Crystal structure of two new bifunctional nonsubstrate type thrombin inhibitors complexed with human α -thrombin*

J. FÉTHIÈRE, YUKO TSUDA, RENÉ COULOMBE,
YASUO KONISHI AND MIROSLAW CYGLER

Biotechnology Research Institute, National Research Council of Canada, Montréal,
Québec H4P 2R2, and Montreal Joint Centre for Structural Biology, Canada

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Abstract

The crystal structures of two new thrombin inhibitors, P498 and P500, complexed with human α -thrombin have been determined at 2.0 Å resolution and refined to crystallographic *R*-factors of 0.170 and 0.169, respectively. These compounds, with picomolar binding constants, belong to a family of potent bifunctional inhibitors that bind thrombin at two remote sites: the active site and the fibrinogen recognition exosite (FRE). The inhibitors incorporate a nonsubstrate type active site binding fragment: Dansyl-Arg-(D)Pipicolinic acid (Dns-Arg-(D)Pip), reminiscent of the active-site directed inhibitors MD-805 and MQPA, rendering them resistant to thrombin-induced hydrolysis. The FRE binding fragment of these inhibitors corresponds to the hirudin^{55–65} sequence. They differ in the chemical nature of the nonpeptidyl linker bridging these two functional activities. In both cases, the active site binding fragment is well defined in the electron density. The Dns^{H1}, Arg^{H2}, and (D)Pip^{H3} groups occupy the S3, S1, and S2 subsites of thrombin, respectively, in a way similar to that observed in the thrombin–MQPA complexes. Binding in the active site of thrombin is characterized by numerous van der Waals contacts and ring–ring system interactions. Unlike in the substrate-like inhibitors, Arg^{H2} enters the S1 specificity pocket from the P2 position and adopts a bent conformation to make an hydrogen bond to the carboxylate of Asp¹⁸⁹. In this noncanonical position, its carbonyl points away from the oxyanion hole, which is now occupied by well-ordered solvent molecules. The linkers fit in the groove extending from the active site to the FRE. The C-terminal fragments of both inhibitors bind in the same way as analogous FRE binding elements in previously described complexes.

Keywords: arginine methyl esters, bifunctional inhibitors, crystal structure, thrombin.

Cardiovascular accidents are the major cause of mortality in industrialized countries. Most of these are caused by thrombosis leading to blockage of coronary arteries by a thrombus. Thrombin (EC 3.4.21.5) plays a key role in the blood coagulation cascade (Fenton, 1981; Verstraete, 1993); it is the last protease in this cascade and is responsible for catalyzing the conversion of fibrinogen to fibrin. Natural anticoagulants [tissue plasmino-

gen activator (tPA), urokinase, or streptokinase], which are used clinically as thrombolytics (Coller, 1990; Cairns et al., 1992; Lubin et al., 1992; Tapparelli et al., 1993), act indirectly to inhibit thrombin and are known to induce acute thrombotic reocclusion (Marder, 1988; Verstraete, 1990). The heparin-antithrombin III complex has been the most commonly used therapeutic agent for the treatment of coagulation disorders. Unfortunately, the thrombus-bound thrombin is poorly accessible to the heparin-antithrombin III complex (Angelli et al., 1991), thus limiting the use of such a strategy. These limitations of the existing therapeutic agents have provided the impetus for an intensive search for better anticoagulant agents devoid of the undesirable effects. One can find the current status of the development of antithrombotic compounds in recent reviews (e.g., Lefkovits & Topol, 1994; Stone, 1995; Verstraete & Zoldhelyi, 1995).

Hirudin, the most potent anticoagulant known to date, binds to thrombin in a 1:1 stoichiometry with a K_d of 2.2×10^{-14} M (Stone & Hofsteenge, 1986). It is more effective than the previ-

Reprint requests to: Mirosław Cygler, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, Québec H4P 2R2, Canada; e-mail: mirek.cygler@bri.nrc.ca.
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Abbreviations: MD-805, (2R,4R)-4-methyl-1-[N α -[(3-methyl-1,2,3,4-tetrahydro-8-quinolyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; MQPA, (2R,4R)-4-methyl-1-[N α -[(RS)-3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl]-L-arginyl]-2-piperidine carboxylic acid; PPACK, (D)Phe-Pro-Arg-chloromethyl ketone; μ Adod, 12-aminododecanoic acid; δ Ape, 5-aminopentanoic acid; BAla, β -alanine; Dns-Arg-(D)Pip, dansyl-Arg-(D)Pipicolinic acid.

ously mentioned natural anticoagulants in preventing reocclusion due to its greater accessibility to the thrombus-bound thrombin, (Badimon et al., 1991; Sawyer, 1991) and is a weak antigen (Markwardt et al., 1984). The high resolution structure of the hirudin–thrombin complex provided molecular details on the binding mode of this natural anticoagulant (Grütter et al., 1990; Rydel et al., 1990; Rydel & Tulinsky, 1991). Hirudin interacts simultaneously with two sites on thrombin: the active site and the fibrinogen recognition exosite (FRE). The compact N-terminal fragment (hirudin^{1–48}) blocks access to the active site and the acidic C-terminal fragment (hirudin^{55–65}) shields the FRE; both regions are joined by a linker segment (hirudin^{49–54}) that follows a long groove extending from the active site, making numerous contacts with the thrombin S' subsites.

Hirudin, and, in particular, the hirudin^{45–65} fragment (Krstenansky & Mao, 1987; Mao et al., 1988), was used as a starting model for the design of potent bifunctional inhibitors of thrombin in which the known active site directed inhibitors, e.g., (D)-Phe-Pro-Arg- (PPACK series of analogues, Hauptmann & Markwardt, 1992) were linked to the FRE binding moieties. These are well represented by the hirulog (Maraganore et al., 1990) and the hirutinin (DiMaio et al., 1991, 1992) series of analogues. Details of the interactions of these different inhibitors with the thrombin molecule have been investigated extensively at the atomic level and have revealed their binding mode at the active site and the remote fibrinogen recognition exosite (Banner & Hadváry, 1991; Skrzypczak-Jankun et al., 1991; Brandstetter et al., 1992; Qiu et al., 1992; Zdanov et al., 1993).

The interest in arginine methyl esters as potential anticoagulants grew from the initial observation that synthetic arginine ester derivatives such as *N*^α-tosyl-L-arginine methyl ester inhibited thrombin (Okamoto et al., 1980). A variety of modifications at the amino and carboxyl sides of the arginine were tested to obtain more potent and specific active site thrombin inhibitors (Kikumoto et al., 1980a, 1980b; Okamoto et al., 1980). The structures of representative compounds of this class, MD-805 complexed with human α -thrombin and hirudin^{55–65} (Banner & Hadváry, 1991), and MQPA complexed with bovine thrombin (Brandstetter et al., 1992), was determined by X-ray crystallography and showed a different binding mode from that exhibited by PPACK (Bode et al., 1989, 1992).

We have incorporated compounds containing the nonsubstrate type active site blocking segment dansyl-Arg-(D)Pipicolinic acid in the design of bifunctional inhibitors of thrombin (Tsuda et al., 1994) and have obtained potent inhibitors with affinity of the order of 10^{-11} M, exceeding by 1–2 orders of magnitude affinities of hirutonins and hirulogs. This report describes the crystal structure of human α -thrombin complexed with two such inhibitors, P498 and P500 (Fig. 1 and Kinemage 1). Their active site binding segments are similar to MD-805 and MQPA, which on their own have $K_i = 1.9 \times 10^{-8}$ M (Okamoto et al., 1981), and are linked to the hirudin^{55–65} exosite binding element for a better comparison with structures determined previously. Both the chemically different linkers are 16 atoms long.

Results and discussion

The thrombin molecule

The thrombin molecules in the two complexes are very similar (Kinemage 1); the RMS deviation (RMSD) for all C α atoms is

Fig. 1. Primary structure of P500 and P498 in comparison with hirutinin-2 and MD-805. MD-805 was crystallized in the presence of hirudin^{55–65} and MQPA formed a binary complex with thrombin.

within the experimental error and is similar to that between the thrombin molecules in these complexes and thrombin in the hirutinin-2 complex (PDB code 1ihs). This latter is a typical representative of noncovalent, bifunctional inhibitors with (D)-Phe-Pro-Arg- bound to the active site (Zdanov et al., 1993) and will be used throughout the text for comparison purposes. We will refer to this inhibitor class as dFPR type inhibitors. Structures of thrombin complexes with active site-directed inhibitors similar to P498/P500 have been reported. One of them, MD-805 (Fig. 1), was described by Banner and Hadváry (1991), who solved the structure of a ternary complex of human thrombin MD-805 and hirudin^{55–65} at 3 Å resolution. Brandstetter et al. (1992) reported a 2.3 Å resolution structure of bovine thrombin complexed with MQPA, a stereoisomer of MD-805. Their comparison with complexes presented here shows that the binding of the inhibitors in the active site is similar. The only significant variations in thrombin structures are observed at the C-termini of A and B chains, remote from the binding site. These deviations are likely due to crystal packing differences between the complexes. As in other thrombin–inhibitor complexes, the A and B chains are covalently linked by a disulfide bridge between Cys¹-Cys¹²², and the usual salt bridges Asp¹⁴...Arg¹³⁷, Glu^{14C}...Lys²⁰², Glu^{14E}...Lys¹³⁵ and Glu⁸...Lys²⁰² are present.

The B-chain contains all the structural elements of the active site and the fibrinogen recognition exosite. P498 and P500 have identical active site binding elements and these regions of the thrombin molecules are virtually identical; for all the atoms of 20 residues falling within 4.5 Å from the Dns^{H1}-Arg^{H2}-(D)Pip^{H3} fragment of the inhibitor, the RMSD is 0.13 Å. Residues that form the groove joining the two functional sites of thrombin, and especially Gln³⁸-Leu⁴¹ on one side and Asn¹⁴³, Gln¹⁵¹, Arg⁷³, and Glu¹⁹² on the other, take slightly different positions in the two complexes, which may be related to differences in linker mobility of the two inhibitors. Residues of the exosite that interact with the hydrophobic face of the C-terminal 3₁₀-helical turn of the inhibitors also overlap quite well (RMS for 12 residues within 4.5 Å from Asp^{H55}-Gln^{H64} is 0.30 Å). As noted previously (Zdanov et al., 1993), Arg⁷⁵, positioned near the twofold axis, is found in two alternate conformations.

The inhibitors

Overall structure

The two inhibitors differ chemically only in their linkers. The electron density for both inhibitors is very well defined for the active site binding portion as well as for the main chain of the N-terminal part of the FRE binding element, but density is absent for some side chains (Fig. 2). The electron density for the linker (especially for the C-terminal part of the P498 linker) and the C-terminal ends of the inhibitors is somewhat poorer and these atoms show higher temperature factors, indicating increased mobility (Fig. 2). The active site and the exosite binding parts of both inhibitors overlap very well, but their linkers follow distinct although adjoining paths (Fig. 3 and Kinemage 1). Binding to the active site is similar to that of the MD-805 and MQPA inhibitors (Fig. 4). P498 and P500 are in contact with thrombin along nearly their entire length, with the closest contacts occurring within the N- and C-terminal portions of the inhibitors. The inhibitor is not proteolysed because its binding, different from the natural substrate, is such that neither the Arg^{H2}-(D)Pip^{H3} nor (D)Pip^{H3}-linker peptide bonds are positioned for the nucleophilic attack by Ser¹⁹⁵ (see below).

Active site binding

The Dns^{H1}-Arg^{H2}-(D)Pip^{H3} segments of both inhibitors bind to the active site of thrombin in an identical manner (Kinemage 1), with an RMSD for all atoms of this fragment of 0.12 Å (excluding (D)Pip^{H3} carbonyl oxygen). The orientations of the Pip^{H3} carbonyl differ because the linkers take different paths. Comparison with the thrombin-MQPA and the thrombin-MD-805/hirudin⁵⁵⁻⁶⁵ complexes suggests that the presence of the linker and/or the FRE does not significantly affect the interactions between the active-site-directed portion of the inhibitor and thrombin. The Dns^{H1}-Arg^{H2}-(D)Pip^{H3} moiety binds to thrombin in a conformation different from that of the dFPR type inhibitors (Fig. 5). The dansyl group, Dns^{H1}, occupies the S3 subsite of thrombin, the adjacent Arg^{H2} extends into the arginine specific S1 subsite, and (D)Pip^{H3} fills the S2 subsite. In the dFPR inhibitors, (D)Phe fills the S3 site, Pro is in the S2 site, and Arg occupies the S1 site (Fig. 5). The bulkier dansyl group is located more to the surface of the thrombin molecule than the (D)Phe ring in dFPR type inhibitors. The aliphatic dimethylamine substituent of the dansyl ring partially fills the volume occupied by

the (D)Phe ring of hirutonins, whereas its aromatic rings make numerous van der Waals contacts with the side chains of Tyr^{60A}, Trp^{60D}, Glu^{97A}, Asn⁹⁸, Leu⁹⁹, and Trp²¹⁵ (Fig. 5), analogous to MD-805 (Banner & Hadvary, 1991) and MQPA (Brandstetter et al., 1992). Although the dimethylamine substitution on the ring in P498/P500 is not at the same position as the methyl in MD-805 and MQPA (Fig. 4), both these substituents point inside the S3 site and occupy nearly the same volume. This is achieved by a somewhat different orientation of the rings of the H1 residue in these complexes. In the presently reported structures, the dimethylamine is found in two rotamers. In one conformation, the methyl groups point into the S3 subsite, interacting with Glu^{97A}, Asn⁹⁸, and, to a limited extent, Ile¹⁷⁴, whereas in the other conformation they point toward Leu⁹⁹. The carbonyl oxygens of Arg⁹⁷ and Glu^{97A} and the hydroxyl of Tyr^{60A} are less than 6 Å from this dimethylamine group and could possibly be targeted for the formation of additional hydrogen bonds through appropriate substitutions of this group. The sulfonyl group of dansyl faces the bulk solvent and is not interacting directly with thrombin.

Although the Arg^{H2} enters the S1 specificity pocket from a different direction than in the dFPR type inhibitors, this conformation does not impair its interaction with the residues in this pocket; the specificity provided by the interactions with Asp¹⁸⁹ at the bottom of this pocket is still maintained. In addition to the formation of this salt bridge, Arg^{H2} also forms two hydrogen bonds to Gly²¹⁶ through its backbone atoms in an antiparallel fashion: NH^{ArgH2}...O^{Gly216} (2.9 Å) and O^{ArgH2}...NH^{Gly216} (3.2 Å) (Figs. 5, 6). For comparison, in hirutonin-2 (Zdanov et al., 1993), the same hydrogen bonds to Gly²¹⁶ are formed by (D)Phe, the P3 residue, whereas the Arg forms weak hydrogen bonds to Ser²¹⁴ and Gly¹⁹³. Here, Arg^{H2} enters the S1 pocket from the side and adopts a less frequently observed gauche⁻ conformation (Janin et al., 1978). As a consequence, the interactions with the carboxylate of Asp¹⁸⁹ differ from those observed in hirutonin-2. Following the nomenclature of Singh et al. (1987), this Arg...Asp interaction in hirutonin-2 is of a less frequent twin-N/twin-O coplanar type (NH1...OD1, NH2...OD2), with hydrogen bonding distances between the oppositely charged atoms of less than 3.0 Å. The importance of these hydrogen bonds in dFPR type inhibitors has been discussed by Weber et al. (1995). The inhibitors P498 and P500 display the more frequent single-N/single-O type of interaction, with the NH2 interacting with the OD1 of Asp¹⁸⁹ (3.1 Å for both complexes) at the bottom of the specificity pocket (Fig. 6). This latter side chain is, in turn, hydrogen bonded to OH^{Tyr228} and O^{Ala183} through a bridging water molecule (Wat⁵³³) that is conserved in the MQPA and other inhibitor complexes. In the MQPA complex, this interaction is of a single-N/twin-O type. In addition to forming a hydrogen bond to OD1^{Asp189}, the NH2^{ArgH2} atom is only 3.3 Å away from OD2^{Asp189}. The latter atom is also hydrogen bonded to the NH of Asp²²¹.

The (D)Pip^{H3} residue adopts a chair conformation and fits into the S2 subsite of the active site. This piperidine ring stacks between the aromatic rings of Dns^{H1} and His⁵⁷ of thrombin, and contacts, edge on, the rings of Tyr^{60A} and Trp^{60D} (Fig. 4). It ventures deeper into the S2 subsite than the corresponding ring in MQPA (Brandstetter et al., 1992), which bears an equatorial methyl substituent facing the protein. The penetration of this methyl group in the MQPA complex correlates with small shifts of Trp^{60D} and, to a lesser extent, Tyr^{60A}, relative to their posi-

Fig. 2. Omit map of the whole inhibitor molecules in complex with human α -thrombin. The density is contoured at 2σ , with a map cover radius of 2.7 Å. **A:** Thrombin-P498 complex. **B:** Thrombin-P500 complex. This figure and subsequent ones were prepared with the program *oplot* from the *O* package.

tions in other thrombin complexes, which expand the S2 subsite. No such adjustment of the S2 subsite is observed in the P498 and P500 complexes (Fig. 4).

The 3D structures clarify the molecular basis for the observed resistance of these inhibitors to hydrolysis by thrombin (Tsuda et al., 1994); it comes from their unusual mode of binding in the active site, which is different from that of polypeptide substrates (as extrapolated from the binding of dFPR type inhibitors). Specific cleavage normally occurs at the Arg-X amide bond, but the nature of binding of this inhibitor is such that the arginine carbonyl carbon is too far from the reactive Ser¹⁹⁵ (Fig. 5). The peptide bond closest to the nucleophilic Ser¹⁹⁵ of thrombin is the one linking (D)Pip^{H3} to the linker. Although this carbonyl group points in the direction of the oxyanion hole, its orientation and its distance (>4.0 Å away) from Ser¹⁹⁵ precludes the nucleophilic attack.

Solvent structure

Due to the nonsubstrate-like conformation of these inhibitors, additional water molecules fill both the specificity pocket and

the oxyanion hole to bridge hydrogen bonding groups of thrombin and inhibitor. First, two water molecules, Wat⁴⁷¹ and Wat⁴⁷², form hydrogen bonds bridging OD2^{Asp189}, N^{Arg221A}, and O^{Glu217} (Fig. 6). The former water occupies the space taken by the NH₂ of the Arg guanidinium in complexes that bind in the substrate-like mode. A similar network of solvent molecules is observed in the hirudin-thrombin complex (Rydel & Tulinsky, 1991) and in the hirugen-thrombin complex (Skrzypczak-Jankun et al., 1991), but only one or two of these water molecules are present in other complexes. The solvent molecule that in the hirutinin-2 complex bridges the Glu²¹⁷-Asp²²¹ region to the P1 Arg (Zdanov et al., 1993) is absent in P498 and P500 complexes due to a different position of the Arg side chain. Consequently, a direct hydrogen bond between Gly²¹⁹ and NE^{ArgH2} (Fig. 6) is now established. Second, because the inhibitor cannot reach deep into the oxyanion hole, a water molecule is found there (Wat⁵³⁷) in the P500 complex, making hydrogen bonds to atoms normally involved in the stabilization of the transition state: N^{Ser195} (3.0 Å) and N^{Gly193} (2.9 Å), as well as to O^{PipH3} (3.1 Å) of the inhibitor. In the P498 complex, this water (Wat⁵³⁵)

Fig. 3. Superposition of P498 (thick line) and P500 (shaded line) inhibitors based on the best superposition of thrombin molecules. The transformation matrix for best overlap was calculated with the least-square fit option within O. Selected residues from the thrombin molecules are shown in full (thick lines, P498; thin lines, P500). Dashed lines indicate hydrogen bonds between the linkers in the inhibitors and the protein.

moves away from the oxyanion hole and bridges the O^{BAlaH4} and N^{Gly193} due to a different orientations of the O^{PipH3} and the linker. Another well-ordered solvent (Wat^{534}) occupies the oxyanion hole, and is hydrogen bonded to the $NH1$ of Arg^{H2} (3.0 Å), the O^{Ser214} (3.0 Å), the OG^{Ser195} (2.7 Å), and the O^{PipH3} (3.0 Å) (Fig. 6). It fills out a cavity that corresponds to

the site of entry of the Arg in the dFPR type inhibitors (Fig. 5) and which here is left unoccupied by the inhibitor due to a nonsubstrate-type conformation of the Arg^{H2} . This water is common to both complexes and is also present in the MD-805 and MQPA complexes. Thus, it seems that the tight binding of these nonsubstrate-like inhibitors is enhanced by the participa-

Fig. 4. Superposition of P498 active site binding element (thick lines) and MQPA (shaded lines) in the active site of thrombin based on the best overlap of thrombin residues 173–176, 97–102, 214–220, 189–195, and 57–60D.

Fig. 5. Superposition of P498 (thick lines) and hirutinin-2 (shaded lines) active site binding segments showing the different mode of interaction of these inhibitors. Least-square superposition was done as in Figure 4. Dashed lines indicate hydrogen bonds between the inhibitor and the protein.

tion of water molecules that fill space normally occupied by substrate-like molecules and provide additional hydrogen bonds.

Conformation of the linkers

The chemical structure of the linkers is shown in Figure 1. Both are 16 atoms long and are thus shorter than the hirutinin-2 linker (partially disordered in the crystal), but similar in length

to the hirutinin-6 linker (well ordered, Zdanov et al., 1993). The hydrogen bonding capabilities of these two linkers are significantly different. The P500 linker, made of 12-aminododecanoic acid and a Gly, is very hydrophobic, with the N and O atoms only at its extremities. In contrast, the linker in P498, composed of β -alanine, Gly-Gly, and 5-aminopentanoic acid, has amino and carbonyl groups distributed along its entire length. Yet, despite this hydrogen bonding potential, P498 is a six times less

Fig. 6. Interaction of the Dns^{H1}-Arg^{H2}-(D)Pip^{H3} moiety of P498 in the specificity pocket and the oxyanion hole of thrombin. P498 is in thick lines, and dashed lines represent hydrogen bonds to the thrombin molecule.

potent inhibitor of thrombin ($K_i = 0.13$ nM) than P500 ($K_i = 0.02$ nM) (Tsuda et al., 1994). Kinetic studies using synthetic peptides have shown previously that it is the location of these groups within the linker rather than their number that determines optimal interactions with thrombin and that the binding constant may vary by nearly two orders of magnitude, depending on the choice of the linker (Szewczuk et al., 1993). Combined with molecular dynamics simulations, that study also indicated a correlation between position of a hydrogen bonding group, mobility of the linker and the potency of the related compounds.

So why is it that the incorporation of two additional carbonyl and amino groups does not lead to tighter binding, but instead has the opposite effect? The two linkers take somewhat different paths through the shallow groove connecting the active site with the exosite (Fig. 3 and Kinemage 1). They take different directions at the beginning, with the carbonyl of (D)Pip^{H3} being displaced by 0.8 Å between the two and a maximum distance of 2.6 Å between them near the center. The linker of P500 forms one hydrogen bond near its C-terminal end, between the O^{H4} and NE2^{Gln151} (Table 1). In P498 the central Gly^{H6} forms two hydrogen bonds to the backbone of Leu⁴⁰ (2.8 and 2.9 Å, Fig. 3). Yet the electron density (omit and $3F_o - 2F_c$ maps) for P500 linker is better defined than that for the P498 linker, especially in the central part (Fig. 2). Inspection of the remaining NH groups shows that they are directed toward the solvent, whereas the carbonyls interact more with the protein.

Molecular dynamics simulations for the 12-amino dodecanoic acid linker (shorter than in the inhibitors investigated here) attached to the dFPR type inhibitor performed by Szewczuk et al. (1993) showed large atomic fluctuations in some regions of the linker. The fluctuations were reduced by the introduction of hydrogen bonding groups in some of these regions, suggesting formations of hydrogen bonds, and yielded tighter complex formation with thrombin reflected in lower K_i values (Szewczuk et al., 1993). For P498, despite formation of some hydrogen bonds (Table 1), the mobility of the linker seems not to be reduced. Additionally, some of the interactions are rather unfavorable: the carbonyl of β -Ala is 3.1 Å from the carbonyl of Leu⁴⁰ and it is likely that the sequestration of a linker such as in P498 from the solvent causes decrease in enthalpy that cannot be compensated by interactions with thrombin. The apolar linker of P500 is, on the other hand, transferred from a polar to a less polar environment. The differences in binding constants between these two inhibitors are determined not only by their interactions with thrombin, but also by the transfer of the linker from a polar to a less polar environment, without satisfying all hydrogen bonding groups. In addition, as mentioned above, a solvent molecule in P500 bound near the N-terminal end of the linker (Wat⁵³⁷) provides a more favorable bridging between the inhibitor and thrombin.

The exosite binding

Both complexes have the hirudin⁵⁵⁻⁶⁵ as the FRE recognition element. For both of them, the density is very well defined from Asp^{H55} to Pro^{H60} (except for the side chain of Glu^{H58}), with some discontinuity in the electron density in the remainder of the chain reflected in the higher B -factors for atoms of Glu^{H61}-Leu^{H64}. No density was observed for Glu^{H61} side chain nor could Gln^{H65} be placed reliably in the electron density. As for many other complexes bearing this FRE recognition moiety, the

Phe^{H56} and Ile^{H59} are particularly well ordered. The mode of binding of this fragment to the FRE is similar to many other bifunctional inhibitors containing the same motif, with a characteristic pattern of hydrophobic interactions involving Phe^{H56} and Ile^{H59}. In addition, the presence of a tetrahedrally coordinated water molecule (Wat⁴⁰⁰) linking the backbone of Glu^{H57} to the carbonyl of Thr⁷⁴ and the guanidinium of Arg⁶⁷, is also observed in these complexes. Within the C-terminal 3_{10} -helix, Glu^{H62} is directed toward the bulk solvent, the side chain of Pro^{H60} stacks against the aromatic ring of Tyr⁷⁶ of thrombin, and Tyr^{H63} and Leu^{H64} cluster together with Ile^{H59}. When the thrombin structures deposited in the Protein Data Bank are superimposed, the distribution of the atoms of the inhibitors in the exosite binding fragment is broader than that of the exosite itself. Thus, although the residues forming the exosite change their position very little upon inhibitor binding, the inhibitors themselves display more flexibility in adjusting to this binding site. Largest differences between FRE binding peptides are observed around the first residue of this region, Asp^{H55}, which may partially reflect the fact that in some of these peptides Asp^{H55} is not attached to a linker. Although the linkers do not have, in general, strong interactions with the S' subsites, their presence allows an increase in the efficiency of inhibition and binding by utilizing a remote binding site in addition to the active site region.

Conclusion

The two structures presented here provide evidence for the relative independence of the active site binding and the FRE binding elements in bifunctional inhibitors. It is observed that the presence of a linker moiety, as well as the FRE binding element, does not modify drastically the binding mode of the active site-directed segment compared with similar compounds such as MQPA. However, it is clear that, although the linker does not interact strongly with thrombin, it can affect the affinity by more than one order of magnitude. It contributes to the maintenance of an optimal complementarity between functional domains of the inhibitors and the enzyme for better interaction, thus leading to higher affinity and culminating in more potent compounds. The exosite binding element interacts with the thrombin surface the same way as other FRE recognition segments with a 3_{10} helical turn and a lock and key type interaction with the hydrophobic pocket at the FRE.

Material and methods

Crystallization

Human α -thrombin was obtained from Haematologic Technologies Inc. (Essex Junction, Vermont) and was used for crystallization without further purification. The inhibitors P498 and P500 were synthesized as described previously (Tsuda et al., 1994). Thrombin was concentrated to 6 mg/mL and dialyzed against 50 mM citrate buffer, pH 5.5, and 5.0 mM potassium sulfate. The thrombin-inhibitor complexes were formed by an overnight incubation of thrombin with the inhibitor at a molar ratio of 1:2 at 4 °C. Crystals were grown at 18 °C by the hanging drop vapor diffusion method. For both complexes the reservoir contained 25% PEG 3350, 38 mM potassium sulfate, and 0.1 M citric acid buffered to pH 5.5 with sodium phosphate. The

Table 1. Hydrogen bonds between the inhibitor and thrombin including the bridging solvent molecules^a

Inhibitor atom		Contact atom	(dist, dist) P498 P500		Bridging to atom	(dist, dist) P498 P500
O2 ^{Dsn} H1	...	O ^{Wat} 595	(—, 3.2)	...	OE2 ^{Gln} 192	(—, 2.7)
N ^{Arg} H2	...	O ^{Gly} 216	(2.9, 2.9)			
O ^{Arg} H2	...	N ^{Gly} 216	(3.2, 3.2)			
NE ^{Arg} H2	...	O ^{Gly} 219	(3.0, 3.1)			
NH1 ^{Arg} H2	...	O ^{Wat} 534	(3.0, 3.0)	...	O ^{Pip} H3	(3.0, 2.9)
	...	O ^G ^{Ser} 195	(2.7, 2.5)			
	...	O ^{Ser} 214	(3.0, 2.7)			
NH2 ^{Arg} H2	...	OD1 ^{Asp} 189	(3.1, 3.1)	...	OD2 ^{Asp} 189	(3.3, 3.4)
	...	O ^{Wat} 471	(3.1, 3.3)	...	OD2 ^{Asp} 189	(2.4, 2.5)
O ^{Pip} H3	...	O ^G ^{Ser} 195	(2.9, 3.4)			
	...	O ^{Wat} 537	(3.5, 3.1)	...	N ^{Ser} 195	(2.9, 3.0)
	...	N ^{Gly} 193	(3.1, 2.9)			
N ^{Gly} H6	...	O ^{Leu} 40	(2.8, —)			
O ^{Gly} H6	...	N ^{Leu} 40	(2.9, —)			
O ^{Ada} H4	...	NE2 ^{Gln} 151	(—, 2.8)			
	...	WAT ⁵³⁵	(2.9, —)	...	N ^{Gly} 193	(3.1, —)
OD1 ^{Asp} H55	...	NH1 ^{Arg} 73	(2.9, 3.3)			
OD2 ^{Asp} H55	...	NH2 ^{Arg} 73	(3.4, 3.4)			
N ^{Glu} 57	...	O ^{Thr} 74	(2.9, 3.0)			
OE1 ^{Glu} 57	...	N ^{Tyr} 76	(3.1, 3.4)			
O ^{Glu} 57	...	O ^{Wat} 400	(2.8, 2.8)	...	O ^{Thr} 74	(2.5, 2.6)
	...	NH1 ^{Arg} 67	(3.1, 3.0)			
	...	NH2 ^{Arg} 67	(3.1, 3.0)			
OH ^{Tyr} H63	...	O ^{Wat} 432	(2.8, 3.0)	...	N ^{Ile} 82	(3.0, 3.0)

^a Distances are shown in brackets, first number corresponds to P498 and the second to P500. Dash in place of a number indicates that the corresponding hydrogen bond does not exist in the particular inhibitor. Active site binding fragment, linker, and exosite binding fragment are separated by horizontal lines.

drops were made of 2 μ L of protein solution and 4 μ L of reservoir solution. Crystals suitable for X-ray diffraction analysis appeared within 3–4 days. They are monoclinic, space group C2, with cell dimensions of $a = 71.6$, $b = 72.1$, $c = 73.5$ Å, and $\beta = 101.0^\circ$ for the thrombin–P498 complex, and $a = 71.5$, $b = 72.2$, $c = 73.6$ Å, and $\beta = 101.2^\circ$ for the thrombin–P500 complex. There is one molecule in the asymmetric unit. These crystals are isomorphous to many complexes of thrombin with bifunctional inhibitors (e.g., Skrzypczak-Jankun et al., 1991; Qiu et al., 1992; Zdanov et al., 1993).

X-ray analysis

Diffraction data were collected on a R-axis IIC area detector with a Rigaku RU300 rotating anode source. Data were processed with the R-axis software (Molecular Structure Corporation). For the P500 complex, the crystal was rotated 90 degrees around the c^* -axis with 2 degrees oscillations and a crystal to detector distance of 91 mm. An additional 60 degrees of data were collected after tilting the crystal by approximately 20 degrees. A total of 96,739 observations merged to 22,654 unique reflections with an R_{sym} of 0.079 and completeness of 82.9% to 2.0 Å resolution. The P498 complex was rotated around a^* with 1.5 degree oscillations for 160 degrees. A total of 102,417 observations merged to 21,557 unique reflections with an R_{sym} of 0.070. The completeness of the data to 2 Å resolution was 85%.

Structure determination and refinement

The atomic structure of thrombin taken from the thrombin–hirutinin-2 complex (Zdanov et al., 1993; PDB code 1ihs) was used as a starting model for refinement. The refinement was carried out with the program X-PLOR (Brünger, 1992), and model rebuilding was done using the program O, version 5.9.1 (Jones et al., 1991). Appropriate entries have been added to the dictionaries of both programs to accommodate the nonstandard groups of the inhibitors. The geometries for these groups were based on analogous compounds retrieved from the Cambridge Structural Database.

P498 complex

The initial R -factor for the 8.0–2.0 Å resolution range was 0.27. Rigid body minimization decreased the R -factor to 0.26. Subsequent molecular dynamics (MD) refinement was performed with the slow cooling protocol where, following 60 cycles of minimization, the temperature was decreased stepwise by 4% of the current value from an initial value of 2,000 K to 300 K, with 25 fs of dynamics simulation at each step. This was followed by 60 cycles of conjugated gradient minimization and 20 cycles of individual B -factor optimization. The weighting factor for the crystallographic term, WA , was taken as two thirds of the value

determined by the CHECK procedure. After one round of refinement, the R -factor dropped to 0.227. The $3F_o - 2F_c$ and difference maps calculated at this stage showed clearly the location of most of the inhibitor, except for the Gly^{H5}-Gly^{H6}-δApe^{H7} (Fig. 1) of the linker and the C-terminal part of the FRE binding fragment beyond Pro^{H60}. The well-determined parts of the inhibitor, as well as many solvent molecules, were included in the model and another round of MD refinement was conducted. The electron density was somewhat improved and more of the inhibitor was built in. Three more refinement and rebuilding cycles were performed. The autolysis loop (residues 146–149E) is disordered and is not included in the model. Alternative conformations have been modeled for 11 residues. The difference electron density map showed density extending from Asn^{60G} and corresponding to an N-linked oligosaccharide. However, this density was not sufficiently clear to permit unequivocal identification of the type of sugar present, and thus, no sugar was included in the model, nor was it modeled as discrete solvent molecules. The numbers for the inhibitor residues are prefixed with the letter H. The final model contains residues 1D–14K of the A-chain, residues 16–146 and 150–245 of the B-chain, residues Dns^{H1}-δApe^{H7} and Asp⁵⁵-Leu^{H64} of the inhibitor, and 182 solvent molecules. No electron density was observed for the C-terminal glutamine of the inhibitor. The final R -factor is 0.170 for 21,012 reflections with $I > \sigma(I)$ within the 8–2.0-Å resolution shell. The RMSD for bond lengths is 0.009 Å and for bond angles is 1.46 degrees. The average B -factor for the thrombin molecule is 27.9 Å².

P500 complex

The initial R -factor for the 8.0–2.0-Å resolution range was 0.29. Rigid body minimization decreased the R -factor to 0.27. After the first round of MD refinement, the R -factor dropped to 0.23. At this stage, the Dns^{H1}-Arg^{H2}-(D)Pip^{H3} part of the inhibitor was included in the model. A subsequent round of MD refinement decreased the R -factor to 0.199. The linker portion, as well as part of the exosite binding fragment (Asp^{H55}-Glu^{H61}), could be traced in the $3F_o - 2F_c$ map and were included in the model. At this stage, solvent molecules were also included. Further minimization and model rebuilding to the $3F_o - 2F_c$ and omit maps allowed positioning of the rest of the atoms in the inhibitor, except for the side chains of Glu^{H58}, Glu^{H61}, and Leu^{H64}, and the terminal glutamine residue. The final model includes residues 1D–14K of the A-chain, residues 16–146 and 150–245 of the B-chain, residues Dns^{H1}-μAad^{H4} and Asp^{H55}-Leu^{H64} of the inhibitor, and 188 solvent molecules. There are 17 residues with alternate conformations. The final R -factor is 0.169 for 21,248 reflections with $I > \sigma(I)$ within the 8.0–2.0-Å resolution range. The RMSD for bond lengths is 0.010 Å, and for bond angles is 1.53 degrees. The average B -factor for the thrombin molecule is 29.8 Å².

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