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Antithrombin properties of C-terminus of hirudin using synthetic unsulfated N^{α}-acetyl-hirudin$_{45-65}$

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Unsulfated N^{α}-acetyl-hirudin$_{45\text{-}65}$ (MDL 27 589), which corresponds to the C-terminus of hirudin$_{1\text{-}65}$, was synthesized by solid-phase methods. The synthetic peptide was able to inhibit fibrin formation and the release of fibrinopeptide A from fibrinogen by thrombin. The catalytic site of thrombin was not perturbed by the synthetic peptide as H-D-Phe-Pip-Arg-pNA hydrolysis (amidase activity) was not affected. The binding of synthetic peptide and thrombin was assessed by isolation of the complex on gel-filtration chromatography. A single binding site with a binding affinity (K_a) of approx. 1.0×10^5 M^{-1} was observed for thrombin-hirudin$_{45\text{-}65}$ interaction. The data suggest that the C-terminal residues 45–65 of hirudin contain a binding domain which recognizes thrombin and yet does not bind to the catalytic site of the enzyme.

Thrombin; Hirudin; Thrombin inhibitor; Fibrinogen clot; (Leech)

1. INTRODUCTION

Hirudin is a 65 amino acid peptide isolated from the salivary gland of the blood sucking leech (*Hirudo medicinalis*). The complete amino acid sequence of this peptide has been recently reported [1,2] and is shown in fig.1. Hirudin is a potent anticoagulant forming a tight association ($K_i = 8 \times 10^{-11}$ M) with thrombin and inhibiting the cleavage of fibrinogen by thrombin. It has been proposed, based on homology to fibrinopeptides, that the C-terminal portion of hirudin may be the locus of important binding regions [4]. For example, Tyr-63 is sulfated in the native hirudin molecule; loss of this sulfate group results in a 2-fold loss in potency [4]. Some small C-terminal peptides have been synthesized and their anticoagulant activity assessed [5]. As with hirudin, the unsulfated Tyr-63 analogs have one-half the potency of the sulfated peptide. N^{α}-Acetyl-hirudin 40–48 amide exhibited no thrombin inhibiting activity [6]. In view of the fact that no small oligopeptides have yet been shown to be highly specific for thrombin and that the enzyme may have an extended or multiple binding region that gives it its specificity, the larger C-terminal fragment unsulfated N^{α}-acetyl-hirudin$_{45-65}$ (MDL 27 589) was synthesized. Based on the fibrinopeptide homology, this peptide potentially contains the domains in which one binds to the catalytic site of thrombin and the other binds to another recognition site on the enzyme [4].

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Abbreviations: Boc, *t*-butoxycarbonyl; 2-BrZ, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Chx, cyclohexyl; 2,6-diClZ, 2,6-dichlorobenzyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; FAB-MS, fast atom bombardment mass spectrometry; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; PAM, phenylacetamidomethyl; Pip, piperidine-2-carboxylate; TLC, thin layer chromatography

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2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

The peptide was synthesized by solid-phase methods using 0.5 mmol of a 0.66 mmol/g Boc-Gln-PAM resin. Double symmetrical anhydride couplings were performed with 2.0 mmol N^{α}-Boc-amino acid (Peptides International) except in the case of Boc-Gln and Boc-Asn which were coupled by the DCC/HOBT method. The side chain protection utilized was: Asp(Chx), Glu(Bzl), His(Tos), Lys(2,6-diClZ), Ser(Bzl), Thr(Bzl), Tyr(2-BrZ). Upon completion of the synthesis the N^{α}-Boc protection was removed with 50% trifluoroacetic acid in methylene chloride. The resin was washed three times with methylene chloride, neutralized with three washings of 10% diisopropylethylamine in methylene chloride, washed three times with methylene chloride, acetylated with *N*-acetylimidazole in methylene chloride, washed three times with methylene chloride, and dried in vacuo. The peptide was deprotected and cleaved from the resin with HF containing 2% anisole at 0°C for 35 min. The HF was removed in vacuo at 0°C, the peptide precipitated with ethyl ether, extracted from the resin with 30% aqueous acetic acid and lyophilized.

2.2. *Purification and characterization*

The peptide was desalted on a 92 × 2.6 cm Sephadex G-15 column in 5% aqueous acetic acid and lyophilized. Preparative HPLC was performed on a C18 Vydac 218TP1010 (250 × 10 mm) column with 24% acetonitrile in 0.1% aqueous trifluoroacetic acid at 5 ml/min. The major peak was collected and lyophilized leaving 173 mg of the desired product (28% yield based on initial resin substitution). Homogeneity was determined by HPLC (fig.2) and TLC. HPLC Vydac 218TP54 (250 × 4.6 mm) C18 column, 2 ml/min, t_0 = 1.9 min: time of elution with a 15–40% acetonitrile in 0.1% trifluoroacetic acid linear gradient at 1%/min is 13.3 min; k' in 21% acetonitrile in 0.1% trifluoroacetic acid is 4.61 min. TLC: (Merck 5715 20 × 20 cm silica gel 60 plates, 0.25 mm thickness) *n*-butanol/acetic acid/water/pyridine (6:1.2:4.8:6) R_f = 0.29; isopropanol/conc. ammonium hydroxide/water (3:1:1) R_f = 0.36; *n*-butanol/acetic acid/water (4:5:5) R_f = 0.84. FAB-MS: (M + H) = 2513.9 ± 1 m.u. (calcd 2514.1). Amino acid analysis: (HCl hydrolysis, 24 h, 106°C) 84% peptide content by weight, Asx 2.99(3), Thr 0.99(1), Ser 0.81(1), Glx 5.93(6), Pro 3.02(3), Gly 1.04(1), Ile 0.98(1), Leu 1.05(1), Tyr 0.96(1), Phe 1.02(1), His 1.01(1), Lys 1.02(1), NH_3 3.01(3). ϵ_{280} = 1297.

2.3. *Fibrin clot assay*

Bovine thrombin (Sigma) was titrated to an appropriate concentration so that fibrin clot formation could be monitored by a spectrophotometer within 60 min at 405 nm. For the assay, 50 μl of thrombin (0.2 pmol) were added to microtiter wells (microtiter plate, Falcon) containing 50 μl of hirudin or synthetic hirudin$_{45-65}$ (0–25 nmol). The solution was agitated by a minimix (Fisher) for 1 min at 24°C, after which time 100 μl of diluted human plasma (1:10), collected in a final EDTA concentration of 0.1%, was added and mixed for 20 s. The turbidity of the clot was immediately monitored by a microplate autoreader (EL309, Bio-Tek Instruments) at 405 nm and recorded every 3 min. All the reagents used above were diluted in an assay buffer containing 0.12 M NaCl, 0.01 M sodium phosphate, 0.01% NaN_3 and 0.1% bovine serum albumin (PBS-BSA), pH 7.4.

2.4. *Fibrinopeptide A radioimmunoassay*

The release of fibrinopeptide A from fibrinogen in human plasma following the addition of thrombin was determined by a commercially available kit purchased from Mallinckrodt Chemical Works. Samples in the presence or absence of inhibitors were incubated in the microtiter plate and the reaction condition was identical to that described above. After 30 min incubation, a 10 μl sample from each microtiter well was diluted 1:200 with PBS-BSA buffer containing thrombin inhibitor provided in the kit. The diluted sample (200 μl) was used for fibrinopeptide A determination. The sensitivity of the assay for fibrinopeptide A was between 2–40 ng/ml with inter- and intra-coefficient of variation less than 5%.

2.5. *Amidase activity assay*

The inhibition activity of hirudin and its synthetic peptide was measured by the ability to inhibit the amidase activity of thrombin from releasing *p*-nitroanilide from H-D-Phe-Pip-Arg-pNA (S-2238, Kabi Vitrum, Sweden). The assay

conditions were identical to the clotting assay except 100 μl of H-D-Phe-Pip-Arg-pNA was used instead of human plasma. The increase of absorbance was monitored at 405 nm and data recorded over a 30 min period.

2.6. *Interaction between thrombin and synthetic hirudin$_{45-65}$*

Highly purified thrombin (kindly provided by Dr J.W. Fenton, New York Department of Health), showing one single component by HPLC and polyacrylamide gel electrophoresis in SDS, was used for the binding studies with hirudin$_{45-65}$. In a typical assay, 1 nmol thrombin was incubated with 0–16 nmol hirudin$_{45-65}$ at 24°C for 5 min in a final volume of 150 μl of a PBS buffer without albumin. Hirudin$_{45-65}$ bound and unbound to thrombin were separated by a gel filtration TSK-G3000SW column (LKB, 8 × 300 mm) at 0.5 ml/min. Quantitative binding was determined by the displacement of ^{125}I-labeled hirudin$_{45-65}$ from thrombin with unlabeled hirudin$_{45-65}$. In the absence of thrombin, ^{125}I-labeled peptide co-eluted with unlabeled peptide. ^{125}I-labeled hirudin$_{45-65}$ was prepared by the chloramine-T procedure according to the method described [7]. A Scatchard plot using ^{125}I-hirudin$_{45-65}$ was analyzed for the determination of B/F ratios [8].

1				5	*				10
V	V	Y	T	D	C	T	E	S	G
11			*	15	**				20
Q	N	L	C	L	C	E	G	S	N
21	***			25			**		30
V	C	G	Q	G	N	K	C	I	L
31				35				***	40
G	S	D	G	E	K	N	Q	C	V
41				45					50
T	G	E	G	T	P	K	P	Q	S
51				55					60
H	N	D	G	D	F	E	E	I	P
61				65					
E	E	Y	L	Q					
		|							
		SO_3^-							

Fig.1. Amino acid sequence of hirudin [1,2]. Three disulfide linkages are at the positions designated as *, ** and ***, respectively. Tyrosine at position 63 is sulfated.

3. RESULTS

3.1. *Synthesis, purification and characterization of hirudin$_{45-65}$*

[Tyr-63]-N^{α}-Acetyl-hirudin$_{45-65}$ (fig.1) was synthesized in 28% overall yield by standard solid-phase peptide synthesis techniques and purified by gel filtration and HPLC. The isolated peptide was homogeneous by HPLC (fig.2) and TLC and gave the expected amino acid analysis and FAB-MS (M + H) peak. N- and C-terminal fragmentation in the FAB-MS gave some confirmation of the sequence.

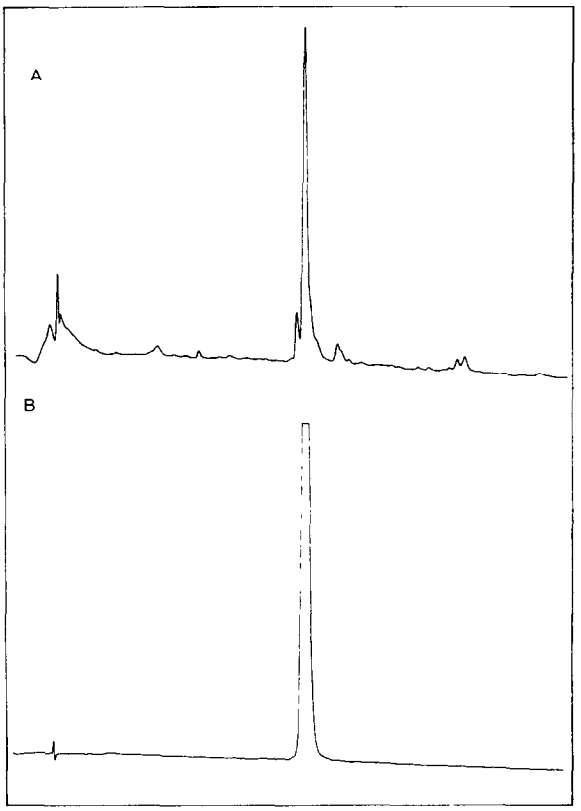


Fig.2. Purification of synthetic hirudin$_{45-65}$ on HPLC. The profiles of peptide monitoring at 215 nm after HF cleavage (A) and after preparative HPLC (B) are shown (see section 2).

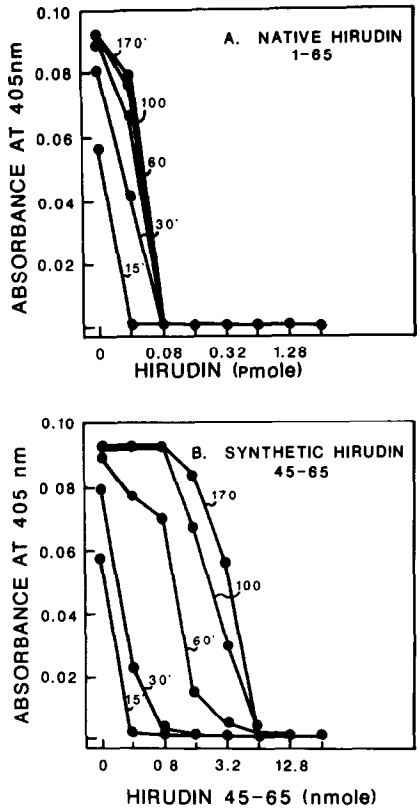


Fig.3. Inhibition of clot activity of thrombin by native hirudin and synthetic hirudin$_{45-65}$. Thrombin (0.2 pmol) was incubated with the inhibitors at 24°C for 5 min in a microtiter plate (see section 2). Turbidity was recorded over a 170 min time period following the addition of human plasma.

3.2. *Inhibition of fibrin clot formation*

The synthetic peptide was initially tested for its ability to inhibit the clotting activity of thrombin. Human plasma was used as a source of fibrinogen for the clot assay. Fibrin clot formation was assessed by the increase of turbidity after the addition of thrombin. Kinetic analysis of the effect of peptide 45–65 on clot formation is shown in fig.3. The maximal turbidity in the absence of inhibitors was reached within a 60 min time period. Both hirudin and the synthetic fragment completely inhibited the clotting activity of thrombin. To achieve 50% inhibition (IC_{50}) at 30 min incubation, 10^4 times as much synthetic hirudin$_{45-65}$ was required as compared to native hirudin.

3.3. *Inhibition of the release of fibrinopeptide A*

Fibrinopeptide A is a major cleavage product following the hydrolysis of fibrinogen by thrombin. The inhibition of fibrinopeptide A formation by hirudin and the synthetic peptide was investigated. The synthetic peptide inhibited the fibrinopeptide A release. However, it required a 10^4 times greater dose than hirudin for 50% inhibition at 30 min (fig.4). The kinetics of the clot formation and fibrinopeptide A release is different. The release of fibrinopeptide A was complete in 5 min (not shown) with 0.2 pmol thrombin. This was much faster than the time required for maximal clot formation monitored by turbidity change (fig.3).

3.4. *Inhibition of amidase activity of thrombin*

It has been suggested that for fibrinogen another recognition site on thrombin exists which is distinct from its catalytic site (responsible for amidase activity) [3]. The recognition binding site may account for the high specificity of thrombin toward certain protein substrates. H-D-Phe-Pip-Arg-pNA was used as a substrate for testing the amidase activity of thrombin. This substrate has a moderate affinity for thrombin ($K_m \simeq 10^{-5}$ M) and can serve as a sensitive probe of the catalytic site. Fig.5

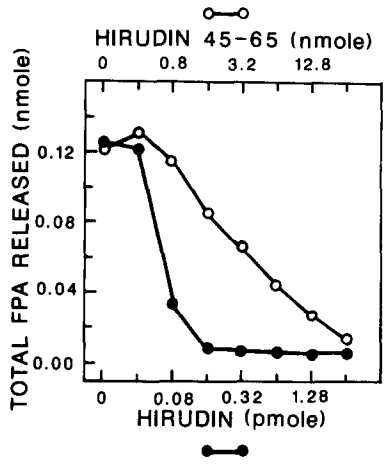


Fig.4. Inhibition of the thrombin-mediated release of fibrinopeptide A by native hirudin and synthetic hirudin$_{45-65}$. Experimental conditions were identical to those described in fig.3. The reaction was terminated after 30 min and the amount of fibrinopeptide A (FPA) released was determined by a radioimmunoassay.

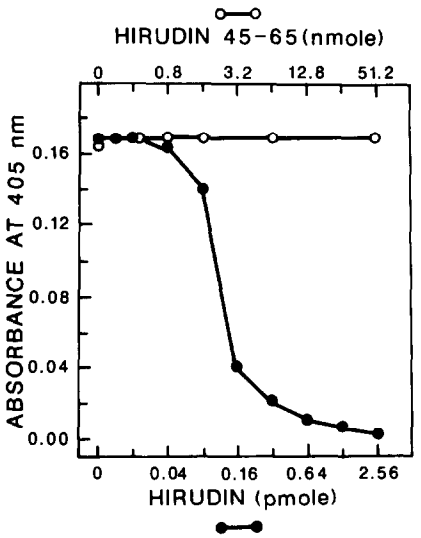


Fig.5. Inhibition of thrombin amidase activity by native hirudin and synthetic hirudin$_{45-65}$. Thrombin activity was determined with Phe-Pip-Arg-pNA as substrate. The experimental conditions were identical to that described in fig.3, except that 100 μl of synthetic substrate was used instead of plasma. The enzyme reaction was monitored for 30 min.

shows that synthetic peptide 45–65 did not inhibit the amidase activity of thrombin at concentrations where clot formation was inhibited. The data indicate that the C-terminal 45–65 region does not block the catalytic site of thrombin, but only binds to a secondary binding region of thrombin and thereby inhibits the fibrinopeptide A release from fibrinogen and subsequent clotting.

3.5. *Direct binding between thrombin and synthetic hirudin$_{45-65}$*

To provide direct evidence that hirudin$_{45-65}$ binds to thrombin, the complex was isolated and characterized by a gel-filtration HPLC technique. ^{125}I-labeled peptide was used to quantitate the interaction. A typical profile for the binding of thrombin and synthetic hirudin$_{45-65}$ is depicted in fig.6. Equilibrium was reached within 1 min as evidenced by no further change of the radioactivity distribution after 4 h. The stoichiometry between thrombin and hirudin$_{45-65}$ was 1:1 as demonstrated by the saturation curve (fig.7). Scatchard plot analysis indicates that there is only a single binding

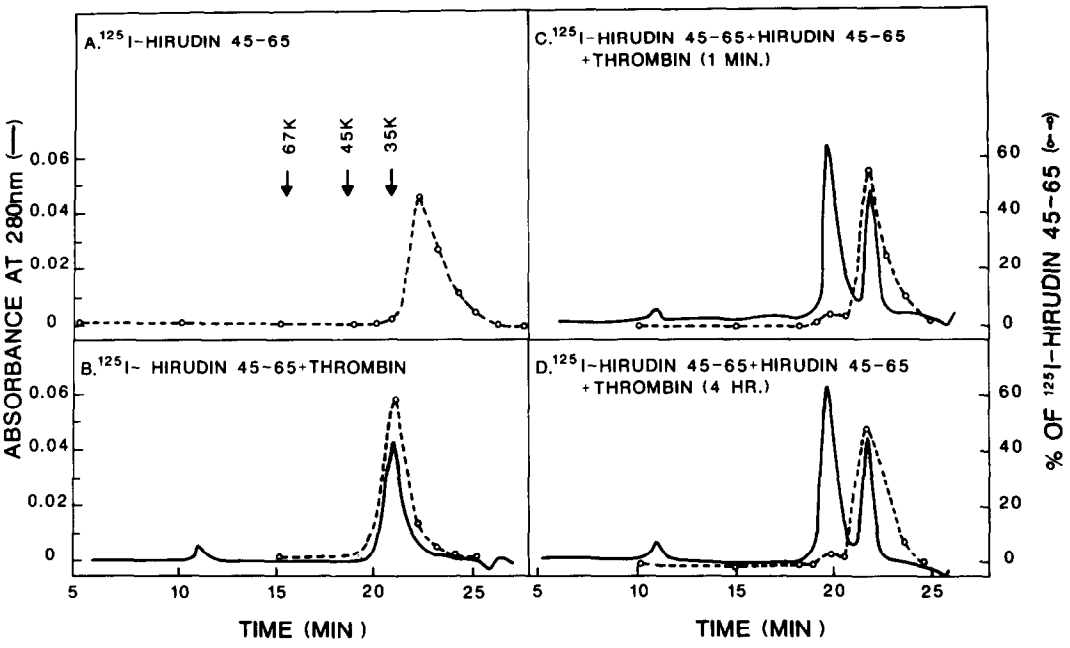


Fig.6. Formation of thrombin-hirudin complex. ^{125}I-labeled hirudin$_{45-65}$ was incubated with thrombin (1 nmol) at 24°C in a final volume of 150 μl. Thrombin-hirudin$_{45-65}$ binding was determined by HPLC on a TSK-3000SW column. (A) Molecular mass markers and ^{125}I-hirudin$_{45-65}$. (B) ^{125}I-hirudin$_{45-65}$ (1 ng, 10^4 cpm) thrombin (1 nmol). (C) ^{125}I-hirudin$_{45-65}$ was displaced from the thrombin in the presence of 4 nmol unlabeled hirudin$_{45-65}$. The mixture was allowed to react for 1 h prior to HPLC separation. The first peak represents the complex of thrombin-hirudin$_{45-65}$, while the second peak represents the unassociated hirudin$_{45-65}$. (D) Same as panel C, but the reaction was allowed to proceed for 4 h prior to HPLC separation.

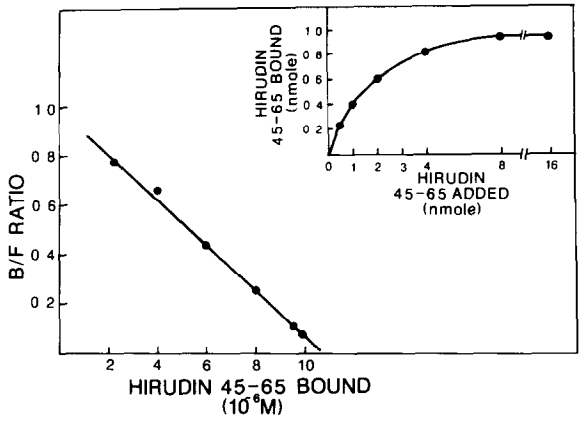


Fig.7. Scatchard plot of thrombin-hirudin$_{45-65}$ interaction. Bound and free ^{125}I-hirudin$_{45-65}$ were determined by HPLC (fig.6). 1 nmol thrombin was bound saturably by 1 nmol hirudin$_{45-65}$ in the presence of hirudin$_{45-65}$ at doses greater than 4 nmol (inset).

site on thrombin for hirudin$_{45-65}$ with a binding affinity (K_a) about 1×10^5 M^{-1} (fig.7).

4. DISCUSSION

Thirty years ago, the blood clotting inhibitor from medicinal leeches, hirudin, was isolated and characterized [9] as a single polypeptide with specific antithrombin activity. Some of the pharmacodynamic and pharmacokinetic properties of hirudin and its influence on various forms of thrombosis in animals have been recently investigated [10]. A quantitative immunoassay for hirudin has also been developed [11]. However, the mode of thrombin-hirudin interaction and functional site(s) of hirudin have not been well established. The tight binding ($K_d \sim 10^{-11}$ M) and almost irreversible inhibition of thrombin by hirudin had led to the speculation that the thrombin-hirudin interaction must comprise multiple binding sites [12]. A study with carboxypeptidase Y suggested that the last 7 amino acids (residues 59–65) are essential for the hirudin-thrombin interaction [4]. It was also proposed that the reactive sites (including the protein binding and catalytic sites) of hirudin to thrombin might be located outside the disulfide-bridged loops (fig.1) of hirudin, namely, residues 47–65 [4]. However, this hypothesis has never been tested experimentally.

In the present study, we show that residues 45–65 inhibit the clotting activity and the release of fibrinopeptide A by thrombin. The synthetic peptide 45–65 binds to thrombin and occupies a single binding site on thrombin (fig.7). The binding affinity (K_a) is about 10^5 M^{-1}, a value only one order of magnitude less than the thrombin-fibrinogen interaction ($K_a \sim 10^6$ M^{-1}). Since the synthetic peptide does not inhibit the hydrolysis of H-D-Phe-Pip-Arg-pNA by thrombin, we suggest that the C-terminal region of hirudin may not be directly involved in the catalytic binding site of thrombin. A similar finding was reported in a recent study with thrombomodulin (M_r 84000), isolated from endothelial membrane [13]. Jakubowski et al. [13] showed that the protein binds to thrombin and inhibits clotting activity and yet does not inhibit the catalytic activity of thrombin.

The importance of the N-terminal portion of hirudin (residues 1–44) which contains 3 disulfide loops is not known. It is possible that a catalytic binding domain is located within these 3 loops, similar to the other proteinase inhibitor proteins: aprolinium, pancreatic secretory trypsin inhibitor, potato inhibitor I, and potato inhibitor II. All the binding domains in these inhibitors are present in the disulfide loops. Oxidation of the disulfide bridges of hirudin by performic acid drastically affects the biological activity of native hirudin to thrombin [14]. The N-terminal region may also mediate the amidase inhibition of thrombin by steric hindrance. Experiments are in progress to synthesize longer peptides in order to determine the possible role of the N-terminus of the hirudin molecule.

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