Structure of the Hirulog 3–Thrombin Complex and Nature of the S′ Subsites of Substrates and Inhibitors†

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ABSTRACT: The X-ray crystallographic structure of the human α-thrombin complex with hirulog 3 (a potent, noncleavable hirudin-based peptide of the "hirulog" class containing a β-homoarginine at the scissile bond), which is isomorphous with that of the hirugen–thrombin crystal structure, was solved at 2.3-Å resolution by starting with a model for thrombin derived from the hirugen–thrombin complex and was refined by restrained least squares methods (R = 0.132). Residues of hirulog 3 were well-defined in the electron density, which included most of the pentaglycine linker and the C-terminal helical turn that was disordered in a related structure of thrombin with hirulog 1. The interactions of D-Phe1′-Pro2′-β-homoArg3′ with the active site of thrombin were essentially identical to those of related structures of PPACK– (D-Phe-Pro-Arg chloromethyl ketone) and hirulog 1–thrombin, with the guanidinium function of the arginyl P1 residue forming a hydrogen-bonding ion pair with Asp189 of the S1 site. A noticeable shift in the CA atom of β-homoArg3′ due to the methylene insertion displaces the scissile bond from attack by Ser195, thus imparting proteolytic stability to the β-homoArg hirulog derivative. Resolution of the pentaglycine spacer, linking N- and C-terminal functional domains into a single oligopeptide bivalent inhibitor, permitted delineation of corresponding S′ subsites of thrombin. The position of Gly4′ (P1′) is stabilized by three hydrogen bonds with His57, Lys60F, and Ser195, while the conformational angles are maintained in a strained, nonallowed configuration for non-glycyl amino acids. The S1′ site inferred from the positioning of the CA atom of Gly4′ is a small cavity lined by amino acids of the back side of the apolar S2 pocket (His57, Tyr60A, Trp60D) and the side chain of Lys60F. This putative S1′ subsite can accommodate small polar amino acid side chains as observed from P1′ amino acids in a number of natural thrombin substrates. A larger surface cavity appears to define the S2′–S3′ sites, lined by Lys60F, Leu41, Phe60H, and Glu39, as determined by the positioning of Gly5′-Gly6′ of hirulog 3. Notwithstanding the absence of tyrosine sulfation in hirulog 3, Pro60′-Leu64′ interacting with the anion-binding exo site of fibrinogen recognition are ordered in a 3_{10} helical turn. As with hirugen– and hirudin–thrombin complexes, this binding interaction is predominantly hydrophobic. In summary, the structural determination and analysis of the thrombin complex with hirulog 3 provide complete molecular detail for hirulog–thrombin interactions. By analogy with thrombin–substrate interactions, the hirulog structure has defined S′ subsites that appear to govern further specificity in thrombin physiologic actions. The fact that Asn53′-Gly54′ are disordered in all the structures of thrombin complexed with hirudin C-terminal peptides is most likely the result of nonenzymatic deamidation of the asparagine residue.

Thrombin is a serine proteinase that converts fibrinogen into clottable fibrin, thus carrying out one of its important functions in thrombosis and hemostasis. The X-ray crystallographic studies of human α-thrombin complexed with hirudin (Rydel et al., 1990, 1991; Grutter et al., 1990) and synthetic peptide inhibitors (Bode et al., 1989, 1992; Skrzypczak-Jankun et al., 1991; Banner et al., 1991) have revealed many details of thrombin–inhibitor interactions at the molecular level that have recently also been summarized (Tulinsky, 1991; Tulinsky & Qiu, 1992).

Hirudin, produced in the salivary glands of the blood-sucking leech *Hirudo medicinalis*, prevents the clotting of ingested blood and is the most potent natural thrombin inhibitor with a K_d reported as low as 20 fM (Stone & Hofsteenge, 1986). Hirudin is a 65-residue protein that forms a 1:1 noncovalent complex with thrombin. The 48-residue globular N-terminal domain, which is stabilized by three disulfide bridges, binds at the active site region, while an extended heptadecapeptide C-terminal domain (which contains a high content of negatively charged amino acids) binds at the fibrinogen recognition "anion-binding" exo site (Rydel et al., 1990, 1991). Hirudin and analogs of its functional domains have been growing in importance in the development of new antithrombotic drugs (Sawyer, 1991). Hirugen[1] and hirulogs are examples of peptide inhibitors designed to mimic thrombin–hirudin in-

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teractions (Maraganore et al., 1989, 1990; Skrzypczak-Jankun et al., 1991; Kline et al., 1991).

The role of the fibrinogen recognition exo site of thrombin has been studied extensively by examining the interaction of thrombin with synthetic hirudin C-terminal analogs (Krstenansky & Mao, 1987; Bourdon et al., 1990; Chang et al., 1990; Jakubowski & Maraganore, 1990; Prescott et al., 1990). Thrombin cleavage of fibrinogen and several other macromolecules can be inhibited effectively by blocking only the fibrinogen recognition exo site without inhibiting the amidolytic function of the thrombin active site for small molecule chromogenic substrates (Krstenansky & Mao, 1987; Naski et al., 1990). Hirugen, the hirudin C-terminal analog, is an exo-site inhibitor. It has a K_d of 150 nM that is decreased by about 1 order of magnitude upon sulfation of Tyr63′ (Maraganore et al., 1989; Hofsteenge et al., 1990; Niehrs et al., 1990). The three-dimensional structure of the human α-thrombin–hirugen complex (Skrzypczak-Jankun et al., 1991) has shown binding associations very similar to that of the C-terminal undecapeptide of hirudin, with the sulfato Tyr63′ of hirugen involved in an extended hydrogen-bonding network utilizing the three sulfato oxygen atoms. The active site of thrombin in this structure is unoccupied, and the positions of catalytic residues and the pattern of hydrogen bonding are essentially identical to that of other serine proteinases. Binding of hirudin or PPACK in the active site produces only a minor effect on the positions of the catalytic triad (Skrzypczak-Jankun et al., 1991).

The thrombin inhibition by hirugen has been improved by mimicking the hirudin–thrombin interaction with the design of a class of bivalent peptides that can interact with both the active site and the anion-binding exo site. Thus, hirulogs link a hirudin C-terminal peptide and an active site inhibitor together using a short oligoglycine spacer, and they show effective inhibition of thrombin at nanomolar concentrations (Maraganore et al., 1990; DiMaio et al., 1990). The structure of the hirulog 1–thrombin complex, with an N-terminal D-Phe-Pro-Arg targeted to bind in the active site, has practically identical interactions with that of the PPACK-thrombin complex in the active site (Bode et al., 1989), and with that of the hirudin– or hirugen–thrombin complexes at the exo site (Skrzypczak-Jankun et al., 1991). However, hirulog 1 is also a substrate so that the Arg3′–Pro4′ bond is cleaved (Witting et al., 1992) although at a slow rate (k_{cat} = 0.3 min^{-1}); moreover, the Pro4′-tetraglycine spacer is disordered in the crystal structure of the complex so that the S1′, S2′, ... sites of thrombin (Laskowski & Kato, 1980; Read & James, 1986) could not be described.[2] The electron density of the C-terminus of hirulog 1 (Pro60′-Leu64′) was also not resolved, which precluded further elaboration of the role of Tyr63′ desulfation on binding in this region.

Hirulog 3 (Figure 1) is a synthetic peptide inhibitor in the hirulog class with β-homoArg at position 3′. Although an amide, the β-homoArg–Gly bond is not a peptide bond and

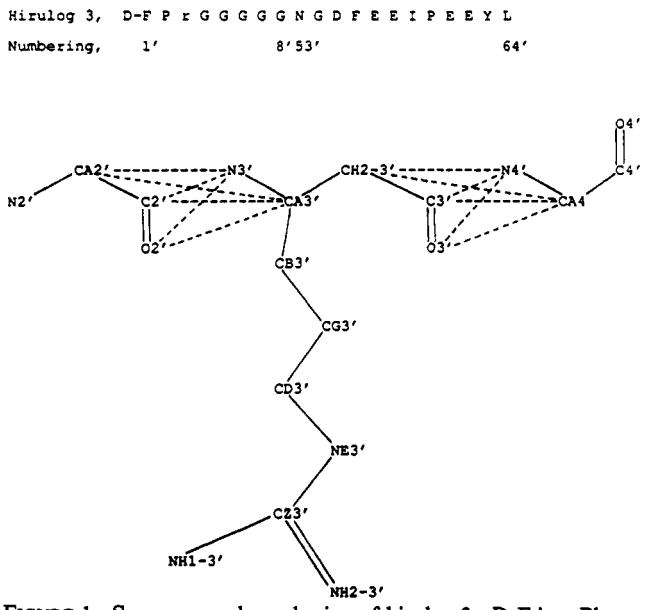

FIGURE 1: Sequence and numbering of hirulog 3. D-F is D-Phe; r is β-homoArg. Since β-homoarginine is not a conventional amino acid, a special set of restraints for bond, angle, and 1,4 planar distances were applied in the least squares program; these are indicated in the figure with broken lines and involve β-homoarginine and its preceding and trailing peptide.

is therefore proteolytically stable (Kline et al., 1991). We presently report here the high-resolution refined structure of hirulog 3 complexed with human α-thrombin. The structure determination has revealed the nature of the S1′, S2′, and S3′ subsites of thrombin that have heretofore been unreported. In addition, the C-terminal pentapeptide is ordered in a helical turn, and Asn53′-Gly54′ are disordered as in the hirudin, hirugen, and hirulog 1 structures; the latter is most likely related to the susceptibility of asparagine to deamidate in an Asn-Gly link (Geiger & Clarke, 1987; Wright, 1991; Bischoff et al., 1992).

EXPERIMENTAL PROCEDURES

The hirulog 3–α-thrombin complex was prepared by placing about a 10-fold molar excess of hirulog 3 solution over a frozen 1-mL sample of human α-thrombin solution at 4 °C (1.48 mg/mL). This was then diluted to 2 mL with 0.1 M sodium phosphate buffer (pH 7.3). The solution of the hirulog 3–thrombin complex was concentrated to about 5 mg/mL using a Centricon-10 miniconcentrator in a refrigerated centrifuge. Crystals were grown by the hanging drop method with 2.5 mg/mL protein complex, 0.075 M sodium phosphate buffer (pH 7.3), 0.19 M NaCl, 12% PEG 8K, and 1 mM NaN_3 in 10-μL drops equilibrating against 1-mL well solutions of 0.1 M sodium phosphate buffer (pH 7.3), 24% PEG 8K, and 1 mM NaN_3. The crystal used for intensity data collection, an approximate cube of 0.4 mm on edge, grew in about 2 weeks.

X-ray diffraction intensity data were collected using a Siemens multiwire area detector. Graphite monochromated Cu Kα radiation was generated by a Rigaku RU200 rotating anode source operating at 5 kW power. The crystal-detector distance was set at 11.7 cm, the detector swing angle was either 12° or 15°, the scan range was 0.2° per frame, and each frame was collected for 75 s. The program XENGEN (Howard et al., 1987) was used to process the raw data to obtain integrated intensities. The crystal diffracted X-rays well to 2.3-Å resolution with the average peak width of a reflection being about 0.6°. The hirulog 3–thrombin crystal was isomorphous to that of the hirugen and hirulog 1 complexes

[1] Abbreviations: hirugen, *N*-acetylhirudin 53′–64′ (NGDFEEIPEEYL) with sulfato Tyr63′ (prime notation designating inhibitor); PPACK, D-Phe-Pro-Arg methyl ketone in PPACK–thrombin or the carboxylic acid counterpart in the cleaved hirulog 1–thrombin complex; hirulog 1, PPACK-Pro-(Gly)$_4$-desulfato-Tyr63′-hirugen (hirulog 2 has fibrinopeptide A 7–16 attached to hirugen, also through a prolyltetraglycine spacer); β-homoArg, β-homoarginine; hirulog 3, D-Phe-Pro-β-homoArg-(Gly)$_5$-desulfato-Tyr63′-hirugen.

[2] Hirulog 2 is also a substrate for thrombin and is cleaved. However, since K_i of fibrinopeptide A is only in the millimolar range (Marsh et al., 1983), crystals of the hirulog 2–thrombin complex simply proved to be those of the hirugen–thrombin complex with the Pro4′-(Gly)$_4$ spacer disordered as with hirulog 1 (unpublished results of this laboratory).

of thrombin (Skrzypczak-Jankun et al., 1991): monoclinic, space group $C2$, four molecules per unit cell with a = 71.44 Å, b = 72.10 Å, c = 73.07 Å, and β = 101.02 (V_m = 2.4 Å^3/Da, protein fraction 51%). After XENGEN data reduction, a total of 13 756 independent reflections were obtained from 59 257 collected reflections. Removing weak reflections with $I/\sigma(I) < 2$ produced a data set of 12 167 unique reflections (77% observed, R_{merge} = 0.045). This data set is complete to 2.5-Å resolution and contains half of the possible reflections between 2.3 and 2.5-Å resolution.

The phases of hirulog 3–thrombin were approximated using the thrombin coordinates of the hirugen–thrombin complex. The initial model included residues Ser1E-Arg15 of the A chain, Ile 16-Leu144 and Gln151-Glu247 of the B chain (chymotrypsinogen numbering; Bode et al., 1989), and the Lys145-Gly150 autolysis loop of the hirudin–thrombin complex (Rydel et al., 1991); hirulog 3 was not included in the first-phase angle calculation. The starting crystallographic R factor ($R = \sum||F_o| - |F_c||/\sum|F_o|$) was 0.31. The first ($2|F_o| - |F_c|$) electron density from 7.0- to 2.8-Å resolution showed good density for most of the thrombin residues, residues D-Phe1′ to Gly4′ of hirulog 3 in the active site, and Asp55′-Ile59′ in the exo site; all were also prominent in the ($|F_o - F_c|$) difference map. However, there was no density for Trp148-Lys149E of the autolysis loop of thrombin, similar to the hirugen and hirulog 1 complexes. The model was improved by fitting the electron density on an Evans & Sutherland PS390 stereographics system with the program FRODO (Jones, 1982) that also included the resolved part of hirulog 3 but excluded the unresolved autolysis loop of thrombin.

The structure was refined using restrained least squares methods with the program PROLSQ (Hendrickson & Konnert, 1980). The program required modification to recognize the β-homoArg residue that has a nonpeptide main-chain structure (Figure 1). Regular amino acids are always -N-CA-C-, so that the program utilized this information by presetting many main-chain restraints. Consequently, if a residue in the chain is a nonpeptide, it cannot be simply overwritten with new restraints in a dictionary file. Restraints were applied to the β-homoArg by treating the residue as a special multiplanar group and excluding it from the peptide chain sequence while applying external distance and planar restraints in the control file. The external restraints include three plane (CA2′-C2′-O2′-N3′-CA3′, $CH_2$3′-C3′-O3′-N4′-CA4′, and NE3′-CZ3′-NH1-3′-NH2-3′) two bond distance, six angle distance, and two 1,4 planar distance restraints (Figure 1).

The PROLSQ refinement was carried out by a series of geometrically "tight–loose–tight" restrained cycles, starting with an overall thermal parameter of 34 Å^2 at 2.8-Å resolution and no solvent and then proceeding to individual thermal parameters and gradually increased resolution (2.8, 2.5, 2.3 Å). Water molecules were introduced at 2.5-Å resolution, and the hirulog 3 model structure was gradually updated to electron density maps with computer graphics, adjusting torsion angles and adding newly resolved residues (Gly5′-Gly7′ and Pro60′-Leu64′ as they developed during the course of refinement). The final structure converged at R = 0.132 with an average thermal parameter of 26 Å^2 using 246 water molecules at 11 408 reflections from 7.0- to 2.3-Å resolution, making this the most accurate structure of thrombin determined thus far. The ω angles of 97% of the peptide bonds of the thrombin part of the structure are within ±6° of planarity, and only four residues are not in the conformationally allowed regions; Glu1C and Asp14L are not well resolved while Phe7 and Glu18 are close to the values reported for the hirudin–thrombin structure (Rydel et al., 1991). An estimate of the mean error in coordinates has been made by examining the R value as a function of scattering angle (Luzzati, 1952), which gives a value of about 0.20 Å. The coordinates of the final structure have been deposited in the Brookhaven Protein Data Bank (Access Number 1ABI).

The crystal structure of PPACK–thrombin was originally determined by Bode et al. (1989). However, since a number of aspects of the refined structure (Bode et al., 1992) differ significantly from those of the hirulog 3–thrombin complex and other thrombin complex structure determinations of this laboratory (resolution, mode of intensity data collection and structure refinement, total number of solvent molecules, etc.), we have determined and refined the PPACK–thrombin structure following a similar protocol used to determine the structures of hirulog 3–thrombin, hirugen–thrombin, and hirulog 1–thrombin (Skrzypczak-Jankun et al., 1991) to keep comparisons more meaningful and between comparably determined structures.[3] The crystallization conditions of PPACK–thrombin were those originally reported by Skrzypczak-Jankun et al. (1989). Although PPACK–thrombin crystals do not scatter as well as hirulog 3–thrombin, the X-ray diffraction intensity data collection was carried out in a manner similar to that described for the hirulog 3–thrombin complex with a crystal about 0.8 × 0.4 × 0.4 mm in size. The crystal was orthorhombic, space group $P2_12_12_1$, four molecules per unit cell with a = 88.26 Å, b = 68.02 Å, and c = 61.22 Å. A set of 10 472 unique reflections at 2.5-Å resolution was obtained (69% observed, R_{merge} = 0.064) by applying similar criteria in processing the raw intensity data as with the hirulog 3 complex. This set contains half of the possible reflections between 2.5- and 2.4-Å resolution. The initial model was that of thrombin of the hirulog 3–thrombin complex with PPACK of PPACK–thrombin (Bode et al., 1992). The autolysis loop was added during the course of least squares refinement. The final structure converged at R = 0.144 with an average thermal parameter of 28 Å^2 using only 208 water molecules and 9536 reflections from 7.0- to 2.4-Å resolution. The structure is practically identical to the reported PPACK–thrombin complex (Bode et al., 1992), except for a few side chains on the surface of the protein molecule. The rms differences between the main-chain and side-chain positions are just 0.30 and 1.19 Å, respectively, while residues Thr1H-Glu1C, Asp14L-Arg15, and Glu247 have no electron density. The final coordinates have also been deposited in the Brookhaven Protein Data Bank (Access Number 1ABJ).

RESULTS

(*a*) *Thrombin.* The thrombin structure is well-defined by the electron density except, as with the isomorphous hirugen and hirulog 1 complexes, for several autolysis loop residues (Trp148-Lys149E), terminal residues of the A chain (Ser1E-Glu1C and Ile14K-Arg15), and the C-terminus of the B chain (Glu247). These residues are on the surface of the protein and appear to be more flexible and loosely arranged. Excluding the disordered residues, the rms differences between thrombin in the hirulog 3 complex and the hirugen and hirulog 1 complexes (Skrzypczak-Jankun et al., 1991) are about 0.3 and 1.0 Å for the main-chain and side-chain positions, respectively (Table II). The rms differences between thrombin in the hirulog 3 and the hirudin complex (Rydel et al., 1991) are larger, most likely resulting from the different crystal packing. There are two peaks in the electron density near Asn60G, which carries a carbohydrate chain, but whose

[3] Comparisons with PPACK–thrombin throughout this paper are with this structure unless otherwise stated.

Table I: rms Deviations of Thrombin between the Hirulog 3–Thrombin Complex and Other Similar Structures

	hirugen	hirudin	PPACK
main chain (Å)	0.3	0.5	0.4
side chain (Å)	1.1	1.4	1.1
sulfur (Å)	0.2	0.3	0.3
55′–64′ (Å)	0.8	1.4	
PPACK (Å)			0.8
P1′–P4′ (Å)		5.8[a]	

[a] Average estimated value between the main chain of Gly4′–Gly7′ and hirudin Glu49′–His51′.

Table II: Hirulog 3 Pentaglycine Spacer–Thrombin Interactions

		distance (Å)	type
Lys60F NZ	Gly4′ O	2.99	hydrogen bond
Gly4′ N	Ser195 OG	3.10	weak hydrogen bond
His57 NE2	Gly4′ N	2.98	hydrogen bond
Gly193 N	Gly5′ O	3.18	possible hydrogen bond
Gly6′ N	Leu41 O	3.53	van der Waals interaction
Leu40 N	Gly7′ O	2.61	hydrogen bond
Gly7′ O	Glu39 OE2	3.57	van der Waals interaction

electron density corresponds well to two water molecules in size so that they are most likely not a partially disordered sugar hexose. The overall solvent pattern of the hirulog 3 complex is also consistent with that of the hirugen and hirulog 1 structures (Skrzypczak-Jankun et al., 1991).

(*b*) *Hirulog 3.* Most of the residues of the hirulog 3 structure are well-defined in the electron density including those of the pentaglycine bridge and most of those of the C-terminal helical turn that was disordered in the hirulog 1 complex (Skrzypczak-Jankun et al., 1991). The side chains of Glu61′ and Glu62′ are directed toward the solvent region and have no electron density, while Tyr63′ and Leu64′ show some discontinuity. There are two water molecules near the Glu62′ side chain and another one in the vicinity of Glu61′. In the complex, hirulog 3 is in an extended conformation, bending at Gly4′ and Asp55′ and then finishing with a 3_{10} helical turn formed by the last four residues. It embraces a full diameter of the thrombin surface (Figure 2) displaying many interactions at both the catalytic site and the fibrinogen anion-binding exo site of thrombin that resemble those formed by an enzyme–substrate complex. The D-Phe-Pro-β-homoArg moiety and the C-terminus of hirulog 3 have similar conformational angles to PPACK in PPACK–thrombin and the C-terminus of hirudin in the hirudin–thrombin complex, respectively (Table I; Bode et al., 1992; Rydel et al., 1991). All the ω angles of hirulog 3 are close to planar with only three residues out of conformationally allowed φ, ψ regions; Glu62′ and Tyr63′ have fair side-chain density while interestingly, and possibly importantly, Gly4′ $\varphi = 100°$, $\psi = -100°$, is far from conformationally allowed regions. The conformational angles of Gly4′ are acceptable for glycine, but this conformation will not be favored for amino acids with side chains. This may be related to the fact that it is adjacent to β-homoArg3′ that displaces it by a bond length from a natural peptide sequence. Not surprisingly, other β-homoArg derivatives with non-glycyl 4′ residues show reduced antithrombin activity (Kline et al., 1991).

Considering the resolution of the structure (2.3 Å), where carbonyl oxygen atoms of the main chain are only marginally defined, there remains a certain degree of ambiguity in interpreting the detailed placement of the glycine residues in the electron density (Figure 3). Thus, although the glycine spacer can be traced confidently, the detailed orientation of each peptide bridge is not as certain as other parts of the structure with well-defined side chains. This has introduced some ambiguity in defining the S3′–S4′ subsites extending from the active site (S1′–S4′ subsites and see Discussion).

As in the hirudin, hirugen, and hirulog 1 complexes (Rydel et al., 1991; Skrzypczak-Jankun et al., 1991), residue Gly8′ and Asn53′-Gly54′ have no electron density in the hirulog 3 structure (Figure 3). This was originally thought to be simple disorder but now appears to be the result of deamidation of Asn53′. Spontaneous aspartyl and asparaginyl deamidation, isomerization, and racemization have been studied extensively (Geiger & Clarke, 1987; Lura & Schirch, 1988; Wright, 1991). The reaction occurs in a matter of days at physiological temperatures and pH and is sequence-dependent: Asn-Gly gives a maximum reaction rate. The reaction goes through a succinimide intermediate and produces structural isomers. The nonenzymic deamidation of recombinant hirudin at Asn53′-Gly54′ has recently also been characterized (Bischoff et al., 1992), showing the formation of both α and γ isomers through the differential cleavage of an unstable succinimide intermediate between Asn53′ and Gly54′. The final α:γ (Asn/iso-Asn) ratio was found to be about 1:3. Since the sequence of Asn53′-Gly54′ also occurs in hirulog 3, and the period and conditions of crystallization are favorable for deamidation, multiple isomeric deamidation products are probably involved in obscuring the resolution of the Gly8′-Asn53′-Gly54′ sequence of the structure.

(*c*) *Hirulog 3–Thrombin Interaction.* (*i*) *Active Site.* The interaction of D-Phe1′-Pro2′-β-homoArg3′ of hirulog 3 at the thrombin active site is practically identical to that of D-Phe-Pro-Arg in the PPACK– and hirulog 1–thrombin structures with only minor positional shifts (Table I, Figure 4; Bode et al., 1989, 1992; Skrzypczak-Jankun et al., 1991). The D-Phe1′ and Pro2′ side chains are in an extensive hydrophobic region formed by Tyr60A, Trp60D, Leu99, Ile174, and Trp215 that is the apolar binding site of thrombin. Even with a β-homoarginine instead of an arginine at the S1 specificity site, the guanidinium group of β-homoArg3′ is still making a good hydrogen-bonding ion pair with Asp189 (2.67 and 2.93 Å) similar to normal arginine. The side-chain conformation of β-homoArg3′ appears to be in a less favored rotomer state, and the CA atom is noticeably shifted (Figure 4). Electron density maps calculated by excluding this residue did not alter the shape of the density in the site, suggesting that the different side-chain conformation is probably an effect of the methylene insertion. By contrast, the binding of hirudin at the thrombin active site is completely different (Rydel et al., 1991), its N-terminus running parallel (rather than antiparallel) to a chain segment of the enzyme with no side chain penetrating the primary specificity site.

The most significant change in the active site of thrombin is associated with Ser195 OG. The χ_1 changes from −70° in hirugen–thrombin and −92° in hirulog 1–thrombin (Skrzypczak-Jankun et al., 1991) to +28° in hirulog 3–thrombin. The χ_1 values of the former would orient OG toward CA3′ of hirulog 3 (Figure 1), which is in the place of the scissile carbonyl group of a substrate (Figure 4). The change of more than 90° in χ_1 in the hirulog 3 complex results in the formation of a hydrogen bond between Ser195 OG and N4′ of hirulog 3 (Table II and Figure 4). With the concomitant disruption of the hydrogen bond between Ser195 OG and His57 NE2 of the catalytic site, this hydrogen bond is replaced with one between Gly4′ N and His57 NE2 in the hirulog 3–thrombin complex. Thus, in addition to being unable to effect catalysis, general base function is disrupted, and considerable hydrogen-bond reorganization takes place in the catalytic site as a result of the introduction of the methylene group at the scissile bond position.

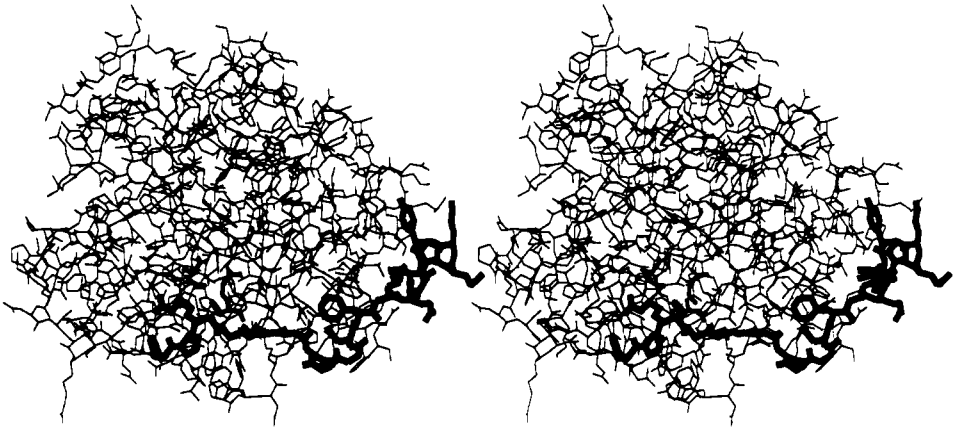

FIGURE 2: Stereoview of the hirulog 3–thrombin complex. Hirulog 3 and the catalytic triad are in bold; S1′, S2′, ... subsites are to the immediate right of the triad; no density is shown for Gly8′-Asn53′-Gly54′, whose structure is only modeled.

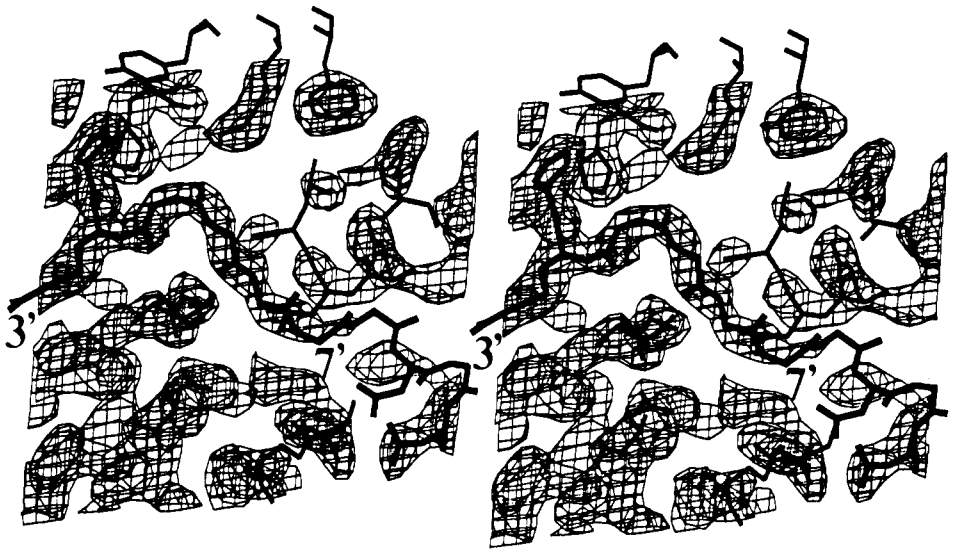

FIGURE 3: Stereoview of the final electron density near the pentaglycine region. Hirulog 3 is in bold in the electron density map; β-homoArg3′ and Gly7′ designed; no density is shown for Gly8′-Asn53′-Gly54′, which is only modeled.

(*ii*) *Hirudin Exo Site.* The interaction of Asp55′-Leu64′ of hirulog 3 at the fibrinogen recognition exo site is also similar to that of the hirudin C-terminus, hirugen, and the resolved portion of the hirulog 1 structure (Figure 5) (Rydel et al., 1991; Skrzypczak-Jankun et al., 1991). The exo site of thrombin is a clifflike wall formed by the Phe34-Leu40 and Arg73-Met84 loops of thrombin, so that Asp55′-Leu64′ is mainly surrounded by residues of these loops plus a few water molecules. The most important interactions include the following: (1) Asp55′ makes a hydrogen-bonded salt bridge with Arg73 (Arg73 NH2–Asp55′ OD1, 2.73 Å), (2) Glu57′ makes an ion pair with Arg75 of a crystallographically 2-fold-related neighboring molecule, and (3) Phe56′, Ile59′, Pro60′, Tyr63′, and Leu64′ are stacked in an extensive apolar cavity formed by Phe34, Gln38, Leu65, Ile82, and Tyr76. In addition, the distances of Lys81 NZ to Tyr63′ OH and Lys36 NZ to the carboxylic group of Leu64′ are less than 4.0 Å, so that Lys81 and Lys36 may be involved in aiding the docking of the C-terminal helical turn in a polar way even though the main interaction of the region is generally undirectional and very hydrophobic.

Notwithstanding the similarities in the structures of hirudin and analogs complexed with thrombin, there are some novel aspects to the hirulog 3 complex in the exo site. In the hirudin structure, Glu58′ makes an ion pair with Arg77A, the autolytic β-cleavage site of thrombin; in the hirugen structure, the Glu58′ side chain was disordered and possibly interacting with the disordered autolysis loop. The hirulog 3 complex is also ambiguous about the side chain of Glu58′, which has no density for CB and CG but with density that may be solvent near a position that could be the carboxylate group. Thus, the protection against β-cleavage at Arg77A by hirugen and hirulog 3 appears to be more by physical obstruction rather than by specific interaction. However, the absence of Gln65′ and lack of sulfation of Tyr63′ in hirulogs do not affect the binding interaction of hirulog 3 significantly even though sulfation produces a 10-fold increase in binding avidity (Dodt et al., 1988; Braun et al., 1988). The crystal structure of the hirugen–thrombin complex reveals that although the sulfato oxygen atoms of Tyr63′ hydrogen bond with thrombin directly or through mediating water molecules, these oxygen atoms make no ion pairs with oppositely charged residues of thrombin. An unexpected result from the hirulog 1–thrombin crystal structure was that the Pro60′-Leu64′ residues of the peptide are disordered. Consequently, detailed structural evaluation of the lack of Tyr63′ sulfate and lack of Gln65′ could not be made. In the hirulog 3 structure, Pro60′-Leu64′ are fairly well resolved although the quality of the density is not as good as that in hirugen. Examining the helical turn in hirulog 3 shows that the binding region is mainly hydrophobic and that the sulfation of Tyr63′ augments the binding. Thus, the disorder of the region in the hirulog 1–thrombin complex is not the result of lack of sulfation or lack of a Gln65′ C-terminus as was previously speculated (Skrzypczak-Jankun et al., 1991).

(*iii*) *S1′–S4′ Subsites.* The methylene insertion in β-homoArg3′ in hirulog 3 prevents proteolysis. Moreover, the

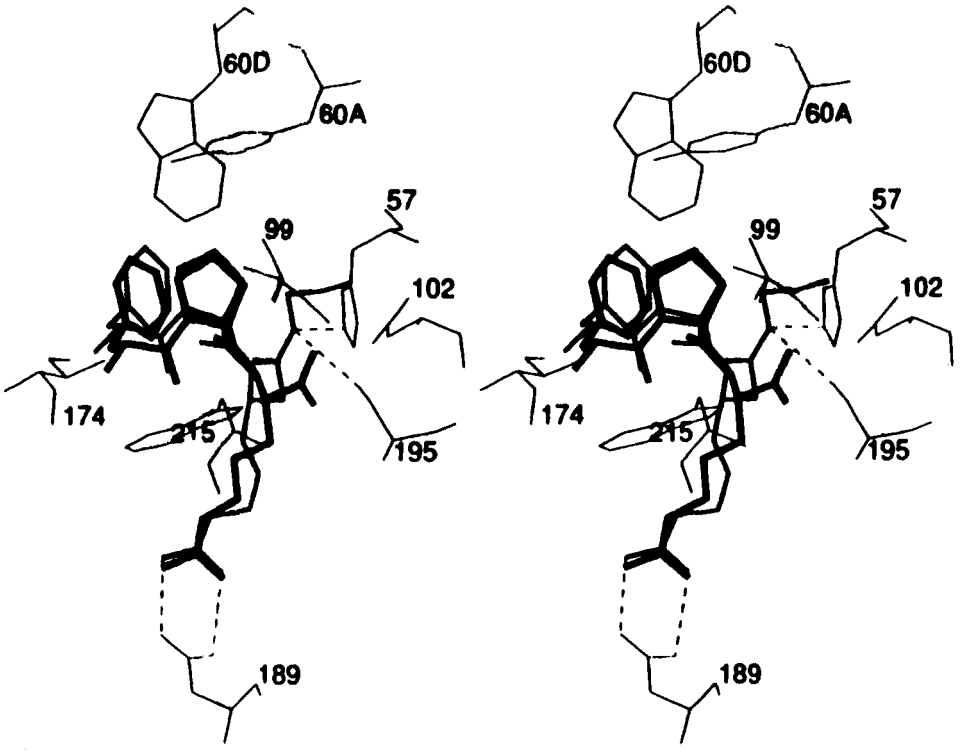

FIGURE 4: Stereoview comparing interactions in the active site between D-Phe-Pro-β-homoArg-Gly in the hirulog 3 complex and PPACK in the hirulog 1 complex. The former are in medium and the latter are in bold lines; hydrogen bonds are shown with broken lines.

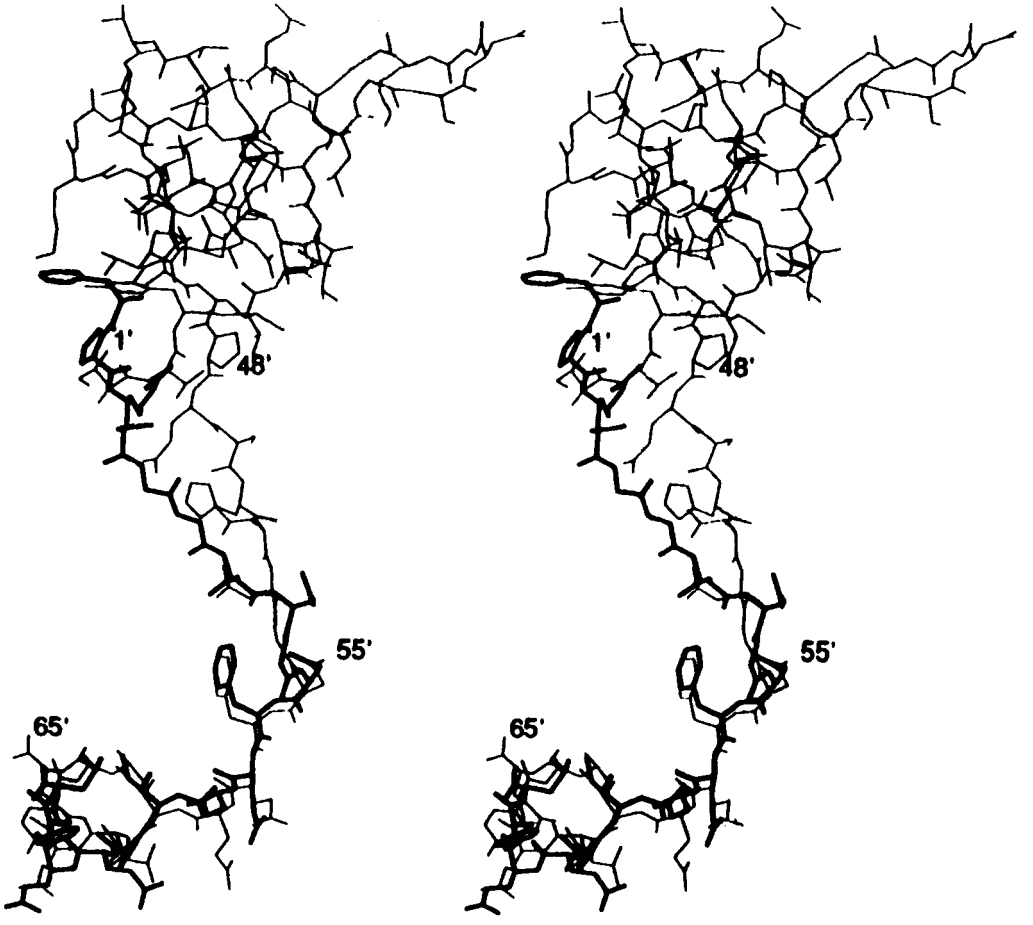

FIGURE 5: Stereoview comparing the location of hirulog 3 and the C-terminal of hirudin in their respective thrombin complexes: hirulog 3, bold; Gly8′-Asn53′-Gly54′ and Asn52′-Asn53′-Gly54′ of inhibitors, only modeled; pertinent residues, numbered.

first four glycine residues of the pentaglycine spacer (Gly4′-Gly8′) are well resolved in the electron density (Figure 3). This is the first structural documentation of the P1′ to P4′ positions of a thrombin substrate bound at the S1′ to S4′ subsites. Hirugen (Skrzypczak-Jankun et al., 1991), PPACK (Bode et al., 1989, 1992), and several other active site inhibitors (Banner et al., 1991; Tulinsky & Qiu, 1992) do not interact with these subsites, while hirudin takes a different path out of the active site where it binds through Ile1′-Tyr3′ (Rydel et al., 1991). In the case of hirulog 1, the Arg3–Pro4′ bond

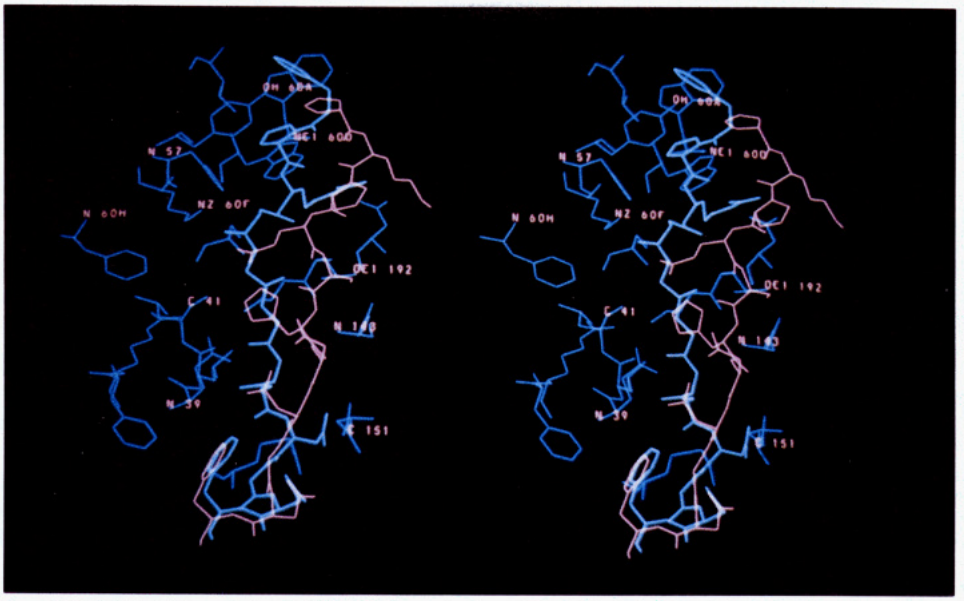

FIGURE 6: Stereoview of S1′–S4′ subsites of thrombin: hirulog 3 (D-Phe1′-Phe56′), light blue; hirudin (Pro46′-Phe56′), pink; thrombin, dark blue; Gly8′-Asn53′-Gly54′ and Asn52′-Asn53′-Gly54′ of inhibitors, only modeled.

is cleaved, like that of a substrate, with the glycine spacer bridge to Asp55′ being totally disordered in the structure (Skrzypczak-Jankun et al., 1991). The importance of the S1′ to S4′ sites in the binding of fibrinopeptide A fragments is indicated by the decrease of K_d by an order of magnitude with the presence of the P3′–P4′ residues (Marsh et al., 1983). The addition of two or three more amino acids of fibrinogen essentially does not alter K_d further; however, this might be dependent upon the nature of the P5′ to P7′ side chains at these positions.

The structure of the pentaglycine spacer of hirulog 3 and nearby thrombin residues is shown in Figure 6. After an initial turn at Gly4′, the remaining spacer residues are in an extended conformation, and since they have no side chains, their interaction with the host only involves main-chain atoms. The position of Gly4′ is firmly anchored by three hydrogen bonds with His57, Lys60F, and Ser195 (Table III), and the S1′ site inferred from the vicinity of the CA position of Gly4′ is lined, in part, by the "back side" of the apolar S2 subsite (His57, Tyr60A, Trp60D). The lysyl side chain of Lys60F, some 5–6 Å from the aromatic residues, completes closing off the region to produce a small cavity. The carboxyl oxygen atom of the next glycine of the spacer makes an apparently weaker hydrogen bond with Gly193 N (Table III). Although the side group of Glu192 is also close by, since there is no electron density for its carboxylate group, it is not clear whether the latter contributes to the Gly5′ interaction. In fact, reorienting the side chain of Glu192 can place the carboxylate group at an alternate position that was interpreted as two water molecules. However, in this case there would be no density corresponding to the CB and CG atoms of Glu192. Thus, Glu192 may be distributed between two alternate positions. A critical role for the position of Glu192 is suggested in protein C activation by the thrombomodulin–thrombin complex, which displays sensitivity to the substrate sequence binding to thrombin (Le Bonniec & Esmon, 1991). This is also in agreement with our own observations of the crystal structures of several different substrate sequences (unpublished results of this laboratory). In either position, Glu192 appears to be too distant to interact with the CA vicinity of P2′ (the present position of Gly5′ CA). Since the position of the latter residue cannot be changed much without affecting Gly4′ significantly, Glu192 is most likely not involved in the S2′ subsite.

Table III: Peptide Sequence around the Cleavage Site of Thrombin-Susceptible Bonds in Proteins from Human Plasma

substrate	specificity site[a]	
fibrinogen A 12–24	GGGVR	GPRVVERH
fibrinogen A 15–27	VRGPR	VVERHQSA
fibrinogen B 10–22	FFSAR	GHRPLDKK
prothrombin 151–163	MVTPR	SEGSSVNL
prothrombin 282–294	FFNPR	TFGSGEAN
factor V 706–718	ALGIR	SFRNSSLN
factor V 1014–1026	PLSPR	TFHPLRSE
factor V 1541–1553	AWYLR	SNNGNRRN
factor VIII 368–380	FIQIR	SVAKKHPK
factor VIII 736–748	AIEPR	SFSQDSRH
factor VIII 1309–1371	TQSKR	ALKQFRLP
factor VIII 1685–1697	NQSPR	SFQKKTRH
factor XIII 33–42	GVVPR	GVNLQEF
factor XIII 509–518	EGVMK	SRSNVDM
platelet receptor 37–50	TLDPR	SFLLRNPN
antithrombin III 389–399[c]	VIAGR	SLNPNR
factor VII 148–157[b]	KPQGR	IVGGKV
factor XI 365–377[c]	KIKPR	IVGGTASR
protein C8–17[c]	QVDPR	LIDGKV

[a] Fibrinogen: Blomback, B., Blomback, M., Hessel, B., & Iwanaga, S. (1967) *Nature 215*, 1445–1448. Prothrombin: Downing, M. R., Butkowski, R. J., Clark, M. M., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 8897–8906. FV and FVIII: Kane, W. H., & Davie, E. W. (1988) *Blood 71*, 539–555. FXIII: Takahashi, N., Takahashi, Y., & Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8019–8023. Platelet receptor: Vu, T. H., Wheaton, V. I., Hung, D. T., Charo, I., & Coughlin, S. R. (1991) *Nature 53*, 674–677. Antithrombin III: Pratt, C. W., & Church, F. C. (1991) *Semin. Hematol. 28*, 3–9. FVII: Hagen, F. S., Gray, C. L., O'Hara, P., Grant, F. J., Saari, G. C., Woodbury, R. G., Hart, C. E., Insley, M., Kisiel, W., Kurachi, K., & Davie, E. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2412–2416. FXI: Fujikawa, K., Chung, D. W., Hendrickson, L., & Davie, E. W. (1986) *Biochemistry 25*, 2417–2424. Protein C: Kisiel, W. (1979) *J. Clin. Invest. 64*, 761–769. [b] Not specific only to thrombin. [c] Cofactor needed.

The residues of Lys60F on one side, Leu41, Phe60H in back, and Glu39 from another side form a much larger surface cavity than that of the S1′ site (Figure 6). This region, which appears capable of harboring two moderately sized side chains, appears to constitute the S2′–S3′ sites of thrombin. In the hirudin complex, Glu49′ and His51′ side chains occupy these positions from a distance and participate in a complicated multicharged interaction involving three positive charges from Arg35 (mediated by a water), Lys60F, and His51′ and negative ones from Glu39, Glu49′, and possibly Glu192, while Ser50′ extends out into solvent and is near Glu192 (Rydel et al.,

1991). Thus, the larger cavity might actually correspond to the S2′ and S4′ sites with the S3′ position of substrate simply involving a surface interaction with thrombin in the vicinity of Asn143 and Glu192, which is in agreement with the effects of substituting glutamine for Glu192 in thrombin (Le Bonniec & Esmon, 1991). However, Glu39 also appears to be a component of the S3′ subsite, at least when P3′ is an aspartate (Le Bonniec & Esmon, 1991).

The spacer interaction continues from Gly5′ with an extended hydrogen bond-like contact between Gly6′ N and Leu41 O (Table III), while Gly7′ is hydrogen bonded to Leu40. If Gly7′ had a side chain, it would be directed toward solvent in the present conformation, similarly to Ser50′ of the hirudin–thrombin complex, but with a twist of the peptide in one direction or another, it could interact with Glu39 on one side or Asn143 and Gln151 on the other. Since precedence has already been set for the former interaction in the hirudin–thrombin complex, Asn143 seems more suitable for assisting S3′ site interactions, while Gln151 is closer to Asn53′ at the S6′ site. The electron density of the last glycine spacer residue deteriorates in the vicinity of the Asn53′-Gly54′ disorder (Figure 3), rendering the S5′–S7′ sites unclear.

DISCUSSION

Hirulogs are a class of potential antithrombin drugs that can block thrombin coagulant activities at nanomolar concentrations by interacting with both the fibrinogen anion-binding exo site and the active site (Maraganore et al., 1989; Witting et al., 1992), and they are by far the most efficient thrombin inhibitors outside more extensive natural peptides like hirudin. Hirulog 3 represents an important thrust in the direction of better design of hirulog analog drugs. The crystal structure of thrombin in the presence of different types of inhibitors has revealed that the several binding sites of thrombin are utilized with considerable flexibility (Tulinsky & Qiu, 1992). The hirudin C-terminal binding at the proposed fibrinogen recognition site (Rydel et al., 1991) provides a credible model for fibrinogen binding at the exo site, notwithstanding the fact that the structure of a fibrinogen–thrombin exo-site complex remains to be determined; fibrinogen binding in the active site has been described with the structure of a fibrinopeptide A–thrombin complex (Martin et al., 1992; Stubbs et al., 1992). The PPACK–thrombin structure shows the positioning of an arginine residue in the primary specificity site and the nature of the apolar S2 subsite and is a fine model for substrate binding at the catalytic site (Bode et al., 1989, 1992). A putative heparin binding site has even been inferred for the thrombin structure on the basis of electrostatic potential calculations (Bode et al., 1992; Karshikov et al., 1992), while the present work does much to define the S′ binding sites of substrates immediate to the catalytic site. Even though substrate side-chain interactions with thrombin have not been directly observed because of the use of a pentaglycine spacer, nonetheless, the structure of hirulog 3 is very useful for modeling various inhibitors or substrates at the S′ sites, which have been shown to be important for binding to serine proteinases in general (Laskowski & Kato, 1980; Reed & James, 1986).

Since the P1′–P4′ residues of hirulog 3 are all glycine, their interaction with thrombin is diminished because of the lack of side groups. Therefore, the antithrombotic potency of hirulog 3 or analogs thereof can probably be improved by rationally optimizing the interaction of the spacer residues with thrombin from interactive computer graphics molecular modeling considerations. Side chains at S1′ will necessarily be restricted to be small because of space limitations. This would suggest glycine or alanine as suitable P1′ residues. However, with the location of the ε-amino group of Lys60F in the same vicinity, small polar groups, such as serine, might be better. This is also borne out by examining the P1′ site of natural substrates of thrombin (Table III). Threonine is a possibility, if it is small enough to fit into the cavity. This would be especially so if it were oriented such that its methyl group abuts the back side of the apolar S2 wall made up of His57, Tyr60A, and Trp60D and its hydroxyl group hydrogen bonds or otherwise interacts with Lys60F NZ, as serine must do. Binding in the S2′ subsite is modeled well by a small bulky hydrophobic residue making many van der Waals contacts with the methylene carbon atoms of Lys60F and the ring of Phe60H; the latter lines the interior wall along with Leu41. Valine, leucine, and isoleucine are observed frequently at the P2′ position of natural thrombin substrates (Table III); phenylalanine is also found at the P2′ position, most likely because it is small enough to be accommodated and can enter into an aromatic stacking interaction with Phe60H.

The nature of the S3′-S4′ subsites is much less definitive, primarily because of the uncertainty of the conformation of CA of P3′, which could be directed out from the complex and not interact at all, as with certain hirudin subsites (Tulinsky & Qiu, 1992), or possibly produce surface interactions with Glu192. The S3′ site would seem to be modeled well by His51′ or hirudin, which forms an ion pair with Glu39 (Rydel et al., 1991). Fibrinogen and factor V have an arginine at the P3′ position while other substrates have either a carboxamide, which could hydrogen bond to Glu39, or a small side group (Table III), which could jut out in solvent (but require a twist of the main chain near P3′). The P4′ position appears to be also capable of interacting with S3′ through a long, positively charged side chain or carboxamide. Otherwise, the most likely interactions of P4′ residues with thrombin have to be with Asn143 and/or Gln151.

The structure of the hirudin–thrombin complex (Rydel et al., 1991) provides supportive evidence implicating the S1′–S4′ subsites of thrombin. The superposition of the hirulog 3 and hirudin structures (Table I, Figures 5 and 6) shows that the two are very similar from Asp55′ to Leu64′ but that although the peptide from Lys47′ to Gly54′ is in an extended conformation in both structures, the path of the chain is far from identical and is displaced with respect to thrombin by about 6.0 Å on the average. The main chain of the pentaglycine spacer of hirulog 3 makes a much closer contact with thrombin (Figure 6). The positions of Lys47′-Pro48′ of hirudin do not correspond to the D-Phe-Pro-β-homoArg positions of hirulog 3 because they do not bind in the active site like the latter. Instead, it is the N-terminal Ile1′-Thr2′-Tyr3′ of hirudin that binds there, and this, in a "nonsubstrate" orientation. Thus, the hirudin C-terminus in the vicinity of the S′ subsites is positioned very differently from hirulog 3. Nonetheless, the side chains of Glu49′-His51′ of hirudin interact impressively in an intricate and concerted way with thrombin. The tolerance of thrombin for imprecision in binding has already been pointed out and discussed elsewhere (Tulinsky & Qiu, 1992); the accommodation of Glu49′-His51′ by the S2′-S4′ subsites extends this flexibility to yet an additional region of the fibrinogen recognition site. The only question that remains is whether natural substrates also display the close contact, main-chain hydrogen-bonding interactions of the pentaglycine spacer (Table II). Judging from the general binding behavior of exo-site subsites (Tulinsky & Qiu, 1992), it would seem that the extent of utilization of main-chain interactions would be variable (as with hirudin where there are none) and sequence-dependent.

Two other considerations are pertinent with respect to S′ subsite binding. The first is that the β-homoArg3′ substitution introduces a displacement of the peptide chain of about a bond length. This might be of some consequence in the immediate vicinity of the scissile bond, but its perturbing effect should dwindle and disappear as the distance from the point of catalysis increases. A more puzzling aspect of exo-site binding is the potentially strained conformation of Gly4′. Natural substrates have either Gly or Ser at this position (Table III). Although $\varphi = 90°$, $\psi = -90°$ are unacceptable conformational angles for serine and must be closer to allowed regions, nonetheless, they could be in a strained conformation to enhance catalysis. Conversely, however, the Gly4′ conformation might simply be an artifact of β-homoArg3′ and its additional carbon–carbon bond. This agrees with the tolerance displayed by thrombin for a proline at the P1′ position in hirulogs 1 and 2, both of which are substrates of thrombin, although poor ones (Witting et al., 1992).

In inferring suitable residues for S1′–S4′ subsite interactions, the consideration of the different rotomers that are possible from free bond rotations was kept to a minimum. Thus, other side groups or orientations may also be fairly suitable in addition to those indicated. It is of note that bulky Leu/Ile groups appear at the S1′ subsite, but only with substrates requiring a cofactor (Table III). The present work would seem to suggest that this would only be possible with a conformational change, in this case, accompanying cofactor binding.

The thrombin–substrate interactions of the hirulog 3–thrombin complex provide revealing conclusions concerning details of the S′ subsites at the molecular level immediate to the active site. A most evident factor emerging from all the foregoing analysis is the corresponding P′ amino acid residues of substrates and inhibitors must confer substantial additional specificity and stability to thrombin action. In this context, the correlation between the complementary of the S′ sites and the corresponding P′ positions of natural substrates (Table III) is particularly striking and impressive.

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