method of molecular replacement (Rossmann, 1972) was used to determine the structure. The atomic model of human thrombin (Bode et al., 1989), without inhibitor or the hirudin included, was used as the starting model. The MERLOT (Fitzgerald, 1988) molecular replacement procedure gave single solutions with initial R values of 58% and 52% for BMS-186282 and BMS-189090, respectively. The initial positions of the inhibitors was immediately apparent in both the ($|F_o - F_c|$, α_{calc}) and the ($|2F_o - F_c|$, α_{calc}) electron density maps.

The coordinates for the hirudin peptide were extracted from the C-terminal region of the thrombin–hirugen structure (Rydel et al., 1990) and added to the thrombin model with the computer graphics program CHAIN (Sack, 1988). Two models of the BMS-186282 complex were constructed by Dr. Wan Lau as described previously (Tabernero et al., 1995). Refinement of both models of the bound conformation of BMS-186282 were attempted; one of these models could not be made to fit the observed density and was discarded. The initial model of BMS-189090 was constructed by modifying the refined BMS-186282 model.

Refinement was performed with X-PLOR (Brünger et al., 1987; Brünger, 1989), version 3.1, using the force field parameters developed by Engh and Huber (1991). In this procedure, both conventional conjugate gradient minimization and simulated annealing refinements were done. The temperature factors for all atoms in the initial model were set to 18 Å^2. For refinement, all reflections greater than 2σ were used, although the higher-resolution shells were incomplete. The structure was subjected to a 200-step conjugate gradient minimization, a simulated annealing dynamics run in 50 K steps from 3,000 to 300 K, and finally a 25-step individual B-factor refinement. Electron density maps, including $|F_o - F_c|$, a $|2F_o - F_c|$, and omit (a $|2F_o - F_c|$ map, where the inhibitor contribution is left out of the calculation) maps, were examined after each stage of refinement with the program CHAIN on a Silicon Graphics Indigo Elan system, and manual adjustments to the model were made when necessary.

Apart from some disordered surface features, the B-values vary slowly and smoothly throughout the structures. Water molecules, modeled as single oxygen atoms, were added manually with CHAIN to areas that had electron density on both the $|F_o - F_c|$ and $|2F_o - F_c|$ maps, and where the water molecule introduced would form hydrogen bonds with the protein. The positions and temperature factors of the water molecules were refined, reexamined, and recycled. The largest temperature factor allowed for a water molecule was 45 Å^2. The omit maps for the final refinements are shown in Figure 4.

The refined model for the BMS-186282 complex contained 28 residues (of 36) for the thrombin A chain, 258 residues (of 259) for the thrombin B chain, 8 residues (of 11) for the hirugen peptide, the inhibitor, and 42 water molecules, for a total of 2,472 non-hydrogen atoms. The model for the BMS-189090 complex

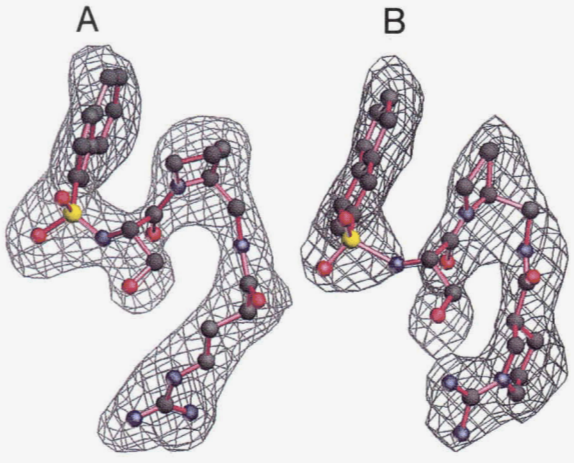

Fig. 4. Final $|2F_o - F_c|$ omit electron density maps at the active site of α-thrombin complexed with (**A**) BMS-186282 and (**B**) BMS-189090 superimposed with the final three-dimensional structures of the inhibitors. Electron density maps were calculated using X-PLOR (Brünger, 1989).

contained 26 residues for the thrombin A chain, 257 residues for the thrombin B chain, 8 residues for the hirugen peptide, the inhibitor, and 45 water molecules, for a total of 2,415 non-hydrogen atoms. The residues at the beginning of the A chain, the end of the B chain, and the end of the hirudin peptide were poorly defined in density and were omitted from the refinements of both complexes. The atomic coordinates have been deposited in the Protein Data Bank (Bernstein et al., 1977) as IBMM and IBMN.

The overall structure of thrombin and the binding of the hirugen are substantially similar to that described in previous reports (Bode et al., 1989, 1992; Rydel et al., 1990, 1991; Skrzypczak-Jankun et al., 1991). The Ramachandram plot shows two residues at the beginning of the thrombin A chain of the BMS-186282 complex (Thr 1C and Ala 1B) in unfavorable conformations.

The most problematic feature in refinement of both of these structures has been the position of the γ-cleavage loop, Glu 146–Lys 149E. In the BMS-186282 complex, electron density in this region is well defined except for the side chain of Trp 148, but in the BMS-189090 complex, electron density for most of the residues of this loop is poorly defined. In both crystal forms, this loop is involved in intermolecular contacts to the residues of the thrombin A chain with a symmetry-related molecule.

Although the electron density in the region His 57–Asn 60G is well defined for both inhibitor complexes, no interpretable residual density is seen for the carbohydrate attached to the Asn 60G side chain.

Acknowledgments

We are indebted to the members of the thrombin active site project team, which is a collaborative effort between Cardiovascular Chemistry, led by Dr. S. David Kimball; Cardiovascular Biochemistry, led by Dr. S. Seiler, and Dr. Wan Lau of the Computer Assisted Drug Design group. We acknowledge the unfailing support of Dr. Jack Z. Gougoutas, whose graphics program ALEX was used to draw the illustrations of the active site binding. Dr. Gougoutas and Dr. Howard Einspahr are thanked for their helpful discussions of the manuscript.

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