Interactions of Hirudin-Based Inhibitor with Thrombin: Critical Role of the Ile^{H59} Side Chain of the Inhibitor†

Yuko Tsuda,‡ Zbigniew Szewczuk,§ Jing Wang, Shi Yi Yue,|| Enrico Purisima,* and Yasuo Konishi*

Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Quebec, Canada H4P 2R2

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ABSTRACT: Hirudin is the most potent and specific thrombin inhibitor from medicinal leech with a K_i value of 2.2×10^{-14} M. It consists of an active site inhibitor segment, $hirudin^{1-48}$, a fibrinogen-recognition exosite inhibitor segment, $hirudin^{55-65}$, and a linker, $hirudin^{49-54}$, connecting these inhibitor segments. The role of the side chain of the hirudin 59th residue, Ile, is studied by using a series of synthetic bivalent thrombin inhibitors, which mimic the binding mode of hirudin. The synthetic inhibitors based on the hirudin sequence have a general sequence of Ac-(D-Phe)-Pro-Arg-Pro-(4-aminobutyric acid)-(7-amino-heptanoic acid)-Asp-Phe-Glu-Glu-Xaa-Pro-Glu-Glu-Tyr-Leu-Gln-OH, in which the 59th residue, Xaa, is substituted by various natural and unnatural L-amino acids. For example, substitution of Ile^{H59} by Val, which is equivalent to removing the δ-methyl group of Ile^{H59}, reduces the affinity of the inhibitor 5.7-fold ($\Delta\Delta G° = 1.0$ kcal/mol) to a K_i value of 4.7 nM compared to that ($K_i = 0.82$ nM) of the corresponding inhibitor with Ile^{H59}. Removal of the entire side chain of Ile^{H59}, i.e., a substitution of Ile^{H59} by Gly, reduces the affinity of the inhibitor 6300-fold, revealing the critical role of the Ile^{H59} side chain in the inhibitor binding. Theoretical free energy calculation successfully reproduces the binding free energy of most of the analogs. It suggests that intra- and intermolecular van der Waals interactions of δ-CH_3, γ-CH_3, and γ-CH_2 of Ile^{H59} play the major role in the binding affinity. Further search of possible methyl group(s) which may be incorporated in the side chain of the 59th residue results in the substitution of Ile^{H59} with *tert*-butylalanine, improving the inhibitor affinity 2.1-fold ($K_i = 0.39$ nM).

Thrombin (EC 3.4.21.5), a serine protease, is a key enzyme in blood coagulation and hemostasis. Thrombin is generated by proteolytic cleavages of its circulating zymogen, prothrombin. Thrombin as a coagulation enzyme cleaves specifically Arg-Xaa peptide bonds of fibrinogen to convert the soluble fibrinogen to the insoluble fibrin clot. Thrombomodulin converts thrombin to an anticoagulation enzyme by changing its specificity to activate protein C, which then inactivates coagulation enzymes, factors V and VIII (Esmon et al., 1986). Another anticoagulation process in plasma is the inhibition of thrombin by antithrombin III, forming a one-to-one covalent complex in the presence of heparin (Davie et al., 1991). Thrombin is mainly cleared through the thrombin−antithrombin III complex.

Besides these plasma anticoagulants, blood-sucking animals have various types of anticoagulants. Hirudin from leech *Hirudo medicinalis* (Markwardt, 1970) is a small protein of 65 amino acid residues and is the most potent thrombin inhibitor with a K_i value of 2.2×10^{-14} M (Stone & Hofsteenge, 1986). The inhibition mechanism of hirudin is unique compared to other protease inhibitors. The N-terminal domain, $hirudin^{1-48}$, binds to the thrombin active site, whereas the C-terminal domain, $hirudin^{55-65}$, binds to FRE[1] (Rydel et al., 1990; Grütter et al., 1990). These two bindings are independent. Indeed, the N-terminal fragment, $hirudin^{1-49}$, binds to the thrombin active site with a K_i value of 7.2×10^{-8} M, and the C-terminal fragment, Ac-$hirudin^{55-65}$, binds to the thrombin FRE with a K_d value of 1.6×10^{-6} M (Chang, 1990; Mao et al., 1988; Hopfner et al., 1993). The binding energy of these fragments additively contribute when they are linked by a linker, $hirudin^{50-54}$, for simultaneous binding, resulting in a K_i value of 2.3×10^{-13} M for r-hirudin (Braun et al., 1988). The finding of the independent binding of the N- and C-terminal fragments motivated designs of hirudin-based FRE inhibitors (Maraganore et al., 1989; Krstenansky et al., 1990) and synthetic bivalent inhibitors which bind to both the thrombin active site and FRE simultaneously (Maraganore et al., 1990; DiMaio et al., 1990, 1991; Yue et al., 1992).

$Hirudin^{55-65}$ has a sequence composed of five hydrophobic residues, five negatively charged residues, and a C-terminal carboxyl group. These hydrophobic and acidic residues form an amphiphilic structure when it binds to thrombin FRE, i.e., the hydrophobic residues face toward thrombin's FRE and the side chains of the acidic residues are exposed to solvent (Rydel et al., 1990, 1991). This amphiphilic structure of the FRE inhibitor complements the surface of thrombin FRE, which is characterized by a region of hydrophobic residues

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* Author to whom correspondence should be addressed.

‡ Present address: Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Nishi-ku, Kobe 651-21, Japan.

§ Present address: Institute of Chemistry, University of Wroclaw, ul. Joliot-Curie 14, PL-50-383, Wroclaw, Poland.

|| Present address: Molecular Research Institute, Palo Alto, CA.

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[1] Abbreviations: αAbu, 1-aminobutyric acid; γAbu, 4-aminobutyric acid; Ac, acetyl; allo-Ile, alloisoleucine; AMC, 7-amino-4-methylcoumarin; ζAph, 7-aminoheptanoic acid; Boc, *tert*-butyloxycarbonyl; FRE, fibrinogen recognition exosite; HPLC, high-performance liquid chromatography; Nle, norleucine; NVa, norvaline; r-hirudin, recombinant hirudin; Tba, *tert*-butylalanine; Tbg, *tert*-butylglycine; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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surrounded by basic residues. Electrostatic potential contours of thrombin FRE and recombinant hirudin$^{55-65}$ show complementary potentials (Karshikov et al., 1992). Indeed, the negatively charged residues contribute to the association rate constant in the hirudin−thrombin complex (Braun et al., 1988; Stone et al., 1989; Betz et al., 1991a). The dissociation rate constant, on the other hand, is fairly constant against the ionic strength of the solution or against substitutions of Glu residue(s) by Gln (Stone & Hofsteenge, 1986; Braun et al., 1988; Stone et al., 1989). Thus, interactions other than ionic interaction must play an important role in the dissociation rate constant and, consequently, stabilize the thrombin−hirudin complex.

A small fraction of hirudin$^{55-65}$ forms by itself a structure similar to its active conformation with a hydrophobic cluster composed of Phe^{H56}, Ile^{H59}, Pro^{H60}, Tyr^{H63}, and Leu^{H64} as observed in an NMR study (Ni et al., 1992).[2] Among these hydrophobic residues, substitution of each residue by Gly reveals that the Ile^{H59} side chain is critical for binding (Yue et al., 1992). In the complex with thrombin, the Ile^{H59} side chain forms van der Waals contacts with Tyr^{H63} and Leu^{H64} intramolecularly and with Phe^{34}, Leu^{65}, Tyr^{76}, Ile^{82}, and the aliphatic part of Arg^{67} of thrombin intermolecularly (Zdanov, et al., 1993).[3] It is also situated at the center of the hydrophobic core in the complex. A detailed study on the contribution of the Ile^{H59} side chain to the binding was carried out using synthetic bivalent inhibitors, Ac-dFPRP-ζAhp-γAbu-DFEE-X-PEEYLQ-OH, where X is Ile or various amino acid residues. Theoretical free energy calculation was also applied to these analogs to investigate the critical role of the Ile^{H59} side chain in the binding.

EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin (3000 NIH units/mg), bovine fibrinogen (~70% protein, 85% of protein clottable), Tos-Gly-Pro-Arg-AMC·HCl salt, poly(ethylene glycol) 8000, and Tris were purchased from Sigma. AMC was obtained from Aldrich. Boc-αAbu, Boc-γAbu, Boc-ζAph, and Boc-allo-Ile were purchased from BaChem. Boc-Nva was obtained from STAR Biochemicals, Inc. All other amino acid derivatives for peptide synthesis were purchased from Advanced ChemTech. The side chain protecting groups for Boc-amino acids were benzyl for Glu, cyclohexyl for Asp, Tos for Arg, and [(2-bromobenzyl)oxy]carbonyl for Tyr. Boc-Gln-OCH_2-phenylacetylamidomethyl resin (0.7 mmol/g) was purchased from Applied Biosystems Inc. The solvents for peptide synthesis were obtained from B&J Chemicals and Applied Biosystems Inc. Citric acid was purchased from Anachemia. HF and TFA were purchased from Matheson and Halocarbon Products Co., respectively.

Peptide Synthesis. The peptides reported in this paper were prepared by manual solid-phase synthesis according to a method described elsewhere (Szewczuk et al., 1992). Only single coupling was performed followed by acetylation with acetic anhydride at each cycle. Peptides were cleaved from the resin using HF in the presence of anisole (5% volume) and dimethyl sulfide (5% volume) at −5 °C for 60 min. After evaporation of HF, the peptides were washed with ethyl ether and extracted with 50% acetic acid followed by water prior to lyophilization.

The peptides were then purified by a preparative HPLC column (Vydac C_4, 4.6 × 25 cm) using a linear gradient of 20−50% acetonitrile in 0.1% TFA (0.5%/min gradient, 33 mL/min flow rate). The final products were lyophilized with 98% or higher purity estimated by an analytical HPLC column (10−70% acetonitrile in 0.1% TFA, Vydac C_{18}, 0.46 × 25 cm column, 1.0%/min gradient, 1.0 mL/min flow rate). The elution profile was monitored by absorbance at 210 nm. The peptides were identified with a Beckman model 6300 amino acid analyzer and a SCIEX API III mass spectrometer. Amino acid analysis was used for peptide content determination. All peptides used in this article have correct amino acid composition and molecular weight. Two peptides, P24 and P151, were reported previously [P24, Szewczuk et al. (1992), and P151, Szewczuk et al. (1993)]. However, we resynthesized P24 and measured its IC_{50} because the lower concentration of thrombin (0.10 NIH unit/mL compared to 0.20 NIH unit/mL in the previous work) was used in this article. All other peptides were synthesized for this article.

Fibrin Clotting and Amidolytic Assays. The fibrin clotting assay was performed in 50 mM Tris·HCl buffer (pH 7.52 at 37 °C) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 with 9.0 × 10^{-10} M (0.1 NIH unit/mL) and 0.03% (w/v) of the final concentrations of human thrombin and bovine fibrinogen, respectively, at 37 °C (Szewczuk et al., 1992). The clotting time was plotted against the inhibitor concentrations, and the IC_{50} was estimated as the inhibitor concentration required to double the clotting time relative to the control.[4]

The inhibition of the amidolytic activity of human α-thrombin was measured spectrophotometrically using Tos-Gly-Pro-Arg-AMC as a fluorogenic substrate in 50 mM Tris·HCl buffer (pH 7.80 at 25 °C) containing 0.1 M NaCl and 0.1% poly(ethylene glycol) 8000 at room temperature (Szewczuk et al., 1992). The final concentrations of the inhibitors, the substrate, and human α-thrombin were 0.5−1000-fold of K_i, 1−8 μM, and 60 pM, respectively. The reaction was monitored on a Varian-Cary 2000 spectrophotometer in the fluorescence mode (λ_{ex} = 383 nm, λ_{em} = 455 nm) or a Hitachi F2000 fluorescence spectrophotometer (λ_{ex} = 383 nm, λ_{em} = 455 nm), and the fluorescent intensity was calibrated using AMC. The kinetic data were analyzed using the methods described by Segel (1975). A nonlinear regression program, RNLIN in the IMSL library (IMSL, 1987) or LMDER in MINPAC library (More et al., 1980), was used to estimate the kinetic parameters (K_m, V_{max}, and K_i).

Calculation of Free Energy Change. The conformational free energy of a system is given by

$$G = -kT \ln Z \quad (1)$$

where k is Boltzmann's constant and Z is a partition function given by

$$Z = \int \exp[-E(\Omega)/kT]d\Omega \quad (2)$$

where $E(\Omega)$ is the energy at a conformational state (Ω) and

[2] The numbering of the inhibitor residues is based on the hirudin sequence, and "H" is added to the residue number, e.g., Ile^{H59}.

[3] The numbering of human α-thrombin residues is based on the chymotrypsin sequence (Bode et al., 1989).

[4] The inhibitor concentrations required to double the clotting time were 2.03-fold higher than those required to inhibit 50% of human α-thrombin at the enzyme concentration of 0.10 NIH unit/mL.

the integration is over all possible conformations of thrombin and inhibitor atoms in the system. Since we are interested in configurational free energy differences, the constants in Z cancel out in the relevant expressions and are not included in eq 2. The binding free energy of an inhibitor is the free energy difference between the dissociated and associated systems of thrombin and the inhibitor:

$$\Delta G_b = -kT \ln(Z_b/Z_f)$$

where Z_b and Z_f are the partition functions of the associated and dissociated states, respectively. The change of the binding free energy due to a substitution of Ile^{H59} in an inhibitor, Ac-dFPRP-ζAhp-γAbu-DFEE-Ile^{H59}-PEEYLQ-OH (P151), by an amino acid, Xaa, is given by

$$\Delta\Delta G_b = -kT \ln[(Z_b)_{Xaa}(Z_f)_{Ile}/(Z_b)_{Ile}(Z_f)_{Xaa}] \quad (3)$$

where $(Z_b)_{Ile}$ and $(Z_f)_{Ile}$ are the partition functions of the associated and dissociated states of the thrombin−P151 system, respectively. Similarly, $(Z_b)_{Xaa}$ and $(Z_f)_{Xaa}$ are the partition functions of the associated and dissociated states of the thrombin−P151 analog system, respectively.

In general it is extremely difficult to evaluate the partition function due to the size of the phase space to be explored. However, a recently developed method (Wang & Purisima, 1995; manuscript in preparation) that combines conformational search and a harmonic well approximation to energy minima (Go et al., 1968; Gibson & Scheraga, 1969a,b) provides an estimate of the contribution of each local minimum to the partition function. In this method, a systematic search is first carried out by varying chosen rotatable bonds (the side chain torsions of Xaa^{H59} in the bound state of the inhibitor and the side chain and backbone torsions of Xaa^{H59} in the free state of the inhibitor) to obtain sterically feasible conformers. These are then energy minimized allowing all atoms in the inhibitor and protein side chains in contact with Ile^{H59} to move. The free state is approximated by the tripeptide Ac-Glu-Xaa-Pro-$NHCH_3$. The energies obtained in the minimization are then used to construct the partition function as

$$Z = \sum_{i=1}^{N} \exp(-E_i/kT) \quad (4)$$

where N is the number of the feasible conformations.

The starting point for the calculation on the thrombin−inhibitor complex is the crystal structure of Zdanov et al. (1993), and Sybyl 6.0 (Tripos Assoc. Inc.) was used for the calculation and visualization of the structure. The technical details of the calculation are described elsewhere (Wang & Purisima, 1995; manuscript in preparation).

RESULTS

Contribution of Each Side Chain Atom of Ile^{H59}. Several bivalent thrombin inhibitors, Ac-(D-Phe)-Pro-Arg-Pro-ζAhp-γAbu-Asp-Phe-Glu-Glu-**Xaa**-Pro-Glu-Glu-Tyr-Leu-Gln, were synthesized, where Xaa is Gly, Ala, αAbu, or Val. The inhibitory activities of these analogs measured by amidolytic and clotting assays are listed in Table 1. Substitution of Ile^{H59} by Val is equivalent to the removal of the δ-CH_3 group of the Ile side chain and results in a 5-fold increase of the K_i value. The free energy difference of the two inhibitors in the affinity to thrombin is

$$\Delta\Delta G^\circ = -RT \ln[(K_i)_{Ile}/(K_i)_{Val}]$$

$$= 1.03 \text{ kcal/mol}$$

Consequently, the removal of the δ-CH_3 group from the 59th side chain has a moderate effect on the binding affinity of the inhibitor. Substitution of Val^{H59} by αAbu removes a γ-CH_3 group, resulting in a 8.3-fold increase of the K_i value ($\Delta\Delta G^\circ$ = 1.25 kcal/mol). Substitution of αAbu^{H59} by Ala also removes a γ-CH_3 group, resulting in a 13-fold increase of the K_i value ($\Delta\Delta G^\circ$ = 1.50 kcal/mol). Thus, the γ-CH_3 group seems to contribute more to the binding than the δ-CH_3. Substitution of Ala^{H59} by Gly removes a β-CH_3 group, resulting in a 11-fold increase of the K_i value ($\Delta\Delta G^\circ$ = 1.40 kcal/mol). Overall, the removal of the Ile^{H59} side chain (a substitution of Ile^{H59} by Gly) reduces the affinity of the inhibitor 6300-fold ($\Delta\Delta G^\circ$ = 5.2 kcal/mol) and demonstrates the critical role of the Ile^{H59} side chain in the activity of the thrombin inhibitor. The K_d of the FRE inhibitor Ac-$hirudin^{55-65}$ is 1.6 ± 0.1 μM, corresponding to 8.2 kcal/mol (by taking 1 mol/L as a standard state) or 10.7 kcal/mol (by taking mole fraction as a standard state) of the binding free energy (Hopfner et al., 1993). Thus, the Ile^{H59} side chain alone contributes 50−60% of the total binding free energy of Ac-$hirudin^{55-65}$.

Contribution of Other Side Chain Atoms of the 59th Residue. The Ile^{H59} residue of the thrombin inhibitor was further substituted to examine the contribution of atoms which are not present in the Ile^{H59} side chain (Table 1). Ile has a chiral center at β-CH and allo-Ile is a diastereoisomer of Ile. Substitution of Ile^{H59} by allo-Ile increases the K_i value 1.9-fold. This small increase of the K_i value suggests a slight but not critical preference of the 59th residue for the configuration of the Ile side chain. Substitution of Ala^{H59} by αAbu or of αAbu^{H59} by Val adds a first or second γ-CH_3 group and reduces the K_i value 12.6- or 8.3-fold, respectively. This suggests a significant contribution of the first and second γ-CH_3 groups to the affinity of the inhibitor. On the contrary, substitution of Val^{H59} by Tbg, which adds a third γ-CH_3 group, reduces the K_i value only 1.4-fold. Thus, two γ-CH_3 groups are sufficient to optimize the interactions of the γ-CH_3 groups of the 59th residue. Substitution of Gly^{H59} by Ala, of Ala^{H59} by αAbu, of αAbu^{H59} by Nva, or of Nva^{H59} by Nle adds a β-CH_3, γ-CH_3, δ-CH_3, or ε-CH_3 group and reduces the K_i value 10.6-, 12.6-, 5.7-, or 1.5-fold, respectively. Thus, propyl is long enough as a side chain of the

Table 1: Activities of Thrombin Inhibitors with a Sequence of Ac-(D-Phe)-Pro-Arg-Pro-ζAhp-γAbu-Asp-Phe-Glu-Glu-**Xaa**-Pro-Glu-Glu-Tyr-Leu-Gln

peptide	Xaa	K_i (nM)	IC_{50} (nM)
P374	Gly	5200 ± 400	27000 ± 2000
P375	Ala	490 ± 5	3700 ± 100
P377	αAbu	39 ± 1	105 ± 10
P376	Val	4.7 ± 0.2	19 ± 2
P151	Ile	0.82 ± 0.02	6.0 ± 0.9
P456	allo-Ile	1.53 ± 0.21	9.2 ± 1.6
P455	Leu	1.37 ± 0.33	5.8 ± 0.6
P379	Nle	7.7 ± 1.1	35 ± 5
P378	Nva	11.3 ± 0.5	49 ± 4
P449	Tbg	3.3 ± 1.0	17.4 ± 5.6
P450	Tba	0.39 ± 0.09	2.0 ± 0.1

Table 2: Activities of Thrombin Inhibitors with a Sequence of Ac-Asp-Phe-Glu-Glu-**Xaa**-Pro-Glu-Glu-Tyr-Leu-Gln

peptide	Xaa	IC_{50} (μM)
P265	αAbu	98 ± 9
P266	Val	16.3 ± 3.0
P267	Nva	24 ± 3
P24	Ile	2.0 ± 0.4
P138	allo-Ile	10.0 ± 0.7
P369	Leu	1.78 ± 0.04
P368	Nle	13.4 ± 1.9

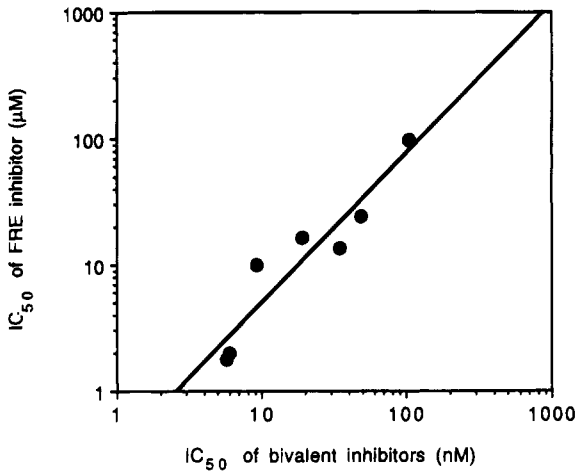

FIGURE 1: IC_{50} values of the FRE inhibitors listed in Table 2 compared to the IC_{50} values of the corresponding substitutions of the 59th residue in the bivalent inhibitors listed in Table 1. The diagonal line shows a linear least squares fitting of the IC_{50} values in log scale with a correlation coefficient of $r = 0.94$.

59th residue, and further prolongation of the side chain does not improve the affinity of the inhibitor to thrombin. The effects of the second and third δ-CH_3 groups are studied by substituting Nva[H59] by Leu and Leu[H59] by Tba, respectively, resulting in a 8.2- and 3.5-fold improvement of the K_i value, respectively. Consequently, the bivalent inhibitor with Tba[H59] shows the highest affinity to thrombin with a K_i value of 0.39 ± 0.09 nM among the substitutions examined in this article (Table 1).

Substitutions of Ac-Hirudin[55-65]. Some of the substitutions in Table 1 were also performed on the FRE inhibitor Ac-hirudin[55-65] as listed in Table 2. The IC_{50} values of these inhibitors correlate well with those of the bivalent inhibitors with corresponding substitutions (Figure 1). This suggests that the effect of the substitutions of the 59th residue is localized at the FRE inhibitor segment of the bivalent inhibitors and thrombin FRE.

Theoretical Evaluation of Ile[H59] Substitutions. The conformational search technique outlined in the experimental section was applied to the various substitutions of Ile[H59]. The most probable side chain conformations of Ile[H59] analogs in the complex are uniquely defined after energy minimization with the two exceptions of αAbu[H59] and Nva[H59]. The γ-CH_3 group of αAbu[H59] takes three stable configurations free in solution due to the rotation of α-CH−β-CH_2 bond. Two of them are stable in the complex, i.e., a conformer with $\chi_1 = -59.5°$ is the most stable and a conformer with $\chi_1 = 60.7°$ is $\Delta E = 1.6$ kcal/mol less stable than the former one. Similarly, Nva[H59] shows three stable conformers in the complex. The most stable conformer has −45.8° and −55.9° for the χ_1 and χ_2 values, respectively. The second and third conformers have −59.4°, 175.6° and 61.6°, −178.9° for χ_1, χ_2 angles, respectively, and they are 0.53 and 0.64 kcal/mol less stable in ΔE than the most stable conformer. The presence of these slightly less stable conformational isomers contributes −0.04 and −0.39 kcal/mol to the total free energy of the analogs with αAbu[H59] and Nva[H59], respectively. The most stable conformations of αAbu[H59] and Nva[H59] are used in the following analysis except for the $\Delta\Delta G°$ calculation. Table 3 lists the free energy change by the various substitutions of Ile[H59] in comparison to the experimental values. The calculated values agree very well with the experimental values. This gives some confidence in interpreting the structural implication of the simulations.

Table 3: Free Energy Difference of Thrombin Inhibitors with a Sequence of Ac-(D-Phe)-Pro-Arg-Pro-ζAhp-γAbu-Asp-Phe-Glu-Glu-**Xaa**-Pro-Glu-Glu-Tyr-Leu-Gln

		$\Delta\Delta G°_{Xaa-Ile}$ (kcal/mol)	
peptide	Xaa	theoretical	experimental
P374	Gly	6.29 ± 0.08	5.19 ± 0.06
P375	Ala	3.60 ± 0.02	3.79 ± 0.02
P377	αAbu	1.63 ± 0.04	2.29 ± 0.03
P376	Val	1.02 ± 0.06	1.04 ± 0.04
P378	Nva	1.48 ± 0.07	1.55 ± 0.04
P151	Ile	0	0
P456	allo-Ile	0.86 ± 0.06	0.37 ± 0.10
P455	Leu	0.38 ± 0.04	0.30 ± 0.16
P379	Nle	1.50 ± 0.07	1.33 ± 0.10
P449	Tbg	0.51 ± 006	0.83 ± 0.19
P450	Tba	−0.65 ± 0.05	−0.44 ± 0.16

The Ile[H59] side chain has several hydrophobic van der Waals contacts intermolecularly and intramolecularly (Zdanov et al., 1993), i.e., the β-CH group interacts with the hydrophobic side chains of Tyr[76], Ile[82], Pro[H60], and Tyr[H63]; the γ-CH_3 group interacts with the hydrophobic side chains of Arg[67], Tyr[76], and Ile[82]; the γ-CH_2 group interacts with the hydrophobic side chains of Ile[82], Pro[H60], Tyr[H63], and Leu[H64]; and the δ-CH_3 group interacts with the hydrophobic side chains of Phe[34], Leu[65], Ile[82], Tyr[H63], and Leu[H64] (Figure 2). The side chains of Val[H59], αAbu[H59], and Ala[H59] take the configuration similar to the Ile side chain in the complex. The most probable Leu[H59] side chain conformation of P455 is uniquely defined after energy minimization, and its structure is shown in Figure 2. The Leu[H59] side chain maintains most of the Ile[H59] side chain interactions but in a different way. The β-CH_2 group of Leu[H59] occupies the position of the γ-CH_2 group of Ile[H59] and interacts with the hydrophobic side chains of Ile[82], Pro[H60], Tyr[H63], and Leu[H64]. One of the δ-CH_3 groups of Leu[H59] occupies the position of the γ-CH_3 group of Ile[H59] and interacts with the hydrophobic side chains of Arg[67], Tyr[76], and Ile[82]. The other δ-CH_3 group of Leu[H59] occupies the position of the δ-CH_3 group of Ile[H59] and interacts with the hydrophobic side chains of Phe[34], Leu[65], Ile[82], Tyr[H63], and Leu[H64]. The γ-CH group of Leu[H59] does not occupy the position of the β-CH group of Ile[H59] but has a comparable number of hydrophobic interactions with the hydrophobic side chains of Phe[34], Ile[82], and Leu[H64]. Consequently, the majority of the Leu side chain atoms occupies the positions of the Ile[H59] side chain atoms. The side chains of Tba[H59] take a configuration similar to the Leu side chain. These suggest preferred locations of the side chain atoms of the 59th residue in the binding pocket.

Classified by the van der Waals contacts of the side chains of the 59th residue, there are three sites where the majority of the side chain atoms of the 59th residue in Table 1

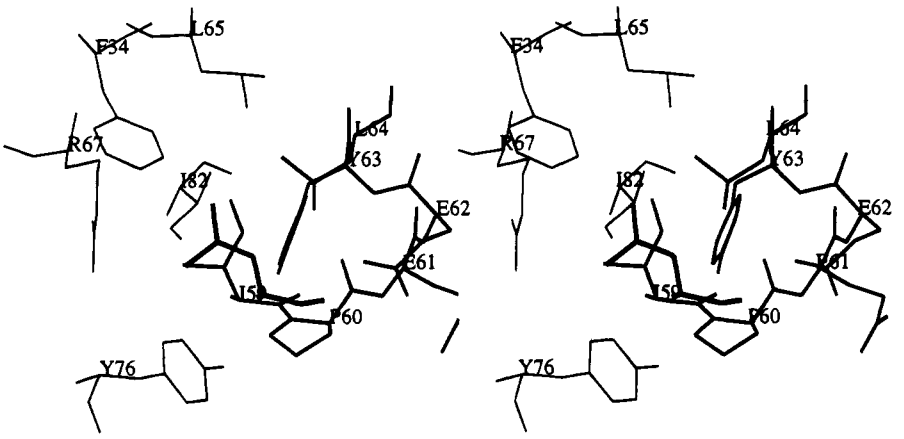

FIGURE 2: Stereoview of Ile^{H59} and the residues of thrombin (thin lines) and inhibitor (thick lines) interacting with the 59th residue of the inhibitor after energy minimization. The starting point for the energy minimization on the thrombin−inhibitor complex is the crystal structure of Zdanov et al. (1993), and Sybyl 6.0 (Tripos Assoc. Inc.) was used for the calculation and the graphic display. Overlaid on top of Ile^{H59} is the Leu^{H59} residue for the inhibitor with Leu^{H59}. The Leu^{H59} side chain has several hydrophobic van der Waals contacts, i.e., the β-CH$_2$ group of Leu^{H59} interacts with δ-CH$_3$ of Ile^{82}, 5-CH$_2$ of Pro^{H60}, β-CH$_2$ of Tyr^{H63}, 2-CH of Tyr^{H63}, γ-CH of Leu^{H64}, and δ-CH$_3$ of Leu^{H64}; the γ-CH group of Leu^{H59} interacts with 3-CH of Phe^{34}, δ-CH$_3$ of Ile^{82}, and δ-CH$_3$ of Leu^{H64}; one of the δ-CH$_3$ groups of Leu^{H59} interacts with 2-CH of Phe^{34}, 3-CH of Phe$_{34}$, δ-CH$_3$ of Leu^{65}, δ-CH$_3$ of Ile^{82}, β-CH$_2$ of Tyr^{H63}, and δ-CH$_3$ of Leu^{H64}; and the other δ-CH$_3$ group of Leu^{H59} interacts with ζ-C of Arg^{67}, 1-CH of Tyr^{76}, 2-CH of Tyr^{76}, and δ-CH$_3$ of Ile^{82}.

Table 4: Locations of the Side Chain Atoms of the 59th Residue in the Complex with Thrombin

peptide	59th residue	site 1	site 2	site 3	other side chain atoms
P374	Gly				
P375	Ala			β-CH$_3$	
P377	αAbu	γ-CH$_3$		β-CH$_2$	
P376	Val	γ_1-CH$_3$		γ_2-CH$_3$	β-CH
P378	Nva	δ-CH$_3$		β-CH$_2$	γ-CH$_2$
P151	Ile	γ_2-CH$_3$	δ-CH$_3$	γ_1-CH$_2$	β-CH
P456	allo-Ile	γ_2-CH$_3$	δ-CH$_3$	γ_1-CH$_2$	β-CH
P455	Leu	δ_2-CH$_3$	δ_1-CH$_3$	β-CH$_2$	γ-CH
P379	Nle	ϵ-CH$_3$		γ-CH$_2$ δ-CH$_2$	β-CH$_2$
P449	Tbg	γ_1-CH$_3$		γ_2-CH$_3$	β-C γ_3-CH$_3$
P450	Tba	δ_1-CH$_3$ δ_2-CH$_3$	δ_3-CH$_3$	β-CH$_2$	γ-C

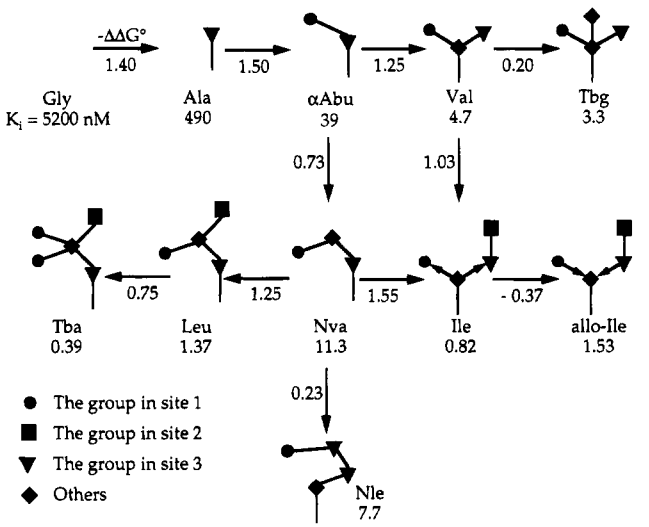

FIGURE 3: Schematic drawing of the side chains of the various 59th residues. The inhibitors have a sequence of Ac-(D-Phe)-Pro-Arg-Pro-ζAhp-γAbu-Asp-Phe-Glu-Glu-Xaa^{H59}-Pro-Glu-Glu-Tyr-Leu-Gln, where Xaa^{H59} is substituted with amino acids shown in the figure. The figure shows only the side chain atoms where the side chain C, CH, CH$_2$, or CH$_3$ group is shown with ●, ■, ▼, or ◆ for the group in site 1, 2, or 3 or others, respectively. The figure also shows the K_i values and the corresponding free energy change ($\Delta\Delta G°$) of the inhibitors. The chiral centers at β-CH of Ile and allo-Ile are distinguished by arrow bonds.

preferably occupies (Table 4). The side chain atoms in site 1 mostly interact intermolecularly with thrombin residues such as 3-CH of Phe^{34}, ζ-C of Arg^{67}, 2-CH of Tyr^{76}, and δ-CH$_3$ of Ile^{82}. The side chain atoms in site 2 interact both intermolecularly and intramolecularly. The typical residues interacting with the atoms in site 2 are 2-CH of Phe^{34}, 3-CH of Phe^{34}, δ-CH$_3$ of Leu^{65}, and δ-CH$_3$ of Ile^{82} for thrombin residues and β-CH$_2$ of Tyr^{H63} and δ-CH$_3$ of Leu^{H64}. The side chain atoms in site 3 interact intramolecularly predominantly. The interacting residues are δ-CH$_3$ of Ile^{82} of thrombin and 5-CH$_2$ of Pro^{H60}, β-CH$_2$ of Tyr^{H63}, 2-CH of Tyr^{H63}, γ-CH of Leu^{H64}, and δ-CH$_3$ of Leu^{H65}. Although the types of interactions of the side chain atoms in these sites are different, they all seem to contribute to the binding of the inhibitors to the thrombin FRE. Figure 3 visualizes the size chain atoms of the 59th residue by the sites occupied in the complex. The figure also includes the K_i and $\Delta\Delta G°$ values to visualize the contribution of the side chain atoms.

DISCUSSION

Thrombin FRE is unique to thrombin and is regularly used for molecular recognition by proteins such as fibrinogen, thrombomodulin, heparin cofactor II, thrombin receptor on platelet, and hirudin. The FRE has two hydrophobic regions; one is composed of Met^{32}, Phe^{34}, and Leu^{40}, and the other is composed of Leu^{65}, Tyr^{76}, Ile^{82}, and Met^{84}. These two regions are separated by Arg^{67} and are surrounded by the basic residues of Arg^{35}, Lys^{36}, Lys^{70}, Arg^{73}, Arg^{75}, Arg^{77A}, Lys^{81}, Lys^{109}, and Lys^{110} (Figure 4). The FRE-binding segments of the above proteins have complementary sequences enriched by acidic and hydrophobic residues. Although the molecular association is dominated by this complementary property, the details are not straightforward.

Hirudin first recognizes thrombin FRE through its complementary electrostatic potentials (Karshikov et al., 1992). However, mutation of Asp^{H55} by Asn, Glu^{H61} by Gln, or Glu^{H62} by Gln in r-hirudin reduced the affinity of r-hirudin

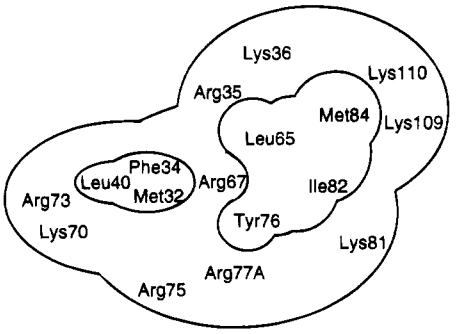

FIGURE 4: Hydrophobic and basic residues at FRE of thrombin. Hydrophobic residues form two clusters at FRE. The two clusters are separated by Arg[67] and surrounded by nine basic residues.

only 1.1−2.6-fold, and mutation of Glu^{H58} in [Gln^{H57}]-r-hirudin by Gln did not change the affinity of the inhibitor (Braun et al., 1988; Betz et al., 1991b). Even the double mutation of Glu^{H58} and Glu^{H61} in Ac-hirudin$^{55-65}$ did not change the affinity of the FRE inhibitor (Szewczuk et al., 1992). Consequently, these four negative charges do not contribute to binding of the inhibitor. An exception is Glu^{H57} (and probably the C-terminal carboxyl group as well). The substitution of Glu^{H57} in r-hirudin by Gln or substitution of Glu^{H57} in Ac-hirudin$^{55-65}$ by Gly reduced the affinity of the inhibitors 10- or 25-fold, respectively (Betz et al., 1991b; Yue et al., 1992). This means that, although the electrostatic fields of thrombin FRE and the FRE inhibitor complement each other (Karshikov et al., 1992), many negative charges on the FRE inhibitor have little contribution to the binding affinity. On the other hand, a strong positive electrostatic potential is not unique to the thrombin FRE surface. For example, the heparin-binding site of thrombin is also enriched by basic residues. Since an ion pair is formed easily by taking basic and acidic residues close to each other, the mechanism(s) by which these complementary sites avoid molecular association is not clear. Furthermore, the FRE-binding segments of other proteins are also enriched by many acidic residues; therefore, these acidic residues seem to have some biological roles which remain to be solved.

Another puzzling point is the Phe^{H56} side chain, which is buried in the thrombin complex and forms an aromatic−aromatic stacking with Phe^{34} and van der Waals contacts with Arg^{73} and Thr^{74} (Rydel et al., 1991). Despite many interactions with thrombin, the removal of the phenyl group, i.e., substitution of Phe^{H56} by Ala, increases the K_i value of recombinant hirudin only 2-fold (Betz et al., 1991b). On the other hand, hirudin$^{57-65}$, i.e., deletion of Phe^{H56}, showed no inhibitory activity toward thrombin (Mao et al., 1988). The free energy calculation used in this article was also applied to the Ala substitution of Phe^{H56}. The conformation of the 55th and 56th residues is largely changed by the mutation, and the backbone of these residues replaces the aromatic side chain of Phe^{H56} to compensate the loss of the phenyl ring (unpublished results). This may explain why the phenyl group of Phe^{H56} can be removed but not the backbone. Besides Phe^{H56}, Tyr^{H63} forms many van der Waals contacts with thrombin FRE residues, such as Lys^{36}, Leu^{65}, and Ile^{82} (Rydel et al., 1991). However, substitution of Tyr^{H63} by Gly increased the IC_{50} only 5.9-fold, suggesting a minor role of the Tyr^{H63} side chain in the binding to thrombin FRE. It also shows a rather high temperature factor in the crystal of thrombin−hirutonin-2 (Zdanov et al., 1993) or is even disordered in the thrombin−hirulog-1 complex (Skrzypczak-Jankun et al., 1991). This provides further indication of a minor contribution of Tyr^{H63} in the molecular association. Thus, many van der Waals contacts of a residue with thrombin do not necessarily mean a strong contribution of the residue to the molecular association.

In contrast, Ile^{H59} clearly plays a critical role in the binding affinity. Its replacement by Gly reduces the thrombin affinity 6300-fold, corresponding to a 5.2 kcal/mol loss in the binding free energy at 25 °C. The K_d of the FRE inhibitor Ac-hirudin$^{55-65}$ is 1.6 ± 0.1 μM at 37 °C (Hopfner et al., 1993), corresponding to −8.2 kcal/mol (taking 1 mol/L as a standard state) or −10.7 kcal/mol (taking mole fraction as a standard state) of association free energy. Consequently, the Ile^{H59} side chain alone contributes 50−60% of the association free energy of the FRE inhibitor. In spite of such critical contribution, Ile^{H59} binds to a rather unusual site which is between the two hydrophobic regions and has fewer hydrophobic contacts with thrombin compared to those of Phe^{H56} and Tyr^{H63} (Rydel et al., 1991). Furthermore, the surface of thrombin FRE is relatively flat, lacking a hydrophobic pocket where Ile^{H59} may be inserted upon the binding. Interestingly, the lack of the binding pocket is compensated by the inhibitor itself. Pro^{H60}, Tyr^{H63}, and Leu^{H64} form a hydrophobic wall and complete the hydrophobic pocket for Ile^{H59} (Rydel et al., 1991). As a result, the side chain of Ile^{H59} forms van der Waals interactions not only with thrombin FRE but also with Pro^{H60}, Tyr^{H63}, and Leu^{H64} of the inhibitor residues and is buried completely upon its binding to the thrombin FRE.

Free energy calculation suggests that the side chains of the 59th residue listed in Table 1 preferably occupy the three sites (1, 2, and 3). Removal of side chain atoms from site 1 occurs in the substitution of αAbu by Ala (Table 4). The corresponding experimental free energy loss in the binding is 1.50 kcal/mol (Table 3). Removal of side chain atoms from site 2 occurs in the substitution of Ile^{H59} by Val or of Leu^{H59} by Nva. The corresponding energy loss in the binding is 1.04 or 1.25 kcal/mol, respectively. Similarly, removal of side chain atoms from site 3 occurs in the substitution of Ala^{H59} by Gly. The corresponding loss of the binding energy is 1.41 kcal/mol. Thus, the contributions of the atoms in these sites to the binding energy are in the range of 1.0−1.5 kcal/mol. This is in good agreement with a finding that removal of a methyl/methylene group for buried hydrophobic side chains alters protein stability by 1.5 ± 0.6 kcal/mol (Serrano et al., 1992). Although the interactions of the side chain atoms in these three sites are quite different (intermolecular for site 1, intramolecular for site 3, and both for site 2) as described in the Results, both inter- and intramolecular interactions more or less equally contribute to the binding affinity. It is obvious how the intermolecular interactions stabilize molecular association. However, the stabilization of molecular association by intramolecular interactions may not be obvious. Assuming that the intramolecular interactions specifically stabilize the active conformation, the free energy of the thrombin−inhibitor complex is lowered by the intramolecular interactions. If an inhibitor in the dissociated state takes predominantly disordered or inactive conformations, the dissociated state is not stabilized by the intramolecular interactions. As a result, the intramolecular interactions increase the free energy difference ($\Delta\Delta G°$) between the dissociated and complex states and stabilize the molecular

association. On the other hand, if the inhibitor already takes a stable, active conformation free in solution, the intramolecular interactions stabilize both the dissociated and complex states. As a result, there would be little or no change in $\Delta\Delta G°$ between the dissociated and complex states. Since a significant population of the FRE inhibitor is disordered free in solution (Ni et al., 1992), both intra- and intermolecular interactions improve the affinity of FRE inhibitors.

The multidisciplinary approach of peptide chemistry, protein crystallography, and molecular modeling gave some insights to the interactions critical to molecular association. It is important to understand these interactions especially for drug design, which reauires two to three critical residues such as Ile^{H59}. Further thermodynamic and solvation studies are in progress.

CONCLUSION

The Ile^{H59} side chain plays a critical role in the binding of the hirudin C-terminal fragment, $hirudin^{55-65}$, to the thrombin FRE, counting approximately one-half of the total binding free energy of the inhibitor. Substitutions of Ile^{H59} with various natural and unnatural amino acids show that Ile is one of the best amino acids at the 59th residue and may be replaced by Leu, allo-Ile, and Tba with comparable affinity to thrombin. Theoretical free energy calculation successfully reproduces the experimental relative binding free energy of various Ile^{H59} analogs, suggesting that van der Waals interactions dominate the binding free energy. According to the energy-minimized conformations of various Ile^{H59} analogs, the side chains of Ile^{H59} analogs preferentially occupy three sites in the binding pocket. The side chain atoms in site 1 such as the γ-CH_3 group of Ile^{H59} predominantly form intermolecular interactions with thrombin FRE residues. The side chain atoms in site 2 such as the δ-CH_3 group of Ile^{H59} form both inter- and intramolecular interactions with thrombin and other inhibitor residues, respectively. The side chain atoms in site 3 such as the γ-CH_2 group of Ile^{H59} form predominantly intramolecular interactions with other inhibitor residues. Both of these inter- and intramolecular interactions contribute to the binding more or less equally.

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