Nonpolar Interactions of Thrombin S′ Subsites with Its Bivalent Inhibitor: Methyl Scan of the Inhibitor Linker‡

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ABSTRACT: We have designed bivalent thrombin inhibitors, consisting of a nonsubstrate type active site blocking segment, a hirudin-based fibrinogen recognition exosite blocking segment, and a linker connecting these segments. The inhibition provided by the bivalent inhibitors with various linker lengths revealed that a minimum of 15 atoms was required for simultaneous binding of the two blocking segments of the inhibitor to thrombin without significant distortion. The crystal structure of the inhibitors with a 16-atom linker showed some conformational flexibility in the linker portion which still lies deep in the groove joining the active site and the fibrinogen recognition exosite. Since the thrombin S′ subsites are not well characterized, we designed a new strategy to search for possible nonpolar interactions between the linker and the thrombin S′ subsites. This strategy, the "methyl scan", is based on the incorporation of a methyl side chain at each atom position of the linker by using sarcosine, D,L-alanine, D,L-3-aminoisobutyric acid, or *N*-methyl-*β*-alanine. The methyl groups on the second and the eighth atom positions of the linker, which correspond to the side chains of the P1′ and the P3′ residues, respectively, improved the affinity of the inhibitors significantly. Further study of the stereospecificity showed that L-Ala at the P1′ residue and D-Ala at the P3′ residue preferably improved the affinity of the inhibitors 20- and 25-fold, respectively. Molecular modeling calculations using a methyl probe were also carried out to identify favorable nonpolar interacting sites on the thrombin surface. Two sites were identified in the vicinity of the P1′ and the P3′ residues, supporting the validity of the methyl scan method. Thus, this study has improved our understanding of the interactions taking place in this groove. In particular, we have been able to show that some specific structural features, such as hydrophobic complementarity between the linker and the thrombin S′ subsites, could be exploited and make these inhibitors trivalent.

Thrombin (EC 3.4.21.5), a serine protease, plays a critical role in thrombosis and hemostasis. Thrombin converts fibrinogen to fibrin and generates an insoluble fibrin clot. The catalytic domain of thrombin is homologous to other plasma proteases involved in thrombosis and hemostasis such as factor Xa, urokinase, tissue plasminogen activator, and protein C as well as trypsin and chymotrypsin. In fact, thrombin exhibits primarily a trypsin-like specificity, i.e., a preference for an Arg−Xaa bond.[1] However, the cleavage of fibrinogen by thrombin is extremely specific; it occurs only at four Arg−Gly bonds among 376 Arg/Lys−Xaa bonds in fibrinogen (Blamback et al., 1967). This exceptional specificity of thrombin is primarily attributed to the multiple interactions between thrombin and fibrinogen at the catalytic active site (AS)[2] and the fibrinogen recognition exosite (FRE) (Bode et al., 1990; Fenton & Bing, 1986). In addition, fibrinogen interacts with thrombin S′ subsites, providing additional specificity (Liem & Scheraga, 1974).[3]

Due to its critical role in thrombosis and hemostasis, thrombin has been the main target for developing anticoagulants. Many of these are active site-directed small inhibitors and are classified into two groups: the substrate type inhibitors and the nonsubstrate type. (D-Phe)-Pro-Arg-H (Bajusz et al., 1990), Ac-(D-Phe)-Pro-boroArg-OH (Kettner et al., 1990), and *N*-methyl-(D-Phe)-Pro-Arg-(2-benzothiazole) (Costanzo et al., 1996) are examples of the first group. Among the aldehyde analogs, *N*-methyl-(D-Phe)-Pro-Arg-H

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[1] The amino acids used in this article are in the L-configuration unless specified as the D-amino acid for the D-isomer and the D,L-amino acid for a racemized mixture of the D- and L-amino acids.

[2] Abbreviations: Ac, acetyl; *β*Ala, *β*-alanine; *γ*Abu, 4-aminobutyric acid; *λ*Adod, 12-aminododecanoic acid; *β*Aib, 3-aminoisobutyric acid; AMC, 7-amino-4-methylcoumarin; *ζ*Ahp, 7-aminoheptanoic acid; *ϵ*Ahx, 6-aminohexanoic acid; *η*Aoc, 8-aminooctanoic acid; *δ*Ape, 5-aminopentanoic acid; AS, active site; *κ*Aud, 11-aminoundecanoic acid; Bbs, 4-*tert*-butylbenzenesulfonyl; Boc, *tert*-butyloxycarbonyl; Cha, *β*-cyclohexylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; FRE, fibrinogen recognition exosite; HPLC, high-performance liquid chromatography; MD-805, (2*R*,4*R*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid; NAPAP, N^{α}-(2-naphthylsulfonylglycyl)-D,L-*p*-amidinophenylalanylpiperidine; Pip, pipecolic acid; Sar, sarcosine; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[3] The substrate/inhibitor and subsite nomenclature was suggested by Schechter and Berger (1967) so that residues of substrates/inhibitors were numbered P1, P2, P3, etc., toward the amino terminus from the scissile peptide bond, and the complementary subsites of the enzyme were numbered S1, S2, S3, etc., respectively. Except here, the residues of the inhibitor analogs of MD-805 and NAPAP are numbered P3, P1, and P2 from its N terminus, reflecting their occupations of thrombin S3, S1, and S2 subsites, respectively, in the complex.

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is orally active and has a prolonged half-life of several hours *in vivo* (Smith et al., 1996; Bajusz et al., 1996). The second group is characterized by its unique binding to the S3, S1, and S2 subsites of thrombin from the N-terminal residue. MD-805 (Kikumoto et al., 1984) and NAPAP (Sturzbecher et al., 1983) are representatives of this class.

Rather large molecules based on the hirudin sequence form a unique class of bivalent inhibitors. Hirudin is a protein of 65 amino acid residues, isolated from the leech *Hirudo medicinalis* (Markwardt, 1970) and is the most potent natural thrombin inhibitor ($K_i = 2.2 \times 10^{-14}$ M; Stone & Hofsteenge, 1986). According to the crystal structure of the thrombin−hirudin complex, the N-terminal residues, hirudin$^{1-48}$, cover the region around the thrombin AS, and the C-terminal residues, hirudin$^{55-65}$, bind to the FRE (Rydel et al., 1990, 1991). Each inhibitor segment is capable of binding to thrombin by itself with K_i values of 7.2×10^{-8} M for hirudin$^{1-48}$ and of 1.6×10^{-6} M for Ac-hirudin$^{55-65}$ (Mao et al., 1988; Chang, 1990; Hopfner et al., 1993). When a spacer, hirudin$^{49-54}$, connects these segments, allowing their simultaneous binding to thrombin, the affinity and the specificity of the bivalent inhibitor are additively enhanced. This drastic effect of the bivalent inhibition has provided the rationale for the design of synthetic inhibitors which mimic the binding mode of hirudin. The initial attempt was to replace the bulky AS inhibitor segment, hirudin$^{1-48}$, with a small substrate-like moiety, (D-Phe)-Pro-Arg-Pro (DiMaio et al., 1990; Maraganore et al., 1990; Witting et al., 1992b). This replacement reduced the molecular mass of the analogs from 7000 to ∼2500 Da and reduced the affinity from the 10^{-14} to the 10^{-9} M range. However, thrombin slowly hydrolyzed the scissile peptide bond of Arg^{H3}−Pro^{H4} (DiMaio et al., 1990; Witting et al., 1992a).[4] The second attempt was undertaken to overcome this proteolytic instability of the inhibitors. Substitution of Arg^{H3} with β-homoarg (Brourdon et al., 1991) and replacement of the Arg^{H3}−Pro^{H4} bond by ArgΨ[CO(CH$_2$)$_n$CO] ($n = 1-4$) (DiMaio et al., 1991, 1992) or ArgΨ[COCH$_2$N$^+$C$_5$H$_4$CH$_2$CO] (Rehse et al., 1995) eliminated the scissile bond and provided a complete proteolytic stability against thrombin.

The elimination of the scissile bond was also achieved by incorporating nonsubstrate type AS inhibitors, with the P1 residue between the P3 and the P2, and by connecting the linker to the P2 residue. This type of inhibitor showed an improved affinity; e.g., dansyl-Arg-(D-Pip)-λAdod-Gly-hirudin$^{55-65}$ (P500) has a K_i value of $(2.6 \pm 1.0) \times 10^{-11}$ M (Tsuda et al., 1994). The crystal structure of this inhibitor in complex with thrombin showed that dansyl, Arg, and D-Pip occupy the S3, S1, and S2 subsites, respectively, and the distance between the carbonyl carbon of D-Pip and the side chain oxygen of Ser^{195} is 4.35 Å, which is too far to form a tetrahedral intermediate for hydrolysis (Féthière et al., 1996).[5]

The linkers in the synthetic bivalent thrombin inhibitors primarily act as spacers; their length has little effect on the affinity of the inhibitor as long as it allows the simultaneous binding of the AS and the FRE inhibitor segments (Szewczuk et al., 1992). The excess atoms in longer linkers seem to be largely exposed to solvent (Zdanov et al., 1993). Shorter linkers, on the other hand, are forced to lie deep in the S′ groove and are close to thrombin residues (Zdanov et al., 1993; Féthière et al., 1996). Consequently, they can be used as templates to lock the P′ residues in the S′ groove of thrombin. Szewczuk et al. (1993) designed short linkers 13 atoms in length with hydrogen bonding potential.[6] This resulted in an 11-fold improvement of the K_i value. In this study, we have further investigated the role of the interactions between the linker and the S′ subsites of thrombin. Our goal is to identify potential sites of nonpolar interactions contributing to the "overall" affinity of the inhibitor. The results showed a 20−25-fold improvement in the affinity after a methyl side chain was inserted at two sites of the linker.

EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin (3000 NIH units/mg), bovine fibrinogen (70% of the protein, 85% of the protein clottable), Tos-Gly-Pro-Arg-AMC·HCl salt, poly(ethylene glycol) 8000, and Tris were purchased from Sigma (St. Louis, MO). AMC, δApe, λAdod, D-Pip, L-Pip, D-βAib, and L-βAib were obtained from Aldrich (Milwaukee, WI). βAla, Boc-γAbu, Boc-ζAhp, Boc-ϵAhx, Boc-L-Pip, and Boc-D-Pip were purchased from BaChem (King of Prussia, PA). ηAoc and κAud were obtained from Fluka (Ronkonkoma, NY). Boc-βAla, Boc-λAdod, Boc-δApe, Boc-ηAoc, Boc-κAud, and Boc-(D or L)-Pip were prepared according to the procedure described by Chaturvedi et al. (1984). All Fmoc-amino acids and all other amino acid derivatives for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY). Boc-Gln-OCH$_2$-phenylacetamidomethyl resin (0.714 mmol/g) was purchased from Applied Biosystems Inc. (Foster City, CA), and Fmoc-D-Glu(OtBu)-Wang resin (0.59 mmol/g) was purchased from Calbiochem-Novabiochem (San Diego, CA). The solvents for peptide synthesis were obtained from B & J Chemicals (Muskegon, MI) and Applied Biosystems Inc. Citric acid was purchased from Anachemia (Rouses Point, NY). HF and TFA were purchased from Matheson (Montgoneryville, PA) and Halocarbon Products Co. (River Edge, NJ), respectively.

Peptide Synthesis. The peptides in this article were synthesized by the solid-phase method on a Model 396 Multiple Peptide Synthesizer (Advanced ChemTech) using a standard Fmoc procedure or were prepared manually using a standard Boc procedure as described elsewhere (Szewczuk et al., 1992). Peptides were cleaved from the resin using Reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethylenedithiol) for 4 h with 25 mL/(g of peptide-resin) (Fmoc method) or using a standard HF cleavage (Boc method; Szewczuk et al., 1992). After precipitation with diethyl ether, the peptides were filtered, dissolved in 50% acetic acid, lyophilized, and purified by HPLC (Vydac C$_4$ preparative column, 4.6 × 25 cm) using a linear gradient of 20 to 50% acetonitrile in 0.1% TFA (0.5%/min gradient, 33 mL/min flow rate). Purity was evaluated

[4] The numbering of the fibrinogen recognition exosite inhibitor segment is based on the hirudin sequence, and H is added to the residue number of the inhibitors, e.g., dansyl^{H1}-Arg^{H2}-(D-Pip)^{H3}-Gly^{H4}-Gly^{H5}-Gly^{H6}-ϵAhx^{H7}-Asp^{H55}-Phe^{H56}-Glu^{H57}-Glu^{H58}-Ile^{H59}-Pro^{H60}-Glu^{H61}-Glu^{H62}-Tyr^{H63}-Leu^{H64}-Gln^{H65}-OH.

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

[6] The linkers are expressed as the number of the atoms contributing to their length; e.g., a linker of 4-aminobutyric acid is a 5-atom linker, and a linker of 12-aminododecanoic acid−4-aminobutyric acid is an 18-atom linker. The linker is connected to the P2 residue in this paper, whereas it was connected to the P1′ residue in the previous paper (Szewczuk et al., 1993).

by an analytical run on a Vydac C_{18} column (0.46 × 25 cm, 0 to 60% acetonitrile in 0.1% TFA, 1.0%/min gradient, 1.0 mL/min flow rate) and was 98% or higher. The elution profile was monitored by an absorbance at 215 and 254 nm for the analytical HPLC and at 220 nm for the preparative HPLC. The final products were lyophilized and identified using a Beckman model 6300 amino acid analyzer (Beckman, Fullerton, CA) and a SCIEX API III mass spectrometer (Sciex, Thornhill, ON). The amino acid analysis was used for the peptide content determination. All peptides used in this paper have correct amino acid composition and molecular mass.

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) final concentrations of human α-thrombin and bovine fibrinogen, respectively (Szewczuk et al., 1992). The IC_{50} was estimated to be the inhibitor concentration required to double the clotting time relative to that of the control.[7]

The inhibition of amidolytic activity of human α-thrombin was measured fluorometrically 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 thrombin were 0.5−1000 times the value of K_i, $1-8 \times 10^{-6}$ M, and 3.0×10^{-11} M, respectively, if $K_i > 10^{-10}$ M; 10−100 times the value of K_i, $5-40 \times 10^{-6}$ M, and 3.0×10^{-11} M, respectively, if 10^{-10} M $> K_i > 10^{-11}$ M; and $3.3-56 \times 10^{-10}$ M, $5-40 \times 10^{-6}$ M, and 3.0×10^{-11} M, respectively, if $K_i < 10^{-11}$ M.[8] The hydrolysis of the substrate by thrombin was monitored on a Varian-Cary 2000 spectrophotometer in the fluorescence mode or a Hitachi F2000 fluorescence spectrophotometer (λ_{ex} = 383 nm and λ_{em} = 455 nm), and the fluorescent intensity was calibrated using AMC. Substrate and inhibitor were premixed at appropriate concentrations (the solution volume was adjusted to 2.99 mL) before adding 10 μL of human α-thrombin (9.0×10^{-9} M). The reaction reached a steady state within 3 min after adding thrombin. The steady-state velocity was then measured for 2−3 min. The kinetic data of the steady-state velocity at various concentrations of the substrate and the inhibitors were analyzed using the methods described by Segel (1975) and Szewczuk et al. (1993) for competitive and hyperbolic inhibitions. A nonlinear regression program, RNLIN in the IMSL library (IMSL, 1987), LMDER in the MINIPACK library (More et al., 1980), or Microsoft Excel, was used to estimate the kinetic parameters (K_m, V_{max}, K_i, and K_r).[9] Fibrin clotting and amidolytic assays were repeated at least three times to obtain the average and the standard deviation of the IC_{50} and K_i values.

Table 1: Activity of Thrombin Inhibitors with Various Linker Lengths

dansyl-Arg-(D-Pip)-[linker]-
Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH

peptide	linker	linker length[a]	K_i x 10^9 (M)	$IC_{50} \times 10^9$ (M)	$1/K_r$
P514	γAbu-Gly	8 (5 + 3)	6800 ± 1600	1170 ± 80	
P526	δApe-Gly	9 (6 + 3)	7300 ± 380	1210 ± 210	
P525	ϵAhx-Gly	10 (7 + 3)	4000 ± 1100	700 ± 180	
P524	ζAhp-Gly	11 (8 + 3)	4500 ± 300	550 ± 110	
P523	ηAoc-Gly	12 (9 + 3)	195 ± 12	155 ± 26	9.0
P499	λAdod	13	15.1 ± 4.0	130 ± 38	37
P528	ηAoc-γAbu	14 (9 + 5)	0.60 ± 0.07		84
P527	κAud-Gly	15 (12 + 3)	0.043 ± 0.010		
P500	λAdod-Gly	16 (13 + 3)	0.026 ± 0.010		
P448	λAdod-γAbu	18 (13 + 5)	0.0170 ± 0.0042		
P513	λAdod-ϵAhx	20 (13 + 7)	0.0155 ± 0.0026		

[a] The length of the linker is expressed by the number of the atoms in the backbone. The length of each ω- and α-amino acid composed is shown in parentheses.

Molecular Modeling. The three-dimensional coordinates for thrombin were taken from the crystal structure of a thrombin−P498 complex (Féthière et al., 1996). The inhibitor and water molecules were removed and hydrogens added using Sybyl 6.2 (Tripos Inc.). A rectangular box containing the binding sites of interest was constructed. The solvated interaction energy of a methyl probe placed at regular orthogonal intervals of 0.5 Å inside the box was then calculated. The AMBER force field (Weiner et al., 1986) was used for the calculations. The methyl probe was treated as an AMBER united atom with zero partial charge and Lennard-Jones parameters of 2.165 and 0.181 for the radius and well depth, respectively. The protein atoms were assigned AMBER all-atom force field parameters. The solvation component was calculated using the solvation method of Stouten et al. (1993). In their method, solvation is treated as an added pairwise interaction term that favors burial of nonpolar groups and exposure of polar groups. We used the sum of the AMBER and solvation energy terms as a score indicating the propensity of a methyl probe to occupy a test point in the rectangular box. Sites with interaction energies below a given cutoff were then displayed. We found a cutoff of −2.0 kcal/mol to be useful in defining potential nonpolar binding sites.

RESULTS

Linker Length. Szewczuk et al. (1993) determined that a linker 13 atoms long is sufficient when the linker is attached to the C-terminal P1′ residue of the AS blocking segment, D-Phe-Pro-Arg-Pro. Since the linker, in this study, is attached to the P2 residue (D-Pip) of the nonsubstrate type AS inhibitor segment, (dansyl or Bbs)-Arg-(D-Pip), the bivalent inhibitors obviously require a longer linker. Table 1 lists the bivalent thrombin inhibitors with various linker lengths. The inhibitors with an 8−11-atom linker (P514, P526, P525, and P524 in Table 1) inhibit thrombin with K_i values of $4-7 \times 10^{-6}$ M and IC_{50} values of $0.5-1 \times 10^{-6}$ M. These K_i and IC_{50} values are comparable to those of the corresponding AS inhibitor, dansyl-Arg-D-Pip-γAbu-NH_2 [$K_i = (9.8 \pm 1.3) \times 10^{-7}$ M and $IC_{50} = (2.6 \pm 0.7) \times 10^{-6}$ M], and the FRE inhibitor, Ac-hirudin$^{55-65}$ [$IC_{50} = (1.7 \pm 0.2)$

[7] 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.

[8] The final concentrations of the inhibitor, the substrate, and human α-thrombin were 10−100 times the value of K_i, $2-4 \times 10^{-5}$ M, and 3.0×10^{-11} M, respectively, for the inhibition measurements of P553. The data were analyzed using the tight binding equations (Stone & Hofsteenge, 1986) and the nonlinear regression program UltraFit (Biosoft).

[9] The bivalent inhibitor rapidly binds to the thrombin FRE and forms a complex, EI (Jackman et al., 1992). Then, the active site inhibitor segment of the inhibitor binds to the thrombin active site and forms a complex, EI*, in which the inhibitor binds to both the FRE and the active site. K_r is the equilibrium constant between EI and EI*.

$\times 10^{-6}$ M], respectively (Szewczuk et al., 1992; Tsuda et al., 1994). Thus, no additive effect is observed with these short linker inhibitors which indicates that they are blocking the AS or the FRE independently (not simultaneously). P523 with a 12-atom linker showed a K_i value of $(1.95 \pm 0.12) \times 10^{-7}$ M which corresponds to an ~20-fold improvement compared to that of the inhibitors with a shorter linker, suggesting that the inhibitor might act at both sites on thrombin simultaneously. The affinity of the inhibitor was further improved to $(1.51 \pm 0.40) \times 10^{-8}$ M (P499 with a 13-atom linker) and $(6.0 \pm 0.7) \times 10^{-10}$ M (P528 with a 14-atom linker) as the linker length was increased. After 15 atoms or more, the affinity of the inhibitors became insensitive to the length of the linker ($1-4 \times 10^{-11}$ M). The results suggested that a minimum of 15 atoms in the linker is required for optimum simultaneous binding to the AS and the FRE. We have used a 16-atom linker as a template to study the hydrophobic interactions between the linker and thrombin.

We further examined the effect of the linker length on the inhibitor binding modes of the AS blocking and the FRE blocking segments using molecular modeling and the crystal structures of the inhibitors. Several bivalent thrombin inhibitors have been crystallized in complex with thrombin, i.e., the inhibitors with a 16-atom linker (Féthière et al., 1996), with a 17-atom linker (Zdanov et al., 1993), with a 19-atom linker (Rehse et al., 1995), and with a 21-atom linker (Qiu et al., 1992; Zdanov et al., 1993; Krishnan et al., 1996). The AS blocking segment and the FRE blocking segment of these bivalent inhibitors show the simultaneous binding to the active site and the FRE of thrombin, respectively. Thus, it is reasonable to assume that the bivalent inhibitors with linkers of 16 atoms or more bind the AS and the FRE of thrombin simultaneously. The length of the 16-atom linker, which is calculated as the distance between the centers of the amide bonds at the N and C termini of the linker, is 17.8 Å in the crystal structure of the thrombin−P500 complex. This is substantially shorter than the corresponding length (19.9 Å) of the linker in the fully extended structure, allowing some conformational flexibility of the linker in the complex (Féthière et al., 1996). The 15-atom linker (κAud-Gly) of P527 has an 18.6 Å length in its fully extended structure which is still sufficient to span the AS blocking segment and the FRE blocking segment. However, the 14-atom linker (ηAoc-γAbu) of P528 is too short (17.4 Å even in the fully extended structure) to span these blocking segments and requires some distorted binding mode of the AS blocking segment or the FRE blocking segment. These distance analyses of the linker support the above assumption that a minimum of 15 atoms in the linker is required for optimum simultaneous binding to the AS and the FRE.

Thrombin−Inhibitor Complexes. Szewczuk et al. (1993) reported that the location of peptide bond(s) in the linker affects the affinity of the bivalent inhibitors with the substrate type AS inhibitor segment. Since the locations of the peptide bonds vary in a series of inhibitors synthesized in "methyl scan", we synthesized reference inhibitors (P1172, P1173, P1174, P1175, and P836) that have the same locations of peptide bonds as those in methyl scan but with no side chain methyl group. The K_i values of these reference inhibitors are listed in Table 2. In opposition to Szewczuk et al. (1993), one atom shift of the amide bonds due to a series of the βAla insertion at the P1′−P5′ residues hardly changed the

Table 2: Reference Peptides of the Methyl Scan and the Effect of the Peptide Bond Location in the Linker

Bbs-Arg-(D-Pip)-[linker]-
Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

peptide	linker	K_i x 10^{11} (M)
P1172	βAla-Gly-Gly-Gly-Gly	2.4 ± 0.3
P1173	Gly-βAla-Gly-Gly-Gly	3.3 ± 0.2
P1174	Gly-Gly-βAla-Gly-Gly	3.1 ± 0.2
P1175	Gly-Gly-Gly-βAla-Gly	1.9 ± 0.2
P836	Gly-Gly-Gly-Gly-βAla	2.4 ± 0.5

K_i values ($1.9-3.3 \times 10^{-11}$ M). In particular, the location of the peptide bonds in the linkers of P1172 and P836 are shifted by one atom, yet both inhibitors had the same affinity for thrombin, suggesting negligible contributions of these peptide bonds. P1172 has a linker similar to that of P498 [dansyl-Arg-(D-Pip)-βAla-Gly-Gly-δApe-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH] except for Gly^{H7}-Gly^{H8} which is replaced by δApe^{H7} in P498. Therefore, the crystal structure of the thrombin−P498 complex, which was reported elsewhere (Féthière et al., 1996), was used as a template as shown in Figure 1. The thrombin nonpolar sites which were suggested by the methyl probes of molecular modeling are emphasized in the figure. Other than two hydrogen bonds between Gly^{H6} of the P498 linker and Leu^{40}, no specific interaction of the linker with thrombin was observed. These two hydrogen bonds may have little impact in the binding affinity as suggested above. Féthière et al. (1996) also reported another crystal structure of a 16-atom linker of P500 [dansyl-Arg-(D-Pip)-λAdod-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH] which formed a hydrogen bond between the carbonyl oxygen of $\lambda Adod^{H4}$ and NE2 of Gln^{151}. Although these linkers follow a pathway somehow different with a maximum distance of 2.6 Å between them near the center, both linkers lie deep in the groove of the thrombin S′ subsites. The forced location in the thrombin S′ groove with sufficient conformational flexibility of the 16-atom linkers confirms the appropriateness of using the 16-atom linker in this study.

Nonpolar Interacting Sites Defined by a Methyl Probe. Figure 1 shows the volume occupied by methyl probes having a high affinity for thrombin. A cutoff of −2.0 kcal/mol for the interaction energy with desolvation was used to define the volumes. Seven nonpolar interacting sites identified by the methyl probes are in the vicinity of the nonpolar residues of P498 as described in the following. (1) The methyl probes around the N-terminal dansyl group have van der Waals contacts (≤4.7 Å) with thrombin nonpolar residues (Tyr^{60A}, Leu^{99}, Ile^{174}, Trp^{215}, and the aliphatic side chain of Glu^{217}) at the S3 subsite, confirming the strong preference of a hydrophobic residue at the S3 subsite. (2) The methyl probes around the piperidine ring of D-Pip are surrounded by the nonpolar residues (His^{57}, Tyr^{60A}, Trp^{60D}, Leu^{99}, and Trp^{215}) of the thrombin S2 subsite. Pro or its analogs are preferred as the P2 residue. (3) Many methyl probes were found in the S1 subsite which is specific to the Arg residue. Surprisingly, the methyl probes were in the vicinity of not only the nonpolar atoms but also the polar guanidino group of the Arg side chain. The thrombin residues (Ala^{190}, the aliphatic side chains of Glu^{217} and of Asp^{194}, Val^{213}, Gly^{216}, Gly^{219}, Cys^{220}, Gly^{226}, and Tyr^{228}) formed van der Waals contacts with these methyl probes. Indeed, Tucker et al.

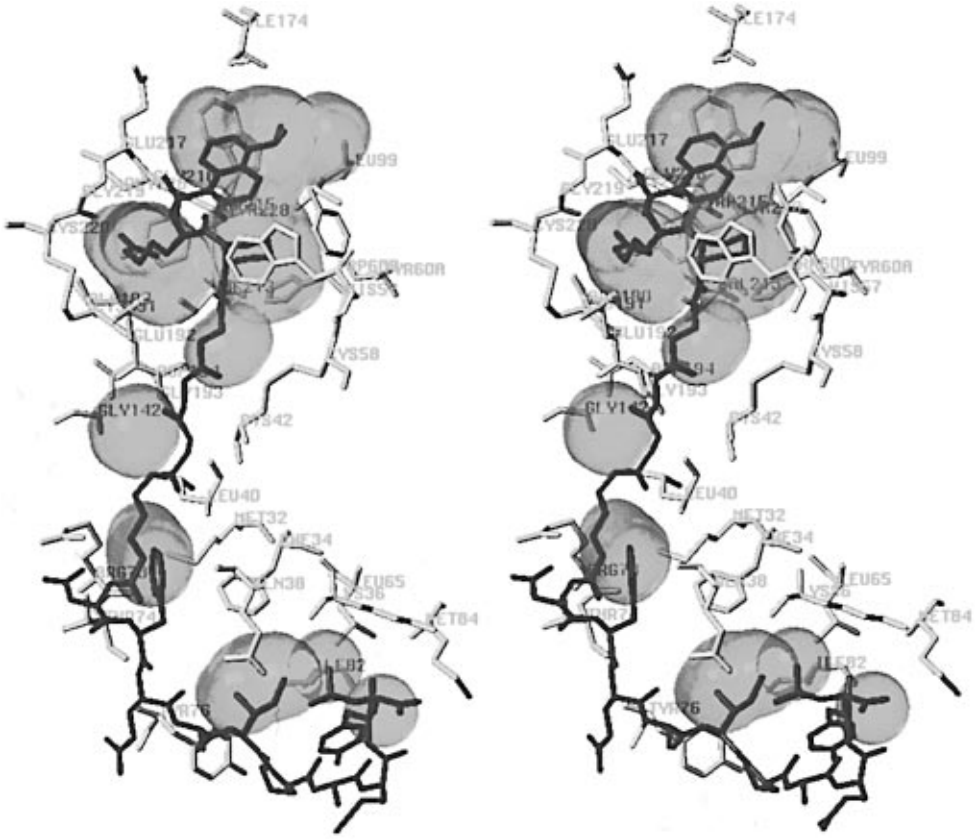


FIGURE 1: Stereoview of the crystal structure of the human α-thrombin−P498 complex and the volume contour (in green) of the methyl probes of molecular modeling. The structure of the inhibitor P498 is represented with bonds in blue. Only the nonpolar residues of thrombin interacting with the methyl probes are shown with bonds in yellow.

(1997) successfully incorporated both phenyl and cyclohexyl groups into the P1 binding pocket simultaneously. (4) The methyl probes were also found in the binding pocket of Phe^{H56} at the FRE and formed van der Waals contacts with Met^{32}, Phe^{34}, Leu^{40}, and the aliphatic side chain groups of Arg^{73} and Thr^{74}. In addition, (5) the methyl probes were found at the Ile^{H59} binding site, interacting with Phe^{34}, Leu^{65}, Tyr^{76}, and Ile^{82}; (6) in the vicinity of Tyr^{H63}, interacting with Ile^{82} and Met^{84}; and (7) in the vicinity of Leu^{H64}, interacting with Leu^{65} and the aliphatic side chains of Lys^{36} and of Gln^{38}. These seven binding sites have strong binding preference to the nonpolar residues of the substrates or inhibitors, except the S1 subsite which also has a specificity to the basic residue (Rydel et al., 1991; Bode et al., 1992). The overlaps of these seven sites with the nonpolar residues of thrombin inhibitors, thus, support the methyl probes of molecular modeling. Besides these seven sites, the methyl probe identified two nonpolar sites at the thrombin S′ subsites where the linkers of the bivalent inhibitors lie. One was in the S1′ subsite, and the methyl probe interacted with Cys^{42}−Cys^{58} residues of thrombin. The other was located between the AS and the FRE, and the methyl probes interacted with Leu^{40}, Gly^{142}, and Gly^{193}.

Methyl Scan. To investigate the nonpolar interactions of the linker with the S′ subsites of thrombin, we have designed a new strategy called methyl scan. This method consists of the incorporation of a side chain methyl group at each atom of a 16-atom linker using Sar, D,L-Ala, D,L-βAib, or *N*-methyl-βAla as shown in Figure 2. In this series, Bbs-Arg-(D-Pip) and Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH were used as the AS inhibitor and the FRE inhibitor segments, respectively. Five reference inhibitors (P1172, P1173, P1174, P1175, and P836) were also prepared (Table 2). These reference peptides have no side chain in the linker but have the same backbone structure as the corresponding inhibitors in Table 4. To investigate the possible nonpolar interactions of both D- and L-isoforms, we used D,L-mixtures of Ala and βAib in the synthesis with an assumption that the D- and L-isoforms were equally incorporated in the peptides. This assumption is reasonable when they reacted with the optically inactive Gly in the linker. An exception is P655 where D,L-Ala was connected to L-Asp^{H55} where L-Asp(tBu) may preferably react with the Fmoc derivative of D- or L-Ala. To avoid this uncertainty in the synthesis, two analogs (P1150 and P1151) of P655 in which D,L-Ala is replaced by D-Ala and L-Ala, respectively, were prepared. Both isoforms did not improve the affinity compared to that of the reference inhibitor P1172 (Table 3); therefore, no further study was performed at the 15th atom position. The K_i values of the inhibitors with a methyl side chain in the linker varied from $(5.4 \pm 0.3) \times 10^{-11}$ M for P646 (the methyl side chain at the fifth atom of the linker) to $(2.1 \pm 0.5) \times 10^{-12}$ M for P643 (the methyl side chain at the second atom of the linker) (Table 4). Correcting the K_i values with those of the corresponding reference inhibitors, the effect of the side chain methyl group is shown in Figure 3. P643 and P649 which have side chain methyl group at the second and eighth atom of the linker, respectively, were ~20-fold more potent than the reference peptide, P836. This suggests potential nonpolar interactions at the second and the eighth atoms of the linker with the corresponding S′ subsites of thrombin. We then examined the stereospecificity of these interactions by comparing the affinity of both isomers of P643 and P649 (Table 3). Both D- and L-isomers

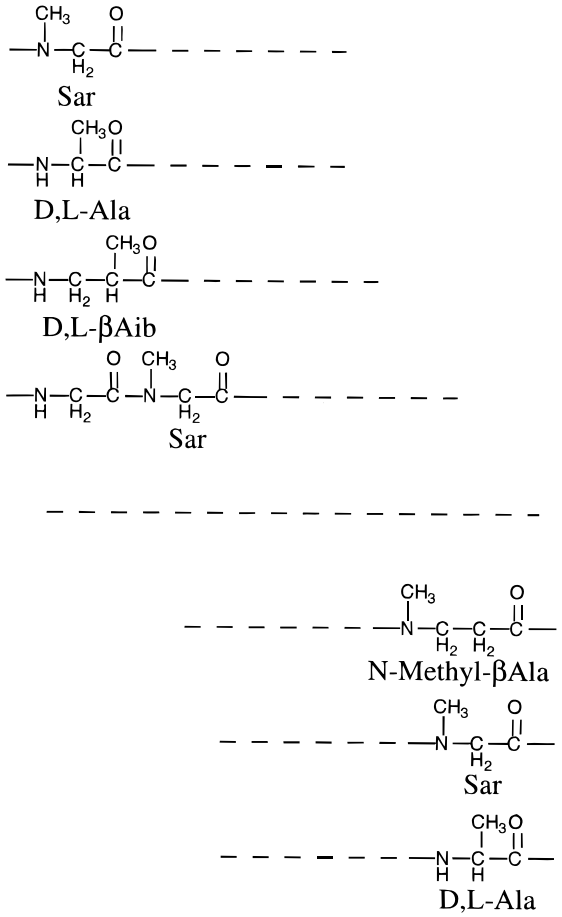


FIGURE 2: Schematic chemical structures of the linkers at the sites where a methyl side chain is introduced. Sar, D,L-Ala, and D,L-*β*Aib were repeatedly used to insert the methyl side chain at each atom site of the linker. *N*-Methyl-*β*Ala is used, as an exception, to insert the methyl side chain at the 13th atom of the linker.

Table 3: Preferred Side Chain Chirality at the Second, the Eighth, and the Fifteenth Atoms of the Linker

Bbs-Arg-(D-Pip)-[linker]-
Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

peptide	linker	K_i x 10^{11} (M)	ΔK_i[a]
P1004	Ala-Gly-Gly-Gly-*β*Ala	0.120 ± 0.040	0.050
P1005	(D-Ala)-Gly-Gly-Gly-*β*Ala	0.42 ± 0.05	0.175
P1054	Gly-Gly-Ala-Gly-*β*Ala	0.87 ± 0.02	0.36
P1055	Gly-Gly-(D-Ala)-Gly-*β*Ala	0.096 ± 0.003	0.040
P1150	*β*Ala-Gly-Gly-Gly-Ala	3.1 ± 0.4	1.29
P1151	*β*Ala-Gly-Gly-Gly-(D-Ala)	0.96 ± 0.05	0.40

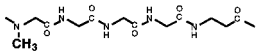
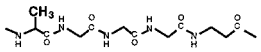
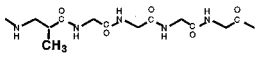
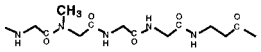
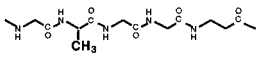
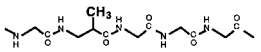
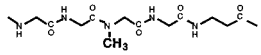
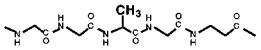
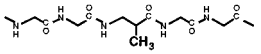
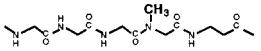
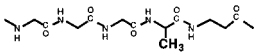
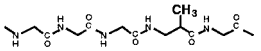
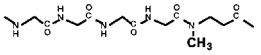
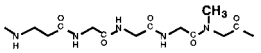
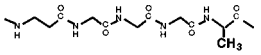
[a] ΔK_i is the K_i value of the inhibitors relative to the reference peptides [P836 [$K_i = (2.4 \pm 0.5) \times 10^{-11}$ M] for P1004, P1005, P1054, and P1055, or P1172 [$K_i = (2.4 \pm 0.3) \times 10^{-11}$ M] for P1150 and P1151].

of Ala improved affinities; however, the best inhibitors were obtained with the L-isomer (P1004) at the second atom and the D-isomer (P1055) at the eighth atom position of the linker. These side chain methyl groups remarkably improved the affinity by 20−25-fold compared to that of the reference inhibitor P836.

Backbone Scan. Searching for nonpolar interactions between the linker and the thrombin S′ subsites was not limited to the side chain. The hydrophobicity of the backbone of the linker can also be increased by inserting any hydrophobic group. In our study, a series of bivalent thrombin inhibitors in which a phenyl or cyclohexyl group was inserted in the backbone of the linker at various positions were prepared as listed in Tables 5 and 6. Since the phenyl and cyclohexyl groups reduce the flexibility of the linker, the length of the linker was increased to 18 atoms instead of 16 atoms used in the methyl scan. P610, P611, and P613 in the phenyl scan and P682 in the cyclohexyl scan showed higher affinities than other inhibitors in the series. Although the lack of the reference inhibitors in the backbone scans made the interpretation of the results difficult, the high affinities of P610, P611, P613, and P682 implied the nonpolar interactions of the linker at around the third through the fifth atoms and sixth or seventh atoms with thrombin. This implication is similar to the results of the methyl scan which suggested potential nonpolar interactions at the second and eighth atoms of the linker.

Table 4: Methyl Scan at the Linker of Bivalent Thrombin Inhibitors

Bbs-Arg-(D-Pip)-[linker]-
Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

Peptide	Linker Structure[a]	K_i x 10^{11} (M)	Reference Peptide
P642		1.8 ± 0.5	P836
P643		0.21 ± 0.05	P836
P644		0.46 ± 0.05	P1172
P645		2.6 ± 0.4	P836
P646		5.4 ± 0.3	P836
P647		3.1 ± 0.4	P1173
P648		1.20 ± 0.2	P836
P649		0.27 ± 0.06	P836
P650		2.4 ± 0.3	P1174
P651		4.1 ± 0.2	P836
P652		5.3 ± 0.5	P836
P653		1.10 ± 0.2	P1175
P1186		1.50 ± 0.2	P836
P654		0.98 ± 0.02	P1172
P655		0.56 ± 0.05	P1172

[a] Ala and *β*Aib used in the linker are D,L-mixtures of the corresponding amino acid.

DISCUSSION

Binding Modes. The length of the linker required for the bivalent inhibition depends on the binding mode (substrate

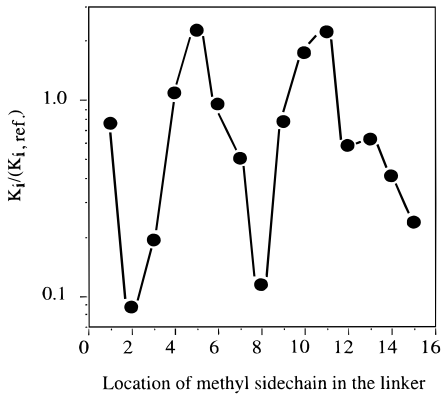
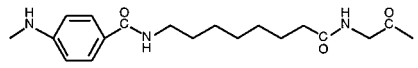
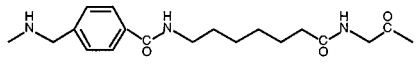
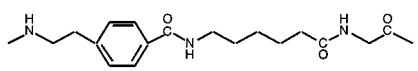
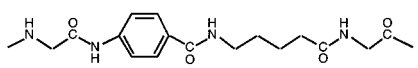
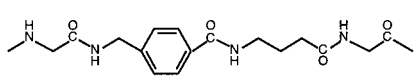
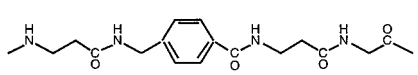
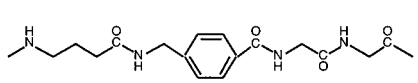


FIGURE 3: Relative K_i values of the bivalent inhibitors, Bbs-R-(D-Pip)-[linker]-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH (see Table 4 for the chemical structures of the linkers). The *X*-axis shows the backbone atom position of the linker to which the methyl side chain was added. The reference peptides listed in Table 2 are the corresponding inhibitors without the methyl side chain at the linker.

Table 5: Phenyl Scan at the Linkers of Bivalent Thrombin Inhibitors

Bbs-Arg-(D-Pip)-[linker]-
Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

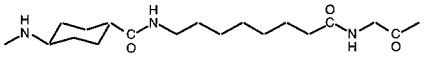
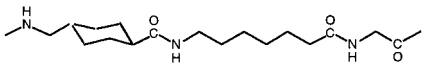
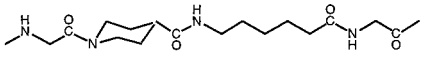
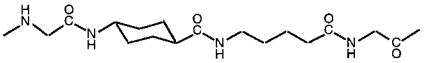
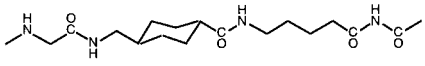
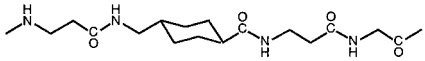
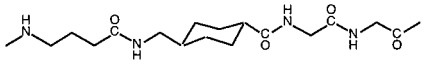
Peptide	Linker Structure[a]	K_i x 10^{11} (M)
P610		0.034 ± 0.005
P611		0.023 ± 0.008
P612		0.21 ± 0.03
P613		0.027 ± 0.008
P614		0.42 ± 0.19
P615		0.51 ± 0.05
P616		1.58 ± 0.22

[a] Due to the insertion of the bulky phenyl group, the length of the linker is extended in 18 atoms.

type or nonsubstrate type) of the AS inhibitor segment. Figure 4 compares the K_i values of these inhibitors as a function of the linker length. The inhibitors with the substrate type AS inhibitor segment (■) requires 12 atoms for high-affinity binding, whereas 15 atoms are required for the inhibitors with the nonsubstrate type binding mode (●). Interestingly, a reduced linker length (∼12 atoms) is required for high-affinity binding when L-Pip replaces D-Pip in the nonsubstrate type inhibitors (▲). This could be explained either by placing L-Pip into the S1′ subsite or by shifting the FRE inhibitor segment toward the AS. In the latter case, the longer linkers would restore the affinity of the inhibitors. The little improvement of the K_i values with the longer linkers excludes the latter binding mode. Dansyl, Arg, and L-Pip would thus take the substrate type binding mode by

Table 6: Cyclohexyl Scan at the Linker of Bivalent Thrombin Inhibitors

Bbs-Arg-(D-Pip)-[linker]-
Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH

Peptide	Linker Structure[a]	K_i x 10^{11} (M)
P681		0.28 ± 0.08
P682		0.041 ± 0.003
P683		0.20 ± 0.03
P684		0.53 ± 0.08
P685		0.71 ± 0.11
P686		1.02 ± 0.21
P687		1.40 ± 0.22

[a] Due to the insertion of the bulky cyclohexyl group, the length of the linker is extended to 18 atoms

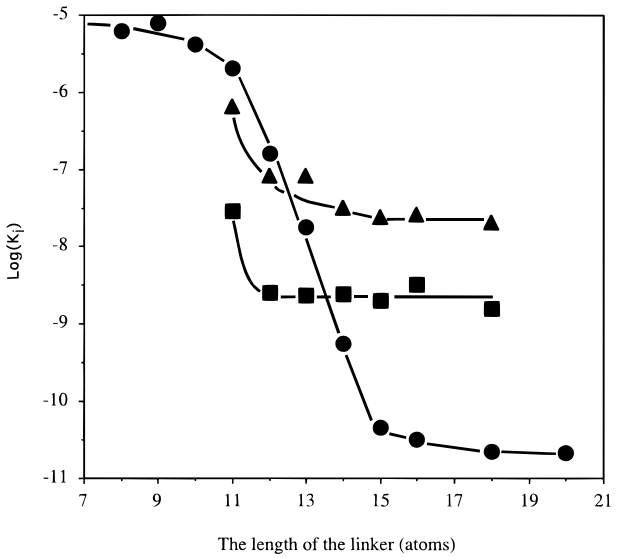


FIGURE 4: Inhibition constant [log(K_i)] of three bivalent thrombin inhibitors, dansyl-Arg-(D-Pip)-linker-hirudin$^{55-65}$ (●), dansyl-Arg-(L-Pip)-linker-hirudin$^{55-65}$ (▲), and Ac-(D-Phe)-Pro-Arg-Pro-linker-hirudin$^{55-65}$ (■) [data from Szewczuk et al. (1993)], as a function of the length of the linker. By taking 1.0 mol/L as a standard state, the K_i values were treated as dimensionless quantities in log(K_i).

occupying S2 and/or S3, S1, and S1′ subsites, respectively. It appears that the chirality of Pip^{H3} determines the binding mode (substrate or nonsubstrate type) of the AS inhibitor segment. Indeed, the replacement of D-Pip of P498 or P500 with L-Pip causes steric conflict in the crystal structures of thrombin−P498 and −P500 complexes. Similar phenomena have also been reported on other thrombin AS inhibitors. For example, the P1 residue is in the *S*-configuration in the substrate type AS inhibitor of D-Phe-Pro-Arg and the nonsubstrate type AS inhibitor of Argatroban but is in the

R-configuration in the other nonsubstrate type AS inhibitor of NAPAP. The P2 residue is in the *S*-configuration in the substrate type AS inhibitor of D-Phe-Pro-Arg but is in the *R*-configuration in the nonsubstrate type inhibitors of Argatroban and NAPAP (Bode et al., 1989; Brandstetter et al., 1992). Furthermore, even a retro-binding mode with L-amino acids is reported in a natural product nazumamide A (Nienaber & Amparo, 1996). This remarkable flexibility of thrombin interactions may reflect the multifunctions of thrombin.

S′ Subsites of Thrombin. Several crystal structures have been reported for the complexes of thrombin and synthetic bivalent inhibitors (Qiu et al., 1992; Zdanov et al., 1993; Rehse et al., 1995; Féthière et al., 1996). The bivalent inhibitors bind to the AS and the FRE of thrombin simultaneously, and the linker, which connects the AS and the FRE inhibitor segments, occupies the thrombin S′ subsites. The linkers with short but sufficient length for the bivalent inhibition lie along the deep canyon (Qiu et al., 1992; Zdanov et al., 1993; Rehse et al., 1995; Féthière et al., 1996), and may be used as templates forcing the P′ residues to be in the S′ subsites. Side chain(s) added on these short linkers may then interact with the thrombin S′ subsites and affect the K_i values of the inhibitors accordingly. In this article, we introduced the smallest side chain, methyl group, as a sensor of the possible nonpolar interactions between the P′ residues and the residues at the thrombin S′ subsites as described below.

Methyl Scan. Ala or Gly substitution is a method of verifying the importance of a particular side chain. This method was applied to the FRE inhibitor, Ac-hirudin$^{55-65}$, and Ile^{H59} was identified as a critical residue for the binding (Yue et al., 1992). Tsuda et al. (1995) then made various substitutions of Ile^{H59} using bivalent inhibitors. The results showed the importance of nonpolar interactions at the FRE. Unlike hydrogen bonds, which require specific functional groups (proton donor and acceptor), orientation, and distance, nonpolar interactions are less specific. Even an addition of a methyl group to Gly^{H59} (a substitution of Gly^{H59} by Ala) improved the affinity of the inhibitor ~10-fold, and the subsequent addition of the methyl groups in this side chain continuously improved the affinity up to ~10000-fold with L-*tert*-butylalanine^{H59}. Thus, the first methyl group inserted (a substitution of Gly^{H59} by Ala) may be a good sensor of nonpolar side chain interactions. Since N-substituted amino acids may interact through the side chain at the backbone nitrogen (Zuckermann et al., 1992), the search should not be restricted to the side chain at the Cα atom, i.e., a substitution of Gly by Ala. We extended the search to the positions of the backbone NH and carbonyl atoms; i.e., Sar introduces the methyl group at the α-amino group, and D,L-Ala introduces the methyl group at the Cα atom. Although a methyl group cannot be inserted on the backbone carbonyl group, using D,L-β-Aib or *N*-methyl-β-Ala can introduce a methyl side chain at the backbone carbonyl position (Figure 2). This insertion of the methyl side chains is designated as the methyl scan and searches the possible nonpolar interactions of both D- and L-isoforms. Indeed, the methyl side chain on the second or the eighth atom of the linker enhanced the affinity of the inhibitor 20−25-fold (Tables 3 and 4). These results encouraged us to investigate the surface of the thrombin S′ subsites further.

Possible Nonpolar Interactions of Thrombin S′ Subsites. Examination of the thrombin crystal structure revealed some nonpolar atoms exposed to the solvent at the S′ subsites. However, they are not necessarily favorable nonpolar binding sites since the binding of a nonpolar group may also desolvate neighboring polar groups. The use of a methyl probe that not only measures van der Waals interaction energies but also adds a desolvation term for both the probe and the protein assists in reliably identifying favorable nonpolar binding sites. The method is similar in spirit to the GRID calculation of Goodford and co-workers (Goodford, 1985; Boobyer et al., 1989) which had a methyl probe that used purely van der Waals interactions. We optimized the parameters of water molecules and the energy cutoff for general use; i.e., 90% of 86 nonpolar molecular association sites of 20 other protein−ligand complexes taken from the Brookhaven Protein Data Bank were identified by this method (manuscript in preparation). After we applied the probe to the thrombin S′ subsites, two sites were identified at the S1′ and the S3′ subsites, consistent with the results of the methyl scan. However, the preferred chirality of the P1′ and the P3′ residues was not predicted correctly if the linker structure of P498 was used as a template; i.e., the methyl side chain of D-Ala and D-Ala overlaps with the methyl probes in Figure 1 at the P1′ and the P3′ residues, respectively, whereas L- and D-isoforms are experimentally preferred for the P1′ and the P3′ residues, respectively. The inconsistency would come from the flexibility of the linker structure. Since the preference for the chirality is rather small (6- and 3-fold improvement in the K_i values at the P1′ and P3′ residues, respectively), no further analysis was performed on the chirality. We must emphasize that the molecular modeling methyl probe is a powerful technique for identifying the possible nonpolar interacting sites on protein surfaces; however, it does not mean that all these sites identified are used *in vivo*. For example, nonpolar amino acids are not preferred as the P1′ residue in the thrombin substrates. Nevertheless, these nonpolar S1′ and S3′ subsites are attractive in designing thrombin inhibitors, and the optimization of the P1′ and P3′ residues will be reported elsewhere (manuscript in preparation).

CONCLUSIONS

We have studied the nonpolar interactions between the thrombin S′ subsites and the linker of the bivalent thrombin inhibitors. The crystal structures of thrombin−bivalent inhibitor complexes revealed that the 16-atom linker length is appropriate for forcing the linkers to lie in the deep canyon of thrombin S′ subsites while retaining their conformational flexibility. We then introduced a methyl scan method as a sensor of possible nonpolar interactions between the S′ subsites and the linker side chain. The methyl side chains on the second and the eighth atoms of the linker, which correspond to the P1′ and the P3′ residues, respectively, improved the affinity of the inhibitors by ~20-fold. Further chirality study of these methyl side chains showed that the methyl group with L- and D-configurations on the second and the eighth atoms effectively improved the inhibitor affinity by 20- and 25-fold, respectively. These two nonpolar sites at the thrombin S1′ and S3′ subsites were independently identified by the methyl probe of the molecular modeling, supporting the validity of the methyl scan.

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