Interaction of Hirudin with Thrombin: Identification of a Minimal Binding Domain of Hirudin That Inhibits Clotting Activity

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Received June 3, 1988

ABSTRACT: Hirudin, isolated from the European leech *Hirudo medicinalis*, is a potent inhibitor of thrombin, forming an almost irreversible thrombin–hirudin complex. Previously, we have shown that the carboxyl terminus of hirudin (residues 45–65) inhibits clotting activity and without binding to the catalytic site of thrombin. In the present study, a series of peptides corresponding to this carboxyl-terminal region of hirudin have been synthesized, and their anticoagulant activity and binding properties to thrombin were examined. Binding was assessed by their ability to displace ^{125}I-hirudin 45–65 from Sepharose-immobilized thrombin and by isolation of peptide–thrombin complexes. We show that the carboxyl-terminal 10 amino acid residues 56–65 (Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln) are minimally required for binding to thrombin and inhibition of clotting. Phe-56 was critical for maintaining anticoagulant activity as demonstrated by the loss of activity when Phe-56 was substituted with D-Phe, Glu, or Leu. In addition, we found that the binding of the carboxyl-terminal peptide of hirudin with thrombin was associated with a significant conformational change of thrombin as judged by circular dichroism. This conformational change might be responsible for the loss of clotting activity of thrombin.

Hirudin is a 65 amino acid anticoagulant peptide produced in the salivary gland of the medicinal leech. It binds tightly to thrombin ($K_d \simeq 10^{-11}$ M), thereby inhibiting the cleavage of fibrinogen and subsequent fibrin clot formation (Markwardt, 1970; Stone & Hofsteenge, 1986). The complete sequence of hirudin has recently been reported and revised (Figure 1) (Dodt et al., 1985; Mao et al., 1987). The unique clustering of acidic amino acid residues in the carboxyl-terminal half of the molecule is conserved in all reported variants of hirudin (Harvey et al., 1986; Tripier, 1987), and this region of the molecule is thought to be involved in its binding to thrombin (Chang, 1983). Studies of the kinetics of the thrombin–hirudin interaction at varying ionic strengths suggest that the initial and rate-limiting interaction between hirudin and thrombin is diffusion controlled and possibly ionic in nature (Stone & Hofsteenge, 1986). However, the exact size of the functional domains of hirudin and their modes of interaction with thrombin in this region have not been identified. In the present study, peptides (unsulfated at Tyr-63) corresponding to 58–65, 57–65, 56–65, 55–65, 54–65, 52–65, 50–65, and 45–65 of the carboxyl-terminal region of hirudin were synthesized, and their binding properties and anticoagulant activities to thrombin were examined. The results show that the carboxyl-terminal 10 amino acid residues are sufficient for binding and inhibition of thrombin-mediated clotting.

Materials and Methods

Peptide Synthesis. The peptides were synthesized by solid-phase methods on an Applied Biosystems Model 430A peptide synthesizer. Boc-Gln-PAM resin (Applied Biosystems; 0.64 mmol/g) was utilized with double symmetrical anhydride couplings, except for Asn, which was coupled by the DCC/HOBT method. N^{α}-*t*-Boc-amino acids were used with the following side chain protections: Asp(Chx), Glu(Chx), His(Tos), Tyr(2-BrZ), Ser(Bzl). After completion of the synthesis, the peptide was cleaved from the resin with liquid HF containing 5% anisole at 0 °C for 40 min. Upon removal of the HF in vacuo, the peptide was precipitated with ether and extracted with 30% aqueous acetic acid or dilute sodium bicarbonate with a small amount of dimethylformamide. The extract was lyopholized, and the residue was desalted on a Sephadex G-15 column (2.6 × 90 cm) in 5% aqueous acetic acid. The peptide was then purified by reverse-phase high-performance liquid chromatography (RP-HPLC)[1] on a Rainin Dynamax C18 column (21.4 × 250 mm) at 10 mL/min using acetonitrile/0.1% aqueous TFA mobile phases. The major peptide peak was collected, and the fraction was lyophilized. The resultant peptide was analyzed for homogeneity by RP-HPLC and TLC and for identity by amino acid analysis using a Beckman Model 6300 amino acid analyzer (Table I).

Anticoagulant Assay. Inhibition of plasma clot formation was determined as previously described (Krstenansky & Mao, 1987). Human plasma from a healthy female (fasting for 12 h) was collected in a final EDTA concentration of 0.1%. The plasma was immediately sterilized by filtration through a 0.2-μm filter disk (Gelman) and stored at −20 °C; all clotting assays were performed with the same plasma preparation. Briefly, 50 μL of (0.2 pmol) bovine thrombin (Sigma) was added to the wells of a 96-well microtiter plate (Falcon) containing 50 μL of a solution of the synthetic peptide to be tested. After 1-min agitation and additional incubation for 10 min at 24 °C, 100 μL of diluted human plasma (1:10) in 0.12 M NaCl/0.01 M sodium phosphate (PBS) was added and vortexed for 20 s. The turbidity of the solution was monitored by an autoreader (EL 309, Bio-Tek Instruments) at 405 nm and read at 5-min intervals. Typically, the turbidity at 30 min for various doses of peptide was used to construct dose response curves for the IC_{50} values. All of the above reagents were diluted in an assay buffer containing 0.12 M sodium chloride, 0.01 M sodium phosphate, 0.01% sodium azide, and 0.1% bovine serum albumin (BSA), pH 7.4. Intra-

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[1] Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; CD, circular dichroism; PPACK, Phe-Pro-Arg chloromethyl ketone.

0006-2960/88/0427-8170$01.50/0

Table I: Amino Acid Analyses of Synthetic Peptides

amino acid	peptides						
	58–65	57–65	56–65	55–65	52–65	50–65	45–65
Asx				1.00 (1)	2.99 (3)	3.01 (3)	2.99 (3)
Glx	4.03 (4)	5.00 (5)	5.01 (5)	5.01 (5)	5.00 (5)	5.11 (5)	5.93 (6)
Thr							0.99 (1)
Ser						0.96 (1)	0.81 (1)
Pro	0.99 (1)	1.01 (1)	0.99 (1)	1.01 (1)	1.00 (1)	0.99 (1)	3.02 (3)
Gly					1.00 (1)	1.00 (1)	1.04 (1)
Ile	0.96 (1)	0.97 (1)	0.97 (1)	0.97 (1)	0.97 (1)	0.96 (1)	0.98 (1)
Leu	1.02 (1)	1.02 (1)	1.03 (1)	1.02 (1)	1.03 (1)	1.04 (1)	1.05 (1)
Tyr	1.00 (1)	1.01 (1)	0.99 (1)	1.00 (1)	1.00 (1)	0.93 (1)	0.96 (1)
Phe			1.01 (1)	0.98 (1)	1.02 (1)	0.97 (1)	1.02 (1)
His						1.03 (1)	1.01 (1)
Lys							1.02 (1)
calcd M_r	1019	1149	1296	1411	1697	1921	2514
FAB-MS[a]	1020	1150	1296	1412	1698	1921	2514

[a] FAB-MS: (M + H) ± 1 mass unit determined by M-Scan, Ltd., England.

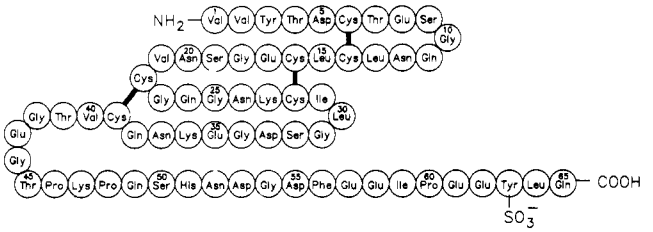

FIGURE 1: Complete amino acid sequence of hirudin (variant 1) determined by Dodt et al. (1985). Tyrosine-63 is sulfated. Mao et al. (1987) report residue 33 as Asn instead of Asp.

and interassay coefficients of variation using hirudin 45–65 were less than 5 and 10%, respectively.

Binding Assay of Synthetic Peptides to Thrombin–Sepharose. Human thrombin (3.7 mg) in 1.5 mL of PBS was dialyzed against 4 L of 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.0, at 4 °C for 4 h. The thrombin was immediately coupled with 1.5 g of CNBr-activated Sepharose (Pharmacia) at 4 °C according to the standard procedures provided by the supplier. More than 90% of the thrombin was bound to Sepharose as determined by its aminidase activity using a synthetic chromogenic substrate (Krstenansky & Mao, 1987). Thrombin–Sepharose was then washed and stored in 15 mL of a buffer containing 0.12 M NaCl, 0.01 M sodium phosphate, and 0.1% BSA, pH 7.4 (PBS–BSA). For the competition binding assay, 100 μL of thrombin–Sepharose was incubated with 100 μL of ^{125}I-labeled hirudin 45–65 containing about 6000 cpm and 100 μL of unlabeled hirudin peptides at 4 °C. After 1-h incubation, 2 mL of PBS–BSA buffer was added, and the gel was pelleted by centrifugation at 1000*g* for 5 min at 4 °C. This wash procedure was repeated. The supernatant fraction, containing unbound ^{125}I-hirudin 45–65, was aspirated, and radioactivity was determined in the pellet.

Gel Filtration. High-performance liquid chromatography (HPLC) was used to isolate peptide–thrombin complexes. Typically, a sample containing thrombin (1 nmol) and peptide (15 nmol) in a final volume of 100 μL of PBS was incubated at room temperature for 30 min. The sample was then injected onto a TSK-G3000SW gel filtration column (8 × 300 mm, LKB) and eluted with PBS at a flow rate of 0.5 mL/min.

Other Procedures. ^{125}I-Labeled hirudin 45–65 (specific activity about 10 μCi/μg) was prepared by using a chloramine T method as described previously (Krstenansky & Mao, 1987). The labeled peptide gave one single peak and coeluted with unlabeled material on HPLC gel filtration described above. Circular dichroic (CD) spectra of thrombin in the absence and presence of hirudin peptides were recorded in PBS buffer using

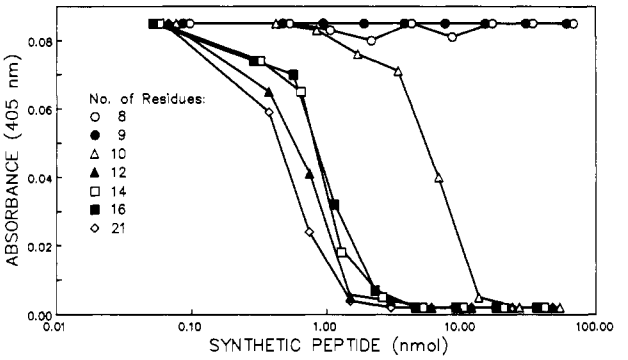

FIGURE 2: Inhibition of clotting activity of thrombin by synthetic hirudin peptides. The clot assay was performed as described under Materials and Methods. Briefly, 50 μL (0.2 pmol) of bovine thrombin (Sigma) was added to the wells of a microtiter plate (Falcon) containing 50 μL of a solution of the synthetic peptide (with varying residues corresponding to the C terminus) to be tested. After 1-min agitation and an additional incubation for 10 min at 24 °C, 100 μL of diluted human plasma (1:10) in 0.1% EDTA was added and vortexed for 20 s. The turbidity of the solution was monitored by an autoreader (EL 309, Bio-Tek Instruments) at 405 nm after 30–40-min incubation.

1-mm cuvettes at 25 °C on a Jasco J-500A spectropolarimeter with a 2-nm slit width. Catalytic activity of thrombin was determined by the release of *p*-nitroanilide from the synthetic substrate H-D-Phe-Pip-Arg-pNA (S-2238; Kabi Vitrum, Sweden). Phe-Pro-Arg chloromethyl ketone (PPACK; Calbiochem, CA) was used as a specific active-site inhibitor for thrombin.

Results and Discussion

Unsulfated N^{α}-acetylhirudin 45–65 has been shown to inhibit fibrinogen cleavage by binding to a noncatalytic site on thrombin (Krstenansky & Mao, 1987). To delineate the minimal sequence responsible for this anticoagulant activity, fragments of the carboxyl-terminal region of hirudin (Figure 1) were chemically synthesized. Figure 2 shows that carboxyl-terminal fragments with 9 amino acids or less (residues 57–65 and 58–65) do not possess anticoagulant activity. With the addition of Phe-56, the peptide completely inhibited thrombin activity. The potency of each peptide increased with chain length and reached maximal inhibition with 12 residues (54–65). Since the minimal sequence for anticoagulant activity was between residues 56 and 65 and necessitated the presence of Phe-56, we next tested the specificity of the thrombin interaction at this residue. Hypothetically (Chang, 1983), the acidic residues of hirudin are important for the recognition

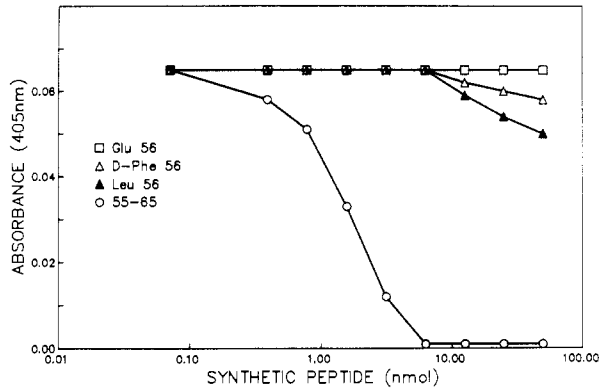

FIGURE 3: Effect of amino acid substitutions at Phe-56 of synthetic hirudin 55–65 on its anticoagulant activity. Phe-56 was substituted by Glu, Leu, or D-Phe, respectively. The inhibition of clotting activity of thrombin was determined by the method described in Figure 2.

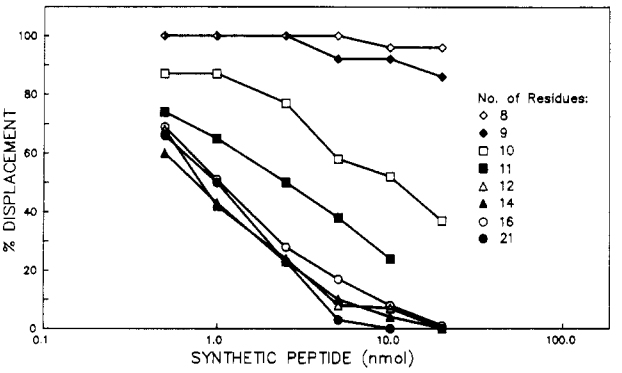

FIGURE 4: Displacement of ^{125}I-labeled unsulfated N^{α}-acetylhirudin 45–65 from thrombin by synthetic C-terminal fragments of hirudin. Two milligrams of thrombin predialyzed in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0, was coupled to 1.5 g of CNBr-activated Sepharose (Pharmacia). More than 90% of the thrombin was covalently bound to Sepharose. ^{125}I-Labeled hirudin 45–65, prepared according to the method previously described (Kvstenansky & Mao, 1987), was incubated with thrombin–Sepharose in the presence or absence of unlabeled peptides at 4 °C for 1 h. Unbound ^{125}I-hirudin 45–65 was aspirated and washed 2 times by phosphate-buffered saline containing 1% BSA.

of a site on thrombin that is composed of clusters of basic Arg and Lys amino acids, residues 62–73 on the B chain of thrombin (Berliner et al., 1985). Either chemical esterification of the acidic residues of hirudin or acylation of the Lys residues of thrombin results in a total loss of affinity between thrombin and hirudin (Tertrin et al., 1967). Therefore, Phe-56 was replaced by Glu, in order to increase the acidic nature of the peptide. [Glu-56]Hirudin 55–65 was inactive (Figure 3). Phe-56 was next replaced by the nonaromatic residue Leu to maintain hydrophobicity. The anticoagulant activity was also abolished by this substitution (Figure 3). Phe-56 was then substituted with D-Phe in order to test the conformational requirements at this residue. The activity was again completely lost (Figure 3). The data indicate that in addition to the acidic residues (Chang, 1983), an L aromatic residue at position 56 is crucial for the interaction of hirudin 56–65 with thrombin.

Previously, we have shown that acetyldesulfatohirudin 45–65 possesses a single binding site on thrombin (Krstenansky & Mao, 1987). To determine the binding properties of the synthetic peptides, thrombin was immobolized on Sepharose, and binding was determined by their ability to displace ^{125}I-hirudin 45–65. Figure 4 shows that peptides with 10 amino acids or longer displaced ^{125}I-hirudin 45–65. Hirudins 54–65, 52–65, 50–65, and 45–65 were equipotent, suggesting that residues Thr-45 to Gly-54 (Figure 1) are not involved in the binding to thrombin. In addition, when the catalytic site

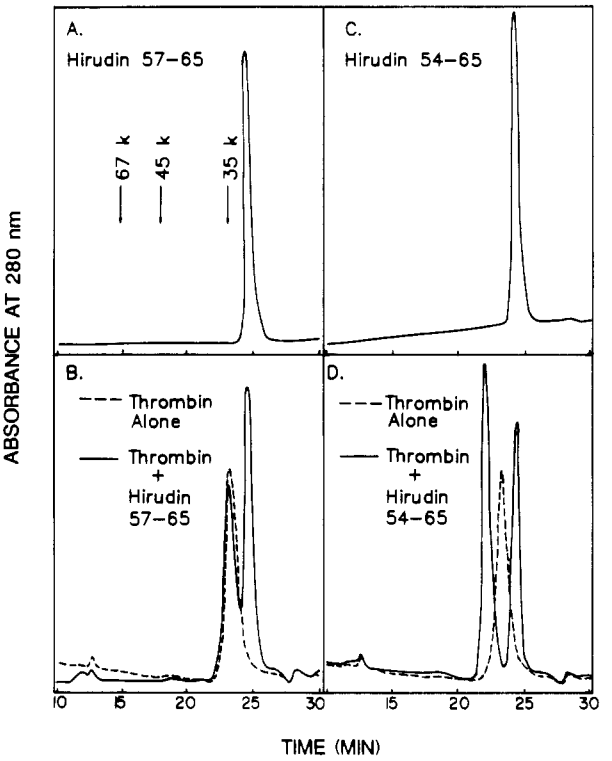

FIGURE 5: Formation of thrombin–hirudin complex on HPLC gel filtration. (A) Hirudin 57–65 alone. (B) Thrombin in the absence (---) and presence of hirudin 57–65 (—). (C) Hirudin 54–65 alone. (D) Thrombin in the absence (---) and presence of hirudin 54–65 (—). Thrombin (1 nmol) was incubated 1 h with synthetic hirudin (15 nmol) at 24 °C in a final volume of 100 μL.

specific inhibitor Phe-Pro-Arg chloromethyl ketone (PPACK) was added to the binding assay, the inhibitor did not displace the synthetic peptide 45–65 (data not shown). Moreover, synthetic hirudin 45–65 with Lys-47 was equipotent to the synthetic hirudin HV-2 peptide 45–65 that possesses an Asn-47 in place of the Lys (data not shown). These results do not support the view that noncleavable residues 46–48 (Pro-Lys-Pro) are involved in the binding to the enzymatic site of thrombin as suggested by others (Chang, 1983; Fenton & Bing, 1986). If the 46–48 residues of hirudin are involved in the thrombin–hirudin interaction, then they may require conformational stabilization from the N-terminal region of hirudin that is not present in our fragment analogues.

To provide direct evidence that the synthetic hirudin peptides bind to solution-phase thrombin, the complex was isolated and characterized by gel filtration HPLC. Figure 5 shows that thrombin forms a complex with hirudin 54–65 but not with 57–65. In the presence of excess hirudin 54–65, the stoichiometry of the isolated thrombin–peptide complex was 1:1. The theoretically calculated molecular weight of the complex is about 35 000, as compared to 33 580 for thrombin alone. The HPLC column (LKB TSK-3000SW) used for isolation of the thrombin–peptide complex would not ordinarily be expected to resolve free thrombin from the hirudin complex. However, as shown in Figure 5, the retention time of the complex was earlier than the expected value, suggesting that the shape of thrombin in the complex has been significantly altered. To confirm this possibility, circular dichroic (CD) spectra were obtained on thrombin with and without hirudin 54–65. At the concentrations that were used, the peptide showed no observable CD absorbance of its own. As shown in Figure 6, the thrombin structure was more disordered in the presence of hirudin 54–65. However, hirudin 57–65 which does not interact with thrombin caused no conformational

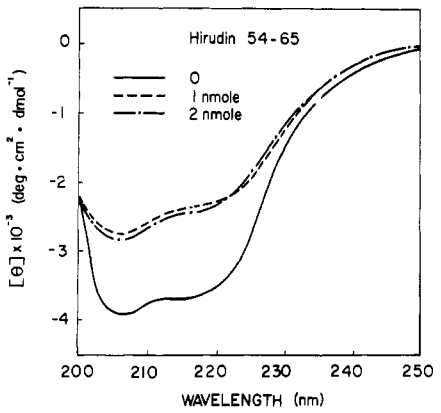

FIGURE 6: Circular dichroic spectra of thrombin in the absence and presence of synthetic hirudin 54–65. Spectra of thrombin (1 nmol) in 250 μL of 0.1 M NaCl/0.01 M sodium phosphate, pH 7.4, were recorded with a 1-mm cuvette. Peptide alone did not show any observable ellipticities with up to 2 nmol of material.

changes. The site-specific inhibitor PPACK also did not affect the CD spectrum of thrombin (data not shown).

This report provides direct evidence that a small portion of hirudin can cause a considerable conformational change in thrombin (from −3600 to −2200 ellipicity at 220-nm wavelength). Hirudin itself has been reported to induce conformational changes in thrombin upon binding (Villanueva et al., 1987). We suggest that the conformational change may be partially responsible for hirudin's broad-range inhibition of thrombin-mediated reactions in addition to its anticoagulant activity (Fenton & Bing, 1986). For example, thrombin also has key roles in nonenzymatic or hormonal activities including monocyte chemotaxis (Bar-Shavit et al., 1983), neutrophil chemotaxis and aggregation (Bizios et al., 1984), lymphocyte aggregation (Bizios et al., 1985), mitogenesis of macrophage-like cells (Bar-Shavit et al., 1983), albumin transport, and stimulation of receptors for increasing cyclic adenosine monophosphate (Carney et al., 1978, 1984). Since the functional domains for these various activities have been proposed to be located in different regions of thrombin (Fenton & Bing, 1986), hirudin may inhibit by directly masking some of these reactive sites, and a change of thrombin conformation upon the binding of hirudin may account for the loss of other activities. Furthermore, it has been suggested that a conformational rearrangement of thrombin may be responsible for the loss of clotting activity for the autoproteolytic products of whole α-thrombin molecule, β- and γ-thrombin (Fenton & Bing, 1986; Villanueva, 1981).

Renewed interest in hirudin is revealing many details of its properties. Recently, the complete cDNA sequence (379 base pairs) for hirudin variant 2 has been reported (Harvey et al., 1986). A 235 base pair DNA, corresponding to the amino acid sequence of hirudin variant 1, has been chemically synthesized and expressed in an *Escherichia coli* system, and the hirudin produced was biologically active (Fortkamp et al., 1986). These recombinant approaches are making available increased quantities of this previously scarce peptide for research and ultimately clinical purposes. The present data using a peptide synthesis approach provide further insight into the nature of the hirudin–thrombin interaction. These hirudin-based peptides consisting of 10–12 amino acids represent a unique class of noncatalytic site thrombin inhibitors potentially useful in antithrombotic therapy.

ACKNOWLEDGMENTS

We thank Dr. R. L. Jackson for suggestions and Dr. J. W. Fenton, New York Department of Health, for the kind gift of purified thrombin. We also thank Susan Treadway for the preparation of the manuscript.

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