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Inhibition of thrombin by synthetic hirudin peptides

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To investigate the role of different regions of hirudin in the interaction with the proteinase thrombin, segments of hirudin containing 15–51 residues were synthesized. The C-terminal segment 40–65 inhibited the fibrinogen clotting activity of thrombin but not amidolysis of tosyl-Gly-Pro-Arg-*p*-nitroanilide. Central peptide 15–42 was insoluble at pH 7, but peptide 15–65 inhibited fibrinogen clotting and amidolysis to an equal extent. The N-terminal loop peptide 1–15 had no inhibitory activity and did not affect the potency of peptide 15–65. These data suggest that the central region inhibits catalysis.

Hirudin; Thrombin; Synthetic segment; Peptide segment

1. INTRODUCTION

Hirudin, a potent anticoagulant from the leech *Hirudo medicinalis* [1,2] is a 65-residue protein that specifically inhibits the enzyme thrombin [3]. The binding of hirudin to thrombin is extremely tight, with an inhibition constant, K_i, of $8 \times 10^{-11} - 2 \times 10^{-14}$ M [4–6], and hirudin can be displaced from thrombin with a high concentration of competing substrate [5,6]. Synthetic hirudin peptide 45–65 inhibits fibrinogen cleavage but not amidolysis of small peptide substrates by thrombin [7], so the region of hirudin that blocks the catalytic site of thrombin is not the C-terminus. The aim of this study was to investigate the role of specific regions of hirudin in the interaction with thrombin using synthetic peptide segments 15–51 amino acid residues in length. Two thrombin assays were used to investigate the inhibitory effects of these peptides, a fibrinogen clotting assay and a tripeptide amidolytic assay [7].

2. EXPERIMENTAL

2.1. *Materials*

Reagents for peptide synthesis were supplied by Applied Biosystems Inc. Trypsin-agarose, thermolysin and SDS molecular weight markers were supplied by Sigma Chemical Co. Human α-thrombin was a gift from John Fenton III. Tosyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH) was supplied by Boehringer Mannheim. Human fibrinogen (96% clottable) was from Kabi Diagnostica.

2.2. *Peptide synthesis and cleavage*

All peptides were assembled by automated solid-phase synthesis using *tert*-butyloxycarbonyl (Boc) protection. Prior to cleavage from the resin the 2,4-dinitrophenyl group on histidine was removed with 2-mercaptoethanol/diisopropylethylamine/dimethyl formamide (2:1:7, v/v) for three 30-min periods [9]. Peptides were cleaved from the resin by treatment with liquid hydrogen fluoride.

2.3. *Peptide purification and folding*

Peptides lacking cysteine were purified by reverse-phase HPLC. Each peptide containing cysteine was converted into the S-sulfonate derivative [10], the sulfonated peptide was purified by HPLC, the sulfonate groups were removed by reduction, and the free peptide was folded by air oxidation in dilute solution.

2.4. *Peptide analysis*

Analytical scale HPLC was performed on an Aquapore RP-300 C8 column (30 × 2.1 mm) using linear gradients of acetonitrile in 0.1% trifluoroacetic acid. Preparative HPLC was performed on an Aquapore OCTYL C8 column (10 × 1 cm). Amino acid compositions were determined after hydrolysis in 6 N HCl at 110°C for 24 h. Peptides were sequenced using an Applied Biosystems Model 470A sequencer. Polyacrylamide gel electrophoresis was performed in the presence of urea [11]. The disulfide-bridged peptides were tested for the presence of free sulfhydryl groups with dithiobis(2-nitrobenzoic acid) as a quantitative colorimetric reagent [12].

2.5. *Clotting assay*

Competition for the secondary fibrinogen-binding site on thrombin was measured by a turbidimetric clotting assay. Solutions contained 0.05 M Tris at pH 7.8, 0.1 M NaCl, 0.1% PEG 6000 and 5 μM fibrinogen. The concentration of Aα chains was 10 μM, which is close to the K_m determined for human Aα cleavage by human thrombin [13,14]. Peptide concentration was verified by quantitative amino acid analysis. Fibrin formation was followed by optical turbidity at 350 nm for 60 min. The concentration of non-inhibited thrombin was determined by comparison to a set of curves obtained by using the same assay with a series of lower thrombin concentrations in the absence of inhibitor. The inhibition constant was calculated as K_i = [free thrombin][peptide]/[complexed thrombin], where [peptide] was the free peptide concentration (not significantly different from the total peptide concentrations under the used conditions), [free thrombin] was taken as one half the concentration of non-inhibited thrombin, calculated from the residual thrombin activity (non-inhibited thrombin being divided equally between free enzyme and fibrinogen-bound enzyme when the substrate concentration equals K_m), and [complexed thrombin] equals the difference between total and non-inhibited thrombin concentrations.

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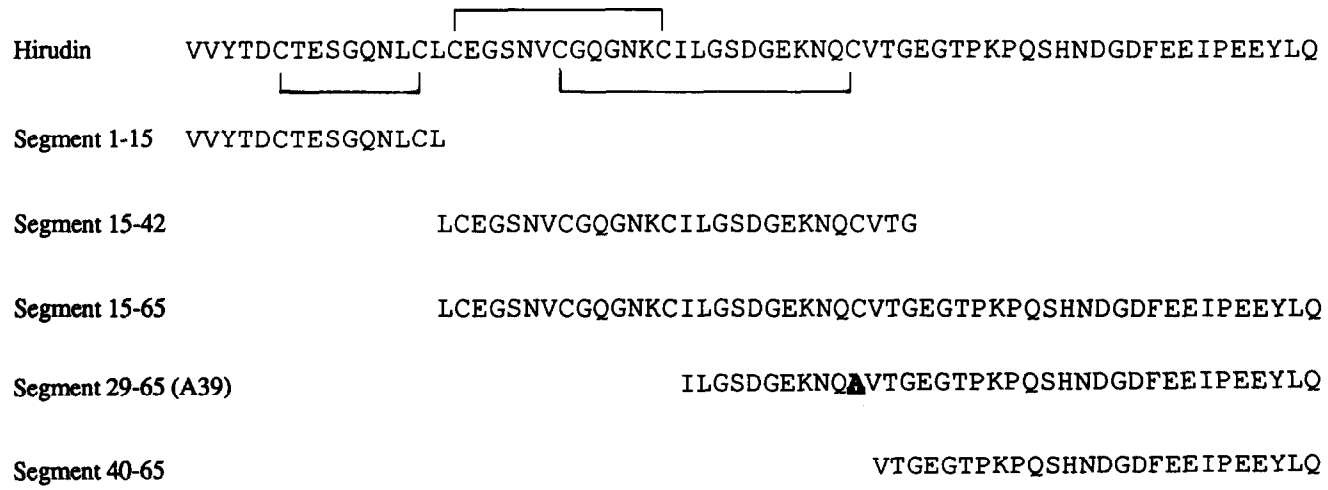

Fig. 1. Diagram of the primary sequence of hirudin and the synthetic segments synthesized in this report. Amino acids are listed according to their standard one-letter code. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; Y, Tyr. The location of the 3 disulfide bridges is indicated. Native hirudin is sulfated at Tyr-63.

2.6. *Amidolytic assay*

Amidolysis of the tripeptide toxyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH) was measured by release of *p*-nitroaniline at 405 nm [15]. In peptide-inhibited samples, peptide was mixed with thrombin prior to the addition of the chromogenic substrate. The K_m and K_i values were determined from a Lineweaver-Burk plot. For each peptide the experiment was repeated at 3 different concentrations to establish the measured K_i was concentration-independent and ensure that the inhibition was simply competitive.

2.7. *Cleavage by agarose-immobilized trypsin and thrombin*

Lyophilized peptide (1.0 mg) was dissolved in 400 μl of clotting buffer. Approximately 120 μl (6 BAEE units) of trypsin-agarose or thrombin was added and the slurry was mixed overnight at room temperature. The supernatant was subjected to analytical HPLC.

3. RESULTS

3.1. *Peptide 40–65*

Sequences of 5 synthetic peptides are shown in Fig. 1. There inhibition constants for fibrinogen cleavage and tripeptide amidolysis are given in Table I and amino acid compositions are given in Table II. Peptide 40–65, the negatively charged 26-residue C-terminal segment of hirudin inhibited clotting of fibrinogen by thrombin with a K_i of 3.0×10^{-6} M (Fig. 2). Peptide 40–65 had no inhibitory effect on cleavage of the tripeptide anilide even at a peptide concentration of 0.2 mM, which was 50 times higher than that showing effect in the fibrinogen cleavage assay. The inhibition constant was too high to measure but it cannot be lower than 5×10^{-4} M. The lack of activity in the amidolytic assay indicated that peptide 40–65 does not contain a sequence capable of blocking the catalytic site of thrombin. Peptide 40–65 contains a single lysine, Lys-47, and incubation with trypsin resulted in cleavage of peptide 40–65 at this position. Thrombin did not cleave the peptide. Either the sequence was not recognized as a site of cleavage by thrombin or a lysine-proline bond cannot be cleaved by thrombin, since a lysine-proline scission has yet to be described for thrombin [16,17].

3.2. *Peptide 29–65(A39)*

The hirudin segment 29–65 contains a single cysteine residue at position 39, that could interfere with the activity assays or lead to peptide dimerization, so peptide 29–65 was synthesized with an alanine residue in place

Table I

Inhibition of thrombin-catalyzed amidolysis and fibrinogen clotting by synthetic hirudin peptides

Peptide	Amidolysis		Clotting		Potency ratio[b]
	K_i (M)	Rel. Potency (%)[a]	K_i (M)	Rel. Potency (%)[a]	
40–65	$>5 \times 10^{-4}$	<0.03	3.0×10^{-6}	3	<0.01
29–65(A39)	3.0×10^{-5}	0.4	1.1×10^{-6}	8	0.05
15–65	1.3×10^{-7}	[100]	0.9×10^{-7}	[100]	[1]
15–42	insoluble		insoluble		
1–15	$>5 \times 10^{-4}$	<0.03	$>5 \times 10^{-4}$	<0.02	

Synthetic peptides were incubated with human thrombin either in the presence of 2.3–50 mM of tosyl-Gly-Pro-Arg-*p*-nitroanilide for measuring amidolysis or 4 μM fibrinogen for measuring fibrinogen clotting inhibition.

[a]Relative to 15–65.

[b]Potency ratio = relative amidolysis potency/relative clotting potency.

Table II

Amino acid analysis of synthetic hirudin segments

	Peptide				
	40–65	29–65(A39)	15–42	1–15	15–65
Asx	2.34 (3)	7.57 (7)	4.19 (4)	1.90 (2)	7.57 (7)
Glx	5.69 (7)	10.85 (11)	4.18 (4)	2.17 (2)	10.85 (11)
Ser[a]	0.70 (1)	2.23 (3)	1.55 (2)	0.95 (1)	2.23 (3)
Gly	2.30 (3)	6.93 (8)	5.95 (6)	1.25 (2)	7.93 (8)
His	0.73 (1)	1.07 (1)			1.07 (1)
Thr	1.93 (2)	2.23 (2)	0.99 (1)	2.00 (2)	2.23 (2)
Pro	3.17 (3)	3.92 (3)			3.92 (3)
Tyr	1.04 (1)	1.35 (1)		1.00 (1)	1.35 (1)
Val	1.09 (1)	1.64 (2)	1.85 (2)	1.69 (2)	1.64 (2)
Ile	1.16 (1)	2.17 (2)	1.97 (2)		2.17 (2)
Leu	1.00 (1)	2.74 (3)		2.27 (2)	2.74 (3)
Phe	1.08 (1)	1.45 (1)	1.96 (2)		1.45 (1)
Lys	1.46 (1)	2.15 (3)			2.15 (3)
Ala		1.00 (1)			
Cys		n.d. (4)	n.d. (4)	n.d. (2)	n.d. (4)

Peptides were hydrolyzed in 6 M HCl for 24 h at 110°C. The amino acids were converted into the PTH derivatives and analyzed by reverse-phase HPLC. The theoretical number is listed in parentheses. n.d., not determined.
[a]Not corrected for decomposition during hydrolysis.

of Cys-39. The expected sequence was confirmed by Edman sequencing. Peptide 29–65(A39) inhibited fibrinogen clotting with a K_i of 1.1×10^{-6} M, which is slightly lower than for peptide 40–65. At a concentration giving marked inhibition in the fibrinogen clotting assay, a small but detectable effect was seen in the amidolytic assay, so it was necessary to increase the peptide concentration 10-fold to quantitate this effect. Fig. 3 shows the effect of peptide 29–65(A39) on tripeptide amidolysis. The decrease in cleavage was consistent with competitive inhibition with a K_i of 3.0×10^{-5} M.

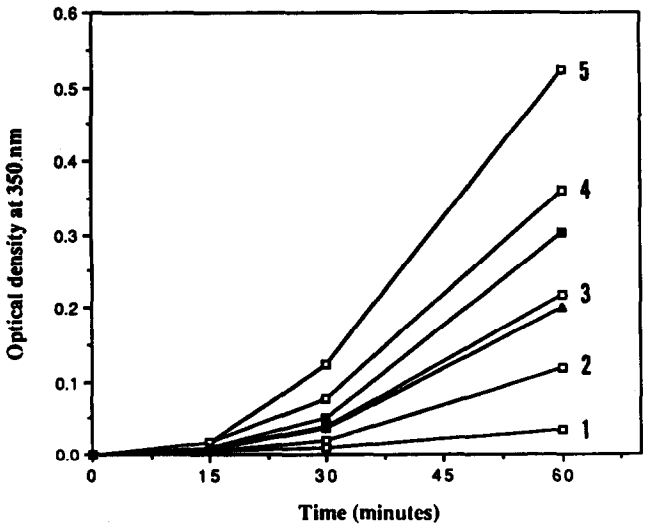

Fig. 2. Inhibition of fibrinogen clotting by hirudin peptide 40–65. Fibrinogen (5 μM, in 0.1 M NaCl, 50 mM Tris, pH 7.8, 0.1% PEG) was clotted by addition of 1–5 μl of a stock solution of thrombin (□: curves 1–5, final thrombin conc. = 0.023–0.115 nM) and the rate of cleavage was measured by the formation of fibrin gel as revealed by turbidity at 350 nm. The inhibition constant of peptide 40–65 was measured by clotting (0.115 nM thrombin) in the presence of peptide (■, 9×10^{-7} M; ▲, 1.8×10^{-6} M).

This peptide was not cleaved by incubation with thrombin.

3.3. *Peptide 15–65*

Peptide 15–65 contained no free sulfhydryl groups (<0.1 mol/mol peptide), indicating that the cysteines had been oxidized to form disulfide bonds. On polyacrylamide gel electrophoresis, peptide 15–65 produced a main band with an apparent molecular weight of 7000 Da. The molecular weight expected from the sequence was 5500 Da, so this band is consistent with a folded monomer. A band of higher molecular weight containing 5–10% of the stain was evident and probably represents a small amount of peptide dimer which formed during folding. The first 30 residues were verified by direct Edman sequencing. Digestion with thermolysin (2 mol%) for 24 h produced several major fragments, which were sequenced and found to be peptide fragments 40–48, 53–63 and 58–63 from the C-terminal region. Such a digestion pattern would be expected if an exposed C-terminus (residues 40–65) were connected to a central region (residues 15–39) cross-linked by two disulfide bonds. Peptide 15–65 inhibited both fibrinogen clotting ($K_i = 0.9 \times 10^{-7}$ M) and tripeptide amidolysis ($K_i = 1.3 \times 10^{-7}$ M) with nearly equal potency. Variants of peptide 15–65 with lysines at position 27 or 36 substituted by alanine were synthesized. The inhibition constants for both thrombin assays did not differ significantly from those of 15–65: K_i for amidolysis and clotting for the Ala-27 variant was 2.2×10^{-7} M and 2.1×10^{-7} M; for the Ala-36 variant was 2.5×10^{-7} M and 3.1×10^{-7} M.

3.4. *Peptide 15–42*

Peptide 15–42, containing the central region of hirudin, did not dissolve in neutral or basic buffer. It

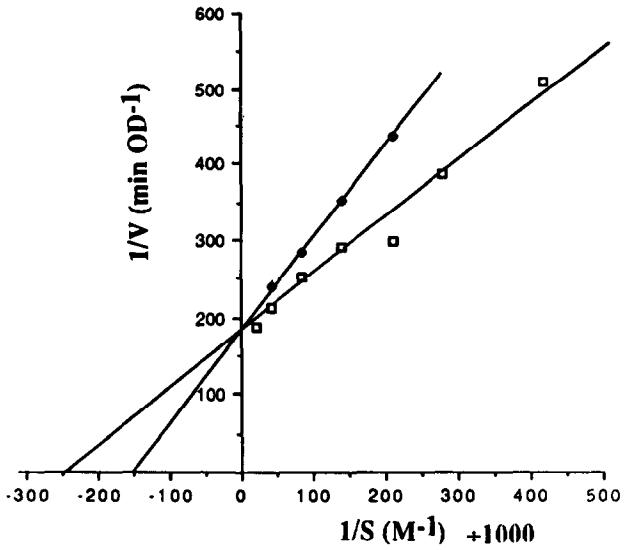

Fig. 3. Competitive inhibition of thrombin amidolytic activity by hirudin 29-65(A39). Lineweaver-Burk plot shows the effect of hirudin on the amidolytic activity of human thrombin. The enzyme activity was measured by cleavage of the chromogenic substrate Chromozym TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide). The release of *p*-nitroaniline was followed by absorbance at 405 nm. Control, □; Inhibited with 2×10^{-5} M peptide, ◆. The apparent K_i determined from these data was 3.0×10^{-5} M.

dissolved completely in highly acidic solutions but precipitated when neutralized by adding dilute ammonia. Since the purified peptide was insoluble at the basic pH used for disulfide formation it was not possible to test its inhibitory effect.

3.5. *Peptide 1–15*

Hirudin peptide 1–15, which contains the N-terminal disulfide loop, had no antithrombin activity in either assay. At peptide concentrations of 1.2–30 mM there was no detectable inhibition of fibrinogen cleavage in the fibrinogen clotting assay and no significant inhibition of amidolysis. A mixture of both fragments 1–15 and 15–65 at a concentration of 1 mg/ml in assay buffer (approximately a 3:1 molar ratio) was tested for activity in both assays. The degree of inhibition corresponded to that produced by peptide 15–65 alone. Despite a 3-fold excess of peptide 1–15, this hirudin segment had no apparent effect in the binding of the remainder of the molecule.

4. DISCUSSION

Hirudin interacts with thrombin at the substrate-binding site and the catalytic site. Binding to the substrate-binding site is demonstrated by the ability of hirudin C-terminal peptides to inhibit fibrinogen clotting [7,18–20]. Binding to the catalytic site is demonstrated by a 10^6-fold decrease in affinity for hirudin on modification of the catalytic histidine in thrombin [21]. Results from this study indicate that the region of hirudin responsible for blocking the catalytic site of thrombin is present in residues 15–39. Amidolysis inhibition appeared when the C-terminal peptide 40–65 was extended by 25 residues to include the central core to give 15–65. Peptide 40–65 did not inhibit amidolysis even at high concentrations but peptide 15–65 had considerable activity. Intermediate peptide 29–65(A39) showed weak inhibition of amidolysis since 20-fold higher concentrations of peptide were needed to inhibit amidolysis than were required to inhibit fibrinogen clotting.

These results for peptide 40–65 are similar to those for peptide 45–65 [7], namely, inhibition of fibrinogen clotting but no effect on the amidolytic activity of thrombin. The increase in relative potency on going from peptide 29–65 to peptide 15–65 was 12-fold for clotting but 250-fold for amidolysis, suggesting that 15–65 blocks the catalytic site of thrombin. The difference in K_i between amidolysis and clotting for peptide 29–65(A39) is most easily explained by flexibility of the bound peptide: bound peptide always occupies the fibrinogen binding site, but this peptide lacks the necessary residues to completely block the catalytic site. Incomplete shielding is reflected in the numerically larger inhibition constant for amidolysis.

The difference between peptides 15–65 and 40–65 is the presence of residues 15–39. The appearance of inhibition against amidolysis when these residues are present indicates that residues in this region block the thrombin catalytic site, however, peptide 15–65 was only 1/200 as potent as desulfato-hirudin ($K_i = 5 \times 10^{-10}$ M) [22]. HPLC indicated that peptide 15–65 contained more than one species but insufficient material was available to determine what fraction was most active and the inhibition constants reported are averages for the mixture, the most conservative case.

N-terminus and central core fragments of recombinant hirudin have been shown to be potent inhibitors of thrombin [23–25]. The hirudin fragment 1–52 has a K_i of 2.4×10^{-8} M [23], fragment 1–47 has a K_i of 6.0×10^{-7} M [24], and fragment 1–43 has a K_i of 3.0×10^{-7} M [25]. The resulting protection of thrombin from elastase digestion and inhibition of amidolytic activity is consistent with binding near the catalytic site of thrombin.

Replacing two of the 3 lysines in peptide 15–65, Lys-27 and Lys-36, separately by alanine had no significant effect on the inhibition of fragment 15–65. Two studies in which each of the 3 lysines in recombinant hirudin were separately substituted by non-basic residues also reported a minimal effect [26,27], suggesting that lysine residues are not essential for hirudin to act as a potent inhibitor. Neither peptide 40–65, which contained a single lysine residue, nor peptide 29–65(A39), which contained two lysine residues, were cleaved by overnight treatment with thrombin.

Acetylation or substitution of the two N-terminal valine residues of hirudin with polar amino acids results in markedly decreased affinity for thrombin [28], indicating the importance of this region in the interaction with thrombin. Peptide 1–15 did not inhibit either fibrinogen clotting or amidolysis. Incubation of thrombin with peptide 15–65 in the presence of peptide 1–15 resulted in a degree of inhibition equivalent to that of peptide 15–65 alone. This suggests that covalent attachment of 1–15 to the rest of the molecule is essential to fulfil its function. For comparison, hirudin fragment 1–52 exhibits a modest, 2-fold cooperativity in the presence of the complementary fragment 53–65 [23].

Peptide 15–42, the isolated central core of hirudin, was insoluble in neutral aqueous buffer. The results reported here indicate that the central core of hirudin, residues 15–39, when solubilized by attachment to the C-terminal part of the molecule, blocks the catalytic site of thrombin. Both results by others [23–25], using fragments of recombinant hirudin and the ones we report in this paper for synthetic fragments, support the binding of the central core of hirudin to the catalytic site of thrombin.

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