Design of Potent Bivalent Thrombin Inhibitors Based on Hirudin Sequence: Incorporation of Nonsubstrate-Type Active Site Inhibitors†

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Received May 10, 1994; Revised Manuscript Received September 20, 1994®

ABSTRACT: Hirudin from medicinal leech 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 blocking moiety, hirudin$^{1-48}$, a fibrinogen-recognition exo-site binding moiety, hirudin$^{55-65}$, and a linker, hirudin$^{49-54}$, connecting these inhibitor moieties. Synthetic inhibitors were designed based on the C-terminal portion of hirudin. The bulky active site blocking moiety, hirudin$^{1-48}$, was replaced by small nonsubstrate-type active site inhibitors of thrombin, e.g., dansyl-Arg-(D-pipecolic acid). The linker moiety was replaced by ω-amino acids of (12-aminododecanoic acid)−(4-aminobutyric acid), and hirudin$^{55-65}$ was used as a fibrinogen-recognition exo-site binding moiety in most of the inhibitors. The crystal structure of the inhibitor in complex with human α-thrombin showed that dansyl, Arg, and D-pipecolic acid of the active site blocking moiety occupy S3, S1, and S2 subsites of thrombin, respectively, and were therefore designated as P3, P1, and P2 residues. The use of dansyl-Arg-(D-pipecolic acid) improved the affinity (K_i) of the inhibitor 10−100-fold (down to 1.70×10^{-11} M) compared to that of the similar compounds having D-Phe-Pro-Arg as their substrate-type inhibitor moiety ($K_i = 10^{-9}-10^{-10}$ M). The linker connected to P2 residue eliminated the scissile peptide bond. The inhibitor was also stable against human plasma proteases. Further inhibitor design revealed that the toxic dansyl group could be replaced by 4-*tert*-butylbenzenesulfonyl group and 1- or 2-naphthalenesulfonyl group for *in vivo* studies. In addition, the replacement of hirudin$^{55-65}$ with [Tyr^{56},-Pro^{58},Ala^{63},Cha^{64},D-Glu^{65}]hirudin$^{55-65}$ improved the affinity of the inhibitors ($K_i = 2.0 \times 10^{-12}$ M) to the level 10-fold less potent than recombinant hirudin ($K_i = 2.3 \times 10^{-13}$ M).

Thrombin (EC 3.4.21.5) plays a central role in the coagulation cascade (Fenton, 1981). The primary function of thrombin is to activate fibrinogen to fibrin to generate an insoluble fibrin clot. In addition, it has regulatory functions in the activation of several proteases such as factor V, factor VIII, factor XIII, and protein C in the coagulation cascade (Fenton, 1981) as well as platelets (Fenton & Bing, 1986). However, beside these important physiological roles, thrombin is known to have severe adverse effects, specially in myocardial infarctus disorders (Badimon et al., 1991). Effectively an acute blockage of coronary arteries by a thrombus almost always leads to myocardial infarction. However, the events leading to such attack can be alleviated by suitable thrombolytic treatment with tissue plasminogen activator, urokinase, or streptokinase. Unfortunately these often induce acute thrombotic reocclusion (Chesebro et al., 1987; Verstraete et al., 1985; While et al., 1989; Wilcox et al., 1988). Although the mechanism of reocclusion is not known, the possible role of thrombus-bound thrombin, which is still active, has been proposed (Francis et al., 1983; Agnelli et al., 1991), indicating the need for potent and specific pharmacological agents.

Heparin, through the formation of a complex with antithrombin III, is known to clear the plasma of free thrombin. It acts as a natural anticoagulant. Unfortunately, the thrombus-bound thrombin is poorly accessible to heparin (Hogg & Jackson, 1989; Weitz et al., 1990). In contrast, small active site inhibitors such as MD-805[1] (Kikumoto et al., 1980b) and benzamidine derivatives (Markwardt et al., 1983; Hauptmann et al., 1980; Krupinsky et al., 1989) effectively inhibit thrombin trapped in the thrombus (Badimon et al., 1991). A major disadvantage that applies to most of these active-site-directed synthetic inhibitors is their short half-life of a few minutes (Badimon et al., 1991). Since thrombolytic therapy activates the coagulation cascade resulting in continuous production of thrombin, the inhibitors

† NRCC Publication No. 36847. We dedicate this article to Professor Maki Yoshifumi on the occasion of his retirement from Gifu Pharmaceutical University in March, 1994.

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® Abstract published in *Advance ACS Abstracts,* November 1, 1994.

[1] Abbreviations: γAbu, 4-aminobutyric acid; Ac, acetyl; Acha, 1-aminocyclohexanecarboxylic acid; μAdod, 12-aminododecanoic acid; AMC, 7-amino-4-methylcoumarin; Bbs, 4-*tert*-butylbenzenesulfonyl; Boc, *tert*-butyloxycarbonyl; Cha, β-cyclohexylalanine; Fmoc, 9-fluorenylmethoxycarbonyl; FRE, fibrinogen recognition exo site; 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; 1-Nas, 1-naphthalenesulfonyl; 2-Nas, 2-naphthalenesulfonyl; Nle, norleucine; OBzl, benzyl ester; Pip, pipecolic acid; PPACK, D-Phe-Pro-Arg chloromethylketone; 3-TAPAP, N^{α}-(4-toluenesulfonyl)-D,L-*m*-amidinophenylalanylpiperidine; 4-TAPAP, N^{α}-(4-toluenesulfonyl)-D,L-*p*-amidinophenylalanylpiperidine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Tos, *p*-toluenesulfonyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/94/0433-14443$04.50/0 Published 1994 by the American Chemical Society

with short half-lives may not be effective agents (Badimon et al., 1991).

Another approach for a selective inhibition of thrombus-bound thrombin is the use of hirudin produced in the salivary glands of the European medicinal leech *Hirudo medicinalis*. Hirudin is a small protein of 65 amino acids that has following potential advantages over other anticoagulants: (1) it is the most potent and specific thrombin inhibitor with a K_i value of 2.2×10^{-14} M (Stone & Hofsteenge, 1986), (2) it blocks the active site and the FRE of thrombin simultaneously (Rydel et al., 1990 & 1991), (3) it inhibits thrombus-bound thrombin as well as free thrombin in solution (Badimon et al., 1991), (4) it has a long half-life of 50 min when given intravenously or subcutaneously (Markwardt et al., 1984), (5) its antigenicity is very weak, and (6) it has no acute side effects.

These advantages have motivated the design of small synthetic thrombin inhibitors based on the hirudin sequence in order to mimic the distinctive mechanism of hirudin. The crystal structure of human α-thrombin−hirudin complex revealed that hirudin is composed of an active site blocking moiety, hirudin$^{1-48}$, a FRE binding moiety, hirudin$^{55-65}$, and a linker moiety, hirudin$^{49-54}$, which connects these two elements (Rydel et al., 1990). The bulky active site blocking moiety, hirudin$^{1-48}$, being difficult to synthesize, was replaced by a smaller peptide, D-Phe-Pro-Arg-Pro (Maraganore et al., 1990; DiMaio et al., 1990; Bourdon et al., 1991). This substitution reduced the size of the inhibitors to 21 residues, which is more reasonable for conventional peptide synthesis; however, it also reduced the affinity 3−4-orders of magnitude due to the low affinity of the tetrapeptide to the thrombin active site. A further replacement of the Arg^{47}−Pro^{48} scissile bond with a pseudo peptide bond (DiMaio et al., 1991 and 1992) or a replacement of the N-terminal D-Phe with D-Cha (Witting, et al., 1992b) restored some of the affinity lost (1.4×10^{-10} M or 7.7×10^{-11} M, respectively).[2] The FRE binding moiety, hirudin$^{55-65}$, which, by itself, mimics anti-thrombin effect of hirudin (Jakubowski & Maraganore, 1990), was also modified to enhance its potency and proteolytic stability while reducing its size. Multiple-substitutions of residues Asp^{55}, Phe^{56}, Glu^{58}, Tyr^{63}, Leu^{64}, and Gln^{65} with succinic acid, Tyr, Pro, Ala, Cha, and D-Glu, respectively, resulted in an 35-fold more potent inhibitor, MDL-28,050 (Krestenansky et al., 1990). The proteolytic stability of the FRE inhibitor (Knadler et al., 1992) was improved by a side chain to side chain cyclization between Glu^{58} and a substituted Lys^{61} with 2−4-fold improvement of the IC_{50} (Szewczuk et al., 1992). Furthermore, the size of FRE inhibitor was reduced from 11 to 7 residues with minimal loss in potency. This truncated FRE inhibitor was successfully incorporated into a bivalent inhibitor (DiMaio et al., 1992). The linker moiety, hirudin$^{49-54}$, has some interactions with the thrombin S′ sites (Rydel et al., 1991). However, the linker in the synthetic bivalent inhibitors has been used as a spacer predominantly. and the sequence was simplified by removing the side chains (Maraganore et al., 1990; DiMaio et al., 1992; Bourdon et al., 1991). Szewczuk et al. (1993) optimized the length of the linker by using a combination of ω-amino acids and improved the inhibition constant up to 11-fold by introducing interactions between the linker and the thrombin S′ sites.

D-Phe-Pro-Arg or its analogs have been used in the design of the active site blocking moiety. The crystal structure of PPACK-thrombin (Bode et al., 1989) suggested that D-Phe-Pro-Arg in the bivalent inhibitors binds to the thrombin active site in the substrate binding mode, where Arg−X is the scissile peptide bond. In fact, the active site blocking moiety, D-Phe-Pro-Arg-Pro, of the bivalent inhibitors was hydrolyzed slowly at Arg−Pro peptide bond (DiMaio et al., 1990; Witting et al., 1992a; Szewczuk et al.,1993). The arginine and benzamidine-based thrombin inhibitors such as MD-805, NAPAP, and TAPAP are other types of active site inhibitors (Okamoto et al., 1980, 1981; Kikumoto et al., 1980a,b, 1984; Stürzebecher et al., 1983, 1984). The characteristic sequence of these compounds is hydrophobic−Arg (or benzamidine analogs)−piperidine (or analogs). The crystal structure of these inhibitors in complex with thrombin reveals that the three residues from the N-terminus occupy S3, S1, and S2 subsites of the thrombin active site, respectively (Brandstetter et al., 1992). Consequently, they are numbered P3, P1, and P2, respectively, in this article.[3] The incorporation of these nonsubstrate type active site inhibitors into the bivalent inhibitors eliminates the thrombin-sensitive peptide bond. Furthermore, a high affinity of the nonsubstrate type inhibitors ($K_i = 1.9 \times 10^{-8}$ and 6.6×10^{-9} M for MD-805 and NAPAP, respectively) may improve the inhibition constant of these bivalent inhibitors.

In this article, we characterized the affinity and proteolytic stability of the bivalent thrombin inhibitors containing nonsubstrate-type active site blocking moiety. Further improvement in the affinity of the inhibitors by incorporating the high affinity exo-site inhibitor, MDL-28,050, is also described.

EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin (3000 NIH units/mg) for enzymatic assays, bovine fibrinogen (~70% protein, 85% clottable protein), Tos-Gly-Pro-Arg-AMC·HCl salt, poly(ethylene glycol) 8000, μAdod, and Tris were purchased from Sigma (St. Louis, MO). Human α-thrombin (approximately 3800 NIH units/mg) for crystallization was purchased from Haematologic Technologies Inc. (Essex Jct., VT) and was dialyzed against 50 mM sodium phosphate buffer, pH 5.5, containing 100 mM NaCl to remove glycerol just before the crystallization. AMC, dansyl chloride, *tert*-butylbenzenesulfonyl chloride, 1-naphthalenesulfonyl chloride, and 2-naphthalenesulfonyl chloride were obtained from Aldrich (Milwaukee, WI). Boc-γAbu, Boc-L-Pip, Boc-D-Pip, and Boc-D-Tic were purchased from BaChem BioScience Inc. (Philadelphia, PA). Acha was obtained from Fluka (Ronkonkoma, NY). Boc-μAdod and Boc-Acha were prepared according to the procedure described by Chaturvedi et al.

[2] The numbering of the inhibitor residues is based on the hirudin sequence.

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

(1984). All other amino acid derivatives for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY). The side chain protecting groups for Boc-amino acids were benzyl for Glu, cyclohexyl for Asp, Tos for Arg, and 2-bromobenzyloxycarbonyl for Tyr. Boc-Gln-OCH_2-phenylacetylamidomethyl resin (0.714 mmol/g) and *p*-methylbenzhydrylamine resin (0.770 mmol/g) were purchased from Applied Biosystems Inc. (Foster City, CA). Boc-D-Glu-(OBzl)-OCH_2-phenylacetylamidomethyl resin (0.31 mmol/g) was purchased from Peninsula Laboratories Inc. (Belmont, CA). The solvents for peptide synthesis were obtained from Anachemia Chemical Inc. (Rouses Point, NY) and Applied Biosystems Inc. Citric acid was purchased from Anachemia Chemical Inc. HF and TFA were purchased from Matheson Gas Products Inc. (Secaucus, NJ) and Halocarbon Products Co. (North August, SC), respectively.

Peptide Synthesis. The peptides were prepared according to the method described elsewhere (Szewczuk et al., 1992). Final products were obtained as lyophilizates with 98% or higher purity estimated by analytical HPLC. The purified peptides were identified by amino acid analysis on a Beckman Model 6300 high performance analyzer and by molecular mass analysis using a SCIEX API III mass spectrometer. Peptide contents in lyophilizates were determined by the amino acid analysis.

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, as reported elsewhere (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 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 0.1−5-fold of K_i, $(1-8) \times 10^{-6}$ M and 6.0×10^{-11} M, respectively, for the data in Table 1. For data in Tables 2 and 3, the corresponding concentrations were 0.5−1000-fold of K_i, $(1-8) \times 10^{-6}$ M, and 3.0×10^{-11} M, respectively , if $K_i > 10^{-10}$ M; 10−100-fold 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.[5] The hydrolysis of the substrate by thrombin was monitored on a Varian-Cary 2000 spectrophotometer in the fluorescence mode ($\lambda_{ex} = 383$ nm; $\lambda_{em} = 455$ nm) or on a Hitachi F2000 fluorescence spectrophotometer ($\lambda_{ex} = 383$ nm; $\lambda_{em} = 455$ nm), and the fluorescent intensity was calibrated using AMC. The reaction reached a steady state within 3 min after mixing thrombin with the substrate and an inhibitor. The steady-state velocity was then measured for a few minutes. P448 was also preincubated with thrombin for 20 min at room temperature before adding the substrate. The steady-state was achieved within 3 min and measured for a few minutes. The kinetic data (the steady-state velocity at various concentrations of the substrate and the inhibitors) of the competitive inhibition was analyzed using the methods described by Segel (1975). A nonlinear regression program, RNLIN in the IMSL library (IMSL, 1987), LMDER in MINPACK library (More et al., 1980), or Microsoft Excel, was used to estimate the kinetic parameters (K_m, V_{max}, and K_i).

Proteolytic Assays. Proteolytic stability of an inhibitor against human α-thrombin (Szewczuk et al., 1993) or human plasma proteases (Szewczuk et al., 1992) was measured as described. Proteolytic stability of an inhibitor against proteases of kidney membranes was measured as follows: Kidney membranes were prepared at 0−4 °C according to the procedure (method 3) of Maeda et al. (1983). The kidneys of Sprague-Dawley rats were finely minced with surgical scissors. The tissue (1 g) was then homogenized in 3 mL of homogenization buffer (10 mM sodium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 30 mM NaCl, 0.02% NaN_3 and 10 μg/L of DNase) with a Polytron homogenizer (Brinkmann). For sufficient cell disruption, the tissue was subjected to five or six bursts for 5 s each at a power setting of 7 separated by 1−2 min of cooling. Approximately 10 mL of the homogenate was layered over 10 mL of a 41% (w/v) solution of sucrose and centrifuged in a Beckman SW27 swinging bucket rotor (100000*g* for 30 min). The interfacial membranes were collected and washed twice with 10 mM Tris-HCl buffer, pH 7.4. Small aliquots of the suspension of the membranes were stored in the same buffer at −80 °C until used. The protein content of the suspension was determined before storage by amino acid analysis. An aliquot of kidney membranes (3 mg) and 3.0×10^{-9} M of the inhibitor were incubated in 0.6 mL of 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 30 mM NaCl, 0.02% NaN_3, and 10 μg/L of DNase, at 37 °C for digestion. Aliquots (0.15 mL) of the reaction solution were taken at 0, 15, and 30 min of the reaction time. The reaction was terminated by heating at 100 °C in boiling water for 2 min. The sample was subsequently spun at 6000*g* for 2 min, and the supernatant was injected onto a Hewlett Packard Model 1090 HPLC to identify the metabolites.

The inhibitors and their proteolytic products were isolated on an analytical C_{18} column (4.6 × 250 mm, Vydac) with a linear gradient of acetonitrile (10−70% in 60 min) containing 0.1% TFA at a flow rate of 1 mL/min. The elution profile was monitored by reading the absorbance at 210 nm. The peptides were collected and identified by amino acid analysis and mass spectrometry (SCIEX API III) (Szewczuk et al., 1992).

Crystallization of Thrombin−Inhibitor Complex. The complex of human α-thrombin and an inhibitor, dansyl-Arg-(D-Pip)-μAdod-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH (P500, $K_i = 2.6 \pm 1.0 \times 10^{-11}$ M), was crystallized by hanging drop method under previously described conditions (Zdanov et al., 1993). The crystals diffracted to 2.2-Å resolution and were isomorphous to other thrombin−inhibitor complexes (Zdanov et al., 1993). Dif-

[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.

[5] The final concentrations of the inhibitor, the substrate, and human thrombin were 10−100-fold 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 a nonlinear regression program UltraFit (Biosoft).

fraction data were collected on R-Axis IIC area detector in a standard way.

RESULTS

Active Site Inhibitors. (2*R*,4*R*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidine carboxylic acid (MD-805), N^{α}-(4-toluene-sulfonyl)-D,L-(*m*- or *p*)-amidinophenylalanylpiperidine (TAPAP), and N^{α}-(2-naphthylsulfonylglycyl)-D,L-*p*-amidinophenylalanylpiperidine (NAPAP) have the characteristic sequence: hydrophobic−Arg (or benzamidine)−piperidine (or its analogs), which bind to S3, S1, and S2 pockets of the thrombin active site, respectively (Brandstetter et al., 1992). The carboxyl group of the P2 residue, (2*R*,4*R*)-4-methylpipecolic acid, of MD-805 is directed to the S1′ subsite in the complex with thrombin and may be connected to the FRE inhibitor through an appropriate linker, thus forming a bivalent inhibitor. Among many MD-805 analogs listed in the patents (Okamoto et al., 1982), dansyl-Arg-Pip is straightforward to synthesize and has a relatively high potency ($IC_{50} = 3.5 \times 10^{-7}$ M) compared to that ($IC_{50} = 3.2 \times 10^{-8}$ M) of MD-805 (Okamoto et al., 1981). Both dansyl-Arg-(D-Pip)-NH_2 (P429) and dansyl-Arg-(L-Pip)-NH_2 (P428) inhibited thrombin competitively (Figure 1). But the D-Pip enantiomer binds tighter to thrombin, i.e., $K_i = (1.58 \pm 0.57) \times 10^{-7}$ M for P429 and $K_i = (1.91 \pm 0.13) \times 10^{-5}$ M for P428 (Table 1). However, an addition of a short linker, 4-aminobutyric acid (γAbu), reduced the potency of the D-isomer (P429) by a factor of 6 (P431), while having almost no effect on that of the L-isomer (P428 vs P430) (Table 1). Since preference of D-Pip over L-Pip was reduced by the addition of a short linker, the stereo requirement of the P2 residue was further examined by incorporating it into a bivalent inhibitor.

Active Site Blocking Moiety in Bivalent Inhibitor. Dansyl-Arg-(D-Pip) and dansyl-Arg-(L-Pip) were incorporated into the bivalent inhibitors (P448 and P447, respectively), in which 12-aminododecanoic acid (μAdod)-γAbu and hirudin$^{55-65}$ were used as a linker moiety and a FRE binding moiety, respectively. This 18-atom linker[6] has sufficient length to link the active site blocking and FRE binding moieties as described elsewhere (Y. Tsuda et al., manuscript in preparation). Under the conditions used (see Experimental Procedures), the inhibitors inhibited thrombin competitively (Figure 1), and thrombin reached a steady state within 3 min. We ensured that the steady state was attained by premixing P448 with thrombin for 20 min before adding the substrate. The steady state was also achieved within 3 min, giving a K_i value of $(1.22 \pm 0.03) \times 10^{-11}$ M. This value is consistent with that [$(1.70 \pm 0.42) \times 10^{-11}$ M] obtained when thrombin was added to the mixture of the substrate and P448. In contrast to the high affinity of P448, the corresponding bivalent inhibitor with L-Pip showed a lower affinity [$K_i = (1.24 \pm 0.18) \times 10^{-8}$ M], demonstrating a strong preference of D-Pip over L-Pip at the P2 position.

Several analogs of P448 were synthesized to investigate the structural requirements of the active site blocking moiety

[6] The linkers were expressed as the number of atoms contributing to the length of the linkers, 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 P2 residue in this paper whereas it was connected to P1′ residue in the previous paper (Szewczuk et al., 1993).

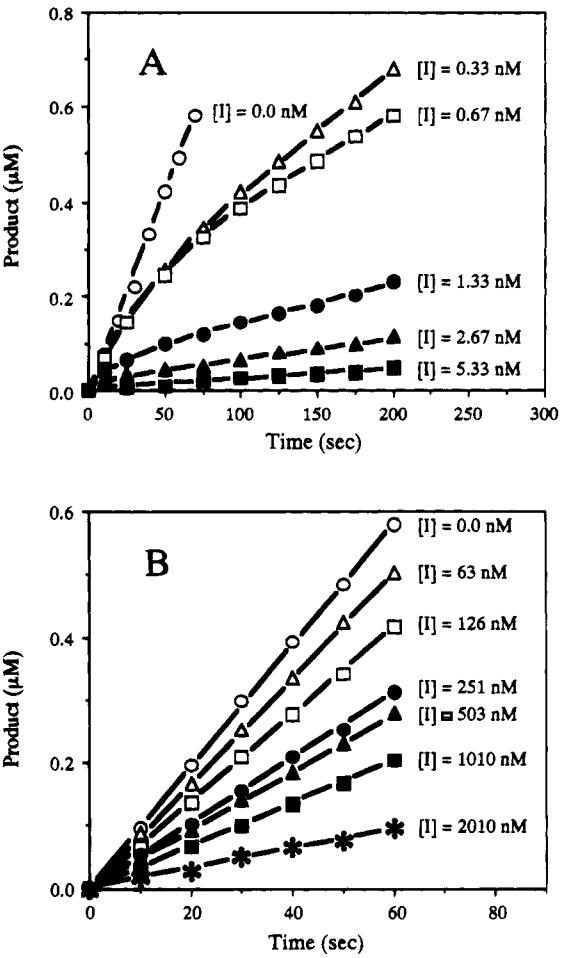

FIGURE 1: Inhibition of human α-thrombin (A; 0.033 NIH unit/mL, B; 0.066 NIH unit/mL) by (A) P448 [$K_i = (1.70 \pm 0.42) \times 10^{-11}$ M] and (B) P429 [$K_i = (1.58 \pm 0.57) \times 10^{-7}$ M], respectively. The fluorogenic assay was performed using Tos-Gly-Pro-Arg-AMC ($K_s = 3.5$ μM and $V_{max} = 1.4$ μM/min) as a substrate at pH 7.8 and room temperature. The amount of the product, 7-amino-4-methylcoumarin, is plotted at a substrate concentration of (A) 40 μM or (B) 4 μM and as functions of inhibitor concentrations and time.

Table 1: Activities of the Thrombin Active-Site-Directed Inhibitors

peptide	sequence	K_i (μM)[a]
P429	dansyl-Arg-(D-Pip)-NH_2	0.158 ± 0.057
P428	dansyl-Arg-(L-Pip)-NH_2	19.1 ± 1.3
P431	dansyl-Arg-(D-Pip)-Abu-NH_2	0.98 ± 0.13
P430	dansyl-Arg-(L-Pip)-Abu-NH_2	11.6 ± 3.3
P396	dansyl-Arg-(D-Tic)-NH_2	0.31 ± 0.02

[a] The inhibitors showed a competitive inhibition, and their inhibition was analyzed by using the method of Segel (1975).

(Table 2). The P2 residue, D-Pip, was replaced by D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic), which increases the hydrophobicity and bulkiness of this site (P471), resulting in a 17-fold loss of affinity [$K_i = (2.9 \pm 0.4) \times 10^{-10}$ M]. However, when a similar substitution was done on the active site inhibitor [P429, dansyl-Arg-(D-Pip)-NH_2, vs P396, dansyl-Arg-(D-Tic)-NH_2], there was only a 2-fold loss of the affinity. Due to the different effect of the substitution on the active site inhibitor and the bivalent inhibitor, the substitutions at the active site blocking moiety were evaluated using the bivalent inhibitor, P448, as a lead prototype (Table 2). The hydrophobic side chain and the amide backbone of the P2 residue were separated using

Table 2: Activity of Thrombin Inhibitors with Various Active Site Inhibitor Segments (Active Site Inhibitor Segment)-μAdod-γAbu-DFEEIPEEYLQ-OH

peptide	structure of active site inhibitor segment	activity (K_i, nM)
P448	dansyl-Arg-(D-Pip)	0.0170 ± 0.0042
P447	dansyl-Arg-(L-Pip)	12.4 ± 1.8
P471	dansyl-Arg-(D-Tic)	0.29 ± 0.04
P472	dansyl-Arg-(D-Cha)	17.1 ± 3.1
P473	dansyl-Arg-Acha	36 ± 10
P476	dansyl-Phe-(D-Pip)	2.6 ± 0.2
P477	dansyl-Cha-(D-Pip)	5.9 ± 1.0
P493	dansyl-Nle-(D-Pip)	5.2 ± 1.3
P492	dansyl-(D-Arg)-(D-Pip)	1.02 ± 0.38
P552	Bbs-Arg-(D-Pip)	0.0165 ± 0.0009
P531	(1-Nas)-Arg-(D-Pip)	0.045 ± 0.001
P532	(2-Nas)-Arg-(D-Pip)	0.032 ± 0.006
P481	Ac-(D-Cha)-Arg-(D-Pip)	9.5 ± 0.2
P482	Ac-(D-Tic)-Arg-(D-Pip)	12.2 ± 3.2
P483	Ac-(D-Phe)-Arg-(D-Pip)	55 ± 7
P484	Fmoc-Arg-(D-Pip)	14.8 ± 1.2

1-aminocyclohexanecarboxylic acid (Acha) and D-β-cyclohexylalanine (D-Cha). Although the separation makes the P2 residue more flexible and may optimize the hydrophobic interaction with the thrombin S2 subsite, these substitutions were not favorable in the bivalent inhibitors, resulting in K_i values of $(3.6 \pm 1.0) \times 10^{-8}$ M and $(1.71 \pm 0.31) \times 10^{-8}$ M for the inhibitors with Acha (P473) and D-Cha (P472), respectively.

Attempts have been made to substitute the P1 residue, Arg, to reduce toxicity (Kikumoto et al., 1980b) and the blood pressure reducing side effect (Kaiser et al., 1987) with some success (Claeson et al., 1992). Since the P1 residue, Arg, was successfully replaced by benzamidine, we replaced it with Phe, the hydrophobic moiety of benzamidine, resulting in a K_i value of $(2.6 \pm 0.2) \times 10^{-9}$ M (P476). It was further substituted with the reduced form, Cha, (P477), to further increase the hydrophobicity. The resulting K_i value showed no improvement [$(5.9 \pm 1.0) \times 10^{-9}$ M]. Similarly, another substitution with an aliphatic side chain, norleucine (Nle), (P493) did not improve the affinity [$K_i = (5.2 \pm 1.3) \times 10^{-9}$ M]. It is interesting to note that a substitution with D-Arg (P492) retained the affinity of the inhibitor in the nanomolar range [$K_i = (1.02 \pm 0.38) \times 10^{-9}$ M]. The nanomolar potency may be acceptable if the substitution improves the properties of the inhibitor *in vivo*.

The dansyl group, which occupies the S3 subsite, is linked to Arg through a sulfonamide bond (SO_2-NH). The P3 residue in all of the nonsubstrate−type inhibitors has the sulfonamide bond including benzamidine-based inhibitors such as NAPAP (Stürzebecher et al., 1983, 1984; Kaiser et al., 1987). The replacement of the sulfonamide bond with an amide bond of D-Tic was unsuccessful: P482, which has (D-Tic)-Arg-(D-Pip), is 700-fold less potent [$K_i = (1.22 \pm 0.32) \times 10^{-8}$ M] than P448. 9-Fluorenylmethoxycarbonyl (Fmoc) is another possible substitute for dansyl, but it introduces a spacer between the aromatic group and the amide bond linkage. Although this spacer may provide more flexibility for the aromatic group to fit into the S3 subsite of thrombin, the affinity of the inhibitor, P484, was 900-fold [$K_i = (1.48 \pm 0.12) \times 10^{-8}$ M] lower than that of P448. It has been known that D-Phe and D-Cha also fit into the S3 subsite (Witting et al., 1992b). However, the substitutions of the dansyl group with D-Phe and D-Cha have increased the inhibition constant of the inhibitors to $K_i = (5.5 \pm 0.7) \times 10^{-8}$ and $(9.5 \pm 0.2) \times 10^{-9}$ M, respectively. This implies that not only the hydrophobicity but also the linkage to the P1 residue, Arg, is critical for the P3 residue. On the contrary, the substitutions of dansyl group with 1-naphthalenesulfonyl (P531) or 2-naphthalenesulfonyl (P532) groups resulted in inhibitors which retained a high affinity [K_i values of $(4.5 \pm 0.1) \times 10^{-11}$ M and $(3.2 \pm 0.6) \times 10^{-11}$ M, for P531 and P532, respectively]. A substitution with 4-*tert*-butylbenzenesulfonyl group (P552) showed no loss of the affinity [K_i value of $(1.65 \pm 0.09) \times 10^{-11}$ M] compared to that of P448. It seems that sulfonamide bond plays an important role in connecting the hydrophobic P3 residue and Arg residue.

Table 3: Activity of Thrombin Inhibitors with Various Active Site Inhibitor Segments (Active Site Inhibitor Segment)-μAdod-γAbu-DYEPIPEEA-Cha-(D-Glu)-OH

peptide	structure of active site inhibitor segment	activity (K_i, nM)
P535	dansyl-Arg-(D-Pip)	0.0020 ± 0.0004
P551	(2-Nas)-Arg-(D-Pip)	0.0042 ± 0.0002
P553	Bbs-Arg-(D-Pip)	0.0021 ± 0.0008

Exo-Site Binding Moiety in Bivalent Inhibitors. Krstenansky et al. (1990) designed a potent thrombin exo-site inhibitor based on the hirudin$^{55-65}$ sequence. MDL-23050 [succinyl-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH] has an IC_{50} value of 1.5×10^{-7} M. We have used this MDL-23050 in the design of our bivalent inhibitors in order to further improve their affinity. For example, the incorporation of MDL-23050 into P448 resulted in the sequence dansyl-Arg-(D-Pip)-μAdod-γAbu-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D-Glu)-OH (P535). This inhibitor, P535, has a K_i value of $(2.0 \pm 0.4) \times 10^{-12}$ M, 8-fold more potent than P448. Similarly, the replacement of hirudin$^{55-65}$ moiety of P532 and P552 improved the inhibition constant 8-fold, resulting in K_i values of $(4.2 \pm 0.2) \times 10^{-12}$ M (P551) and $(2.1 \pm 0.8) \times 10^{-12}$ M (P553), respectively (Table 3). It is noteworthy that the affinity of the inhibitors, P535 and P553, is only one-tenth of that [$K_i = (2.31 \pm 0.06) \times 10^{-13}$ M] of the recombinant hirudin (Wallace et al., 1989).

Proteolytic Stability. Thrombin inhibitors are exposed to three types of proteases *in vivo* (1) thrombin, which forms complexes with the inhibitors and may hydrolyze them (DiMaio et al., 1990; Witting et al., 1992a; Szewczuk et al., 1993), (2) plasma proteases, which the inhibitors encounter in the circulation, and (3) proteases of the kidney, which are involved in the clearance of the peptides. The proteolytic stability of the inhibitor P448, dansyl-Arg-(D-Pip)-μAdod-γAbu-DFEEIPEEYLQ-OH, was examined. No hydrolysis of P448 was observed even after 6 h of incubation with human α-thrombin at 37 °C (Figure 2A). Human plasma contains various proteases associated with living tissue (Fishman & Doellgast, 1975). It digested an FRE inhibitor, succinyl-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH (P137) at several sites (Szewczuk et al., 1992) within 2 h. The bivalent thrombin inhibitor hirutonin-2, N^{α}-acetyl-(D-Phe)-Pro-Arg-(CH_2-CH_2-CO)-hirudin$^{48-65}$, was, on the other hand, highly resistance to plasma proteases with only a minor hydrolysis between Glu^{61} and Glu^{62} after 2 h of incubation (DiMaio et al., 1992). P448 was even more resistance to plasma proteases such that no hydrolysis was observed after

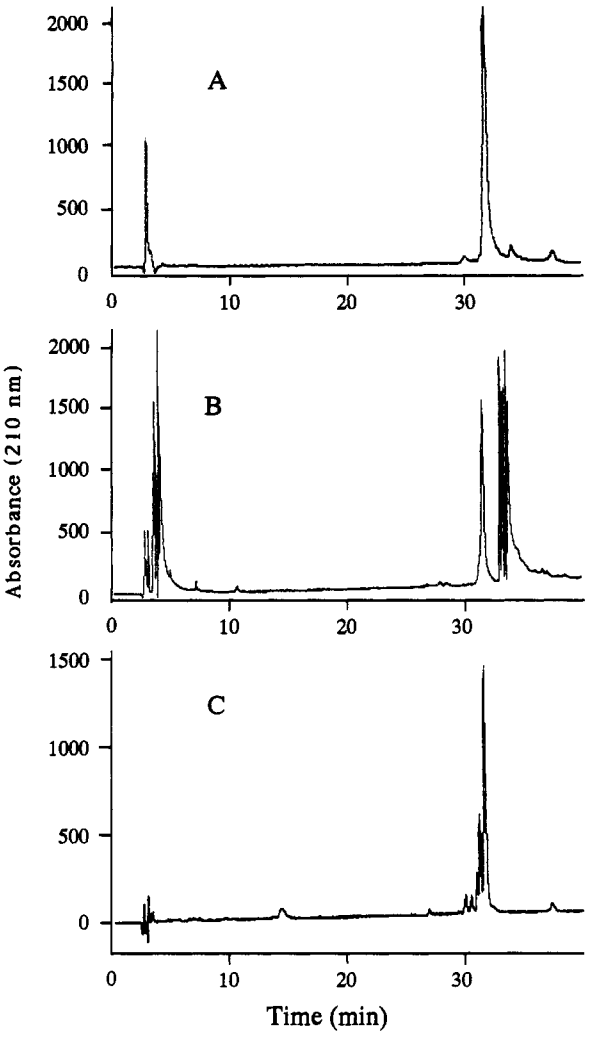

FIGURE 2: HPLC profile of P448 (A) after 6 h of incubation with human thrombin, (B) after 3 h of incubation with human plasma proteases, and (C) after 60 min of incubation with kidney membrane proteases. The peaks at around 3.8 and 33.5 min and small peaks at 7.1, 10.6, 27.8, 28.4, 36.5, and 37.0 min in panel B are due to the proteins in human plasma. The details of the digestions are described under Experimental Procedures.

3 h of incubation at 37 °C (Figure 2B). The proteolytic stability of P448 was further studied using the kidney membrane proteases. The proteases on the kidney membranes digested 40% of P448 by hydrolyzing the Phe^{56}–Glu^{57} peptide bond within 1 h of incubation at 37 °C, producing dansyl-Arg-(D-Pip)-Ada-Abu-Asp-Phe eluting at 30.8 min and Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln eluting at 14.2 min on a C_{18} reverse-phase column (Figure 2C). Further digestion of the C-terminal fragment between Tyr^{63} and Leu^{64} was observed with a longer incubation time (data not shown). Other peaks were too small to be identified, suggesting slow hydrolysis.

Crystal Structure of the Thrombin–P500 Complex. The structure of the thrombin–P500 complex was easily refined, starting from the thrombin model, to the final *R* factor of 0.169. The dansyl-Arg-(D-Pip) moiety has a conformation virtually identical to that observed in the thrombin–MD805 complex (Brandstetter et al., 1992). A stereoview of the active site region of thrombin–P500 complex is shown in Figure 3. The dansyl group occupies the S3 subsite of thrombin lined by the side chains of Tyr^{60A}, Trp^{60D}, Leu^{99}, Ile^{174}, and Trp^{215}.[7] The aromatic ring of dansyl group showed no obvious aromatic–aromatic interaction with any of the aromatic side chains of S3 subsite. Instead, the aliphatic dimethyl amino group of dansyl group is located at the position of the phenyl ring of hirutonin-2 (Zdanov et al., 1993). Arg side chain enters the S1 subsite in the same manner as in MD-805. D-Pip occupies the S2 subsite in a very similar way as in MD-805 (Brandstetter et al., 1992). 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 tetrahedron structure for hydrolysis. Consequently, the amide bond between D-Pip and the linker cannot be hydrolyzed by thrombin as observed in Figure 2. Further detailed structural analysis of the thrombin–P500 complex is beyond the scope of this article and, therefore, will be described elsewhere (J. Féthière et al., manuscript in preparation).

DISCUSSION

The crystal structure of thrombin–hirutonin-2 complex was reported by Zdanov et al. (1993), where hirutonin-2 is a bivalent thrombin inhibitor with the sequence Ac-(D-Phe)-Pro-Arg-[CH_2-CH_2-CO]-QSHNDGDFEEIPEEYLQ. Ac-(D-Phe)-Pro-Arg-[CH_2-CH_2-CO] moiety binds to the thrombin active site in an extended conformation, i.e., D-Phe occupies the S3 subsite and Pro interacts with the S2 subsite. Arg occupies the S1 subsite and forms a salt bridge with Asp^{189}. The carbonyl oxygen of Arg is located in the oxyanion hole formed by the NH groups of Gly^{193} and Ser^{195}, while the carbonyl carbon is positioned at a short distance from the gamma oxygen of Ser^{195}. [CH_2-CH_2-CO] occupies the S1′ subsite, and the lack of an NH group protects the inhibitor from hydrolysis by thrombin. Thus, hirutonin-2 binds to the thrombin active site with the substrate-type binding mode, and the presence of a pseudopeptide bond renders it nonhydrolyzable by thrombin.

On the contrary, the dansyl-Arg-(D-Pip) moiety binds to the thrombin active site in a nonsubstrate-type binding mode, when either alone (Brandstetter et al., 1992) or as part of the bivalent inhibitor (Figure 3). The extension of this fragment on the C-terminal side has little or no effect on its thrombin-bound conformation. The segment, including the side chains, assumes a "Y" shape conformation in the complex. The N-terminal dansyl group is the left arm of the "Y" and occupies the S3 subsite surrounded by Asn^{98}, Leu^{99}, Ile^{174}, and Trp^{215}. The following observations suggest that the hydrophobicity of the P3 residue is the most important requirement for the interaction with the thrombin S3 subsite: (1) the aliphatic dimethylamino group of dansyl is located at the position of the N-terminal aromatic phenyl ring of hirutonin-2 (Figure 3), (2) the aliphatic moiety of the quinoline group of MD-805 deeply nestles in the S3 pocket (Brandstetter et al., 1992), and (3) the substitution of N-terminal D-Phe (P3 residue) of hirulog-1 with D-Cha increases the affinity of the inhibitor by 33-fold (Witting et al., 1992b).

All of the P3 residues of the nonsubstrate-type inhibitors including numerous analogs of NAPAP, TAPAP, and MD-805 are linked through a sulfonamide bond. The substitution of the sulfonamide bond with amide bond reduced the affinity

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

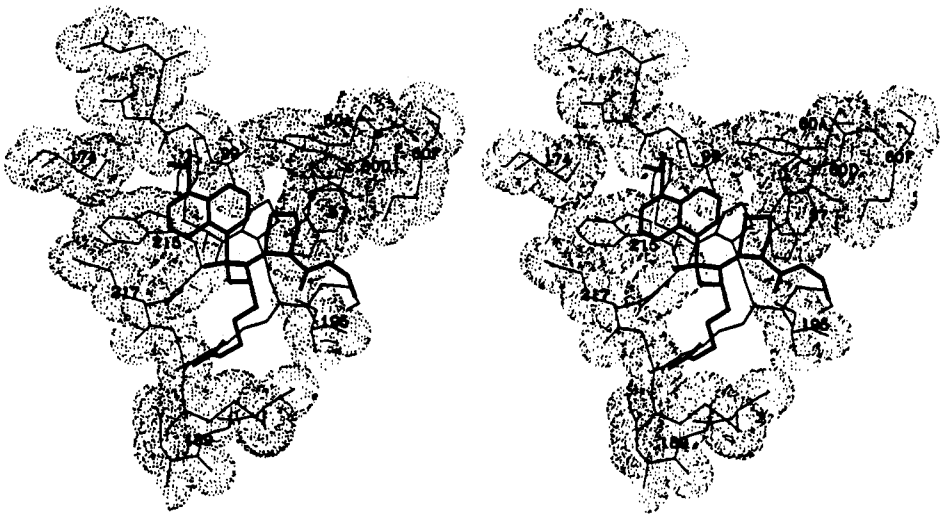

FIGURE 3: Stereoview of the active-site region of human α-thrombin−P500 complex. The thrombin structure (very thin lines) is given with the Connolly surface, and P500 is displayed with thick connection. The active site blocking moiety, (D-Phe)-Pro-Arg, of hirutonin-2 in complex with human α-thrombin is superimposed (thin lines) (Zdanov et al., 1993).

of the inhibitors drastically (Table 2). On the contrary, the P3 residue in the substrate-type inhibitors has been mostly linked to the P2 residue through an amide bond. Interestingly, D-Phe and D-Cha are highly suitable P3 residues in the substrate-type inhibitors but not in the nonsubstrate-type. These results suggest that the P3 residue requires an appropriate linkage to the P1 residue as well as a hydrophobic character for optimal interaction with the thrombin active site.

Although P448 is a potent thrombin inhibitor, the toxicity of the dansyl group reduces the pharmacological value of the inhibitor as an anticoagulant agent (Lenga, 1985). Among the commercially available sulfonyl chlorides, 1-naphthalenesulfonyl, 2-naphthalenesulfonyl, and *tert*-butylbenzenesulfonyl groups could replace the dansyl group with similar affinity. The inhibitors with these groups were synthesized by conventional solid-phase synthesis. Consequently, the synthesis of several analogs for structure−function studies and the scale-up synthesis for *in vivo* studies should not present any difficulty.

The Arg side chain of dansyl-Arg-(D-Pip) moiety forms the pestle of the "Y" and occupies the S1 subsite [Figure 3; also Brandstetter et al. (1992)]. The Arg residue of the active site inhibitors has been reported to be toxic (Kikumoto et al., 1980b) and lower blood pressure (Mattson et al., 1982; Kaiser et al., 1987). Although the replacement of Arg is limited due to the specificity of thrombin for Arg−Xaa bond, attempts were made to substitute it with neutral residues. The affinity loss was as small as 7-fold (Cheng et al., 1991; Claeson et al., 1992; Elgendy et al., 1992). The replacement of Arg by Phe, Cha, or Nle in this study increased the K_i value of the inhibitor to nanomolar range. Interestingly, even D-Arg could replace the Arg residue with the same loss of affinity (P492). Since nanomolar range of K_i values for a drug is still considered valid for therapeutic use, the replacements may be considered if they have beneficial *in vivo* effects.

D-Pip forms the right arm of the "Y" and occupies the S2 subsite formed by Leu^{99}, His^{57}, Tyr^{60A}, and Trp^{60D}. It also interacts with the P3 residue (Figure 3). An attempt to fill the S2 subsite with a more bulky side chain of D-Tic reduced the affinity of the inhibitor 17-fold (P471 in Table 2). As the active site inhibitor, dansyl-Arg-(D-Tic)-NH_2 (P396), has an affinity comparable to that of dansyl-Arg-(D-Pip)-NH_2 (P429) (Table 1), there may be a distorted connection between D-Tic and the linker, resulting in the reduced affinity of the bivalent inhibitor with the D-Tic (P471). D-Pip was further substituted by D-Cha or Acha to reduce the rigidness of D-Pip residue. The substitution resulted in 1000−2000-fold lower affinity, suggesting a negative effect of the flexible P2 residue on the binding affinity.

Hirudin has the distinct property of recognizing simultaneously and independently the active site and the FRE. The N-terminal and the C-terminal fragments of hirudin, on their own, have moderate binding affinities to these sites, i.e., the K_i value of $hirudin^{1-49}$ fragment is 7.2×10^{-8} M (Chang, 1990) and the K_d value of Ac-$hirudin^{55-65}$ is 1.6×10^{-6} M (Hopner et al., 1993). The binding energy is additive when they are covalently connected through the linker, $hirudin^{49-54}$, providing an extremely high affinity ($K_i = 2.3 \times 10^{-13}$ M for recombinant hirudin). The synthetic bivalent inhibitors designed in this study were less potent than the one expected from the energetic additivity of the active site and the FRE inhibitor moieties. For example, the active site blocking moiety, dansyl-Arg-(D-Pip)-NH_2 (P429), and the FRE binding moiety, Ac-$hirudin^{55-65}$, showed a K_i value of 1.58×10^{-7} M and a K_d value of 1.6×10^{-6} M, respectively. If the binding energies contributed additively, the K_i value of the corresponding bivalent inhibitor, P448, should be approximately 2.5×10^{-13} M, which is 67 times more potent than the observed value ($K_i = 1.7 \times 10^{-11}$ M). At least part of the binding energy was lost during the attachment of the linker to the active site blocking moiety, because the addition of an even smaller linker such as, γAbu, reduced the affinity of P429 6-fold (P431 in Table 1). The linker may have pulled out to some extent D-Pip from the S2 subsite to get the appropriate connection, therefore leaving an open space at the S2 subsite with fewer van der Waals interactions.

Nevertheless, the incorporation of the nonsubstrate-type inhibitors into the bivalent inhibitors has the major synthetic advantage of eliminating the scissile amide bond without using a pseudo peptide bond. The oxygen of the Ser^{195} side chain is 4 Å away from the carbonyl carbon of D-Pip, and the use of ω-amino acids as the linker eliminates an amide

bond located close to the side chain oxygen of Ser^{195}. Consequently, thrombin was unable to hydrolyze the amide bond between D-Pip and the linker as observed (Figure 2). Furthermore, replacement of the substrate-type inhibitor moiety, (D-Phe)-Pro-Arg, with the nonsubstrate-type inhibitor moiety such as dansyl-Arg-(D-Pip) improved the affinity of the bivalent inhibitor 10−100-fold (DiMaio et al., 1992), resulting in K_i values in the range of 10^{-11} M. Further modification of the FRE binding moiety enabled us to design a small potent inhibitor, P535, which showed an affinity ($K_i = 2.0 \times 10^{-12}$ M) only one-tenth that of the recombinant hirudin ($K_i = 2.3 \times 10^{-13}$ M) with less than one-third the size (2126 Da of P535 compared to 6970 Da of the recombinant hirudin).

CONCLUSIONS

The nonsubstrate-type active site inhibitor, dansyl-Arg-(D-Pip), was successfully connected to the FRE binding moiety through a linker moiety. The inhibitor, dansyl-Arg-(D-Pip)-μAdod-γAbu-hirudin$^{55-65}$, showed a high affinity with the K_i value of $(1.70 \pm 0.42) \times 10^{-11}$ M due to its concurrent binding to the active site and the FRE of thrombin. The inhibitor was stable against digestion by thrombin and human plasma proteases. Further modifications of the FRE binding moiety improved the affinity of the synthetic bivalent inhibitors [$K_i = (2.0 \pm 0.4) \times 10^{-12}$ M] close to the level of recombinant hirudin, while maintaining the size less than one-third of hirudin.

ACKNOWLEDGMENT

We thank Dr. Zbigniew Szewczuk for the helpful discussions and Yunge Li for crystallization of thrombin−P500 complex. Thanks are also due to Jean Lefebvre for his excellent technical assistance.

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