Production, Properties, and Thrombin Inhibitory Mechanism of Hirudin Amino-terminal Core Fragments*

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Hirudin, a thrombin-specific inhibitor, comprises a compact amino-terminal core domain (residues 1–52) and a disordered acidic carboxyl-terminal tail (residues 53–65). An array of core fragments were prepared from intact recombinant hirudin by deletion of various lengths of its carboxyl-terminal tail on selective enzymatic cleavage. Hir1-52 and Hir1-54 were produced by pepsin digestion at Phe56-Glu57 and Asp59. Gly54. Hir1-52 was generated by Asp-N cleavage at Asn55-Asp56. Hir1-47 was prepared by cleavage of Glu55-Ser56 by chymotrypsin, elastase, and thermolysin. In addition, Hir1-62 (containing part of the carboxyl-terminal tail) was derived from Hir1-85 by selective removal of the three carboxyl-terminal amino acids using carboxypeptidase A.

Hirudin amino-terminal core fragments were stable at extreme pH (1.47 and 12.6), high temperature (95 °C), and resistant to attack by various proteinases. For instance, following 24-h incubation with an equal weight of pepsin, the covalent structure of Hir1-52 remained intact and its anticoagulant activity unaffected.

Unlike intact hirudin (Hir1-65) the inhibitory potency of which is a consequence of concerted binding of its amino-terminal and carboxyl-terminal domains to the active site and the fibrinogen recognition site of thrombin, the core fragments block only the active site of thrombin with binding constants of 19 nM (Hir1-52), 55 nM (Hir1-54), and 72 nM (Hir1-49). As an anticoagulant Hir1-56 is about 2-4fold more potent (on a molar basis) than Hir1-52, Hir1-49, and Hir1-43, respectively. Hir1-56 was also about 15fold more effective than the most potent carboxyl-terminal fragment of hirudin, sulfated-Hir54-65, although they bind to independent sites on thrombin. The potential advantages of hirudin core fragments as antithrombotic agents are discussed in this report.

Hirudin is a thrombin specific inhibitor isolated from the leech Hirudo medicinalis (Markwardt and Walsmann, 1958; Markwardt, 1970; Badgy et al., 1976). The inhibitor acts as a potent anticoagulant by binding to thrombin with high specificity (Brown et al., 1980) and affinity (Stone and Hofsteenge, 1986). Hirudin is distinguished from conventional protease inhibitors by two unique characteristics. (a) Unlike most serine protease inhibitors which are compact molecules, hirudin has a tadpole-like shape, consisting of a compact amino-terminal domain and a disordered carboxyl-terminal tail (Chang, 1983a; Folkers et al., 1989). In fragment form, both domains act as anticoagulants but through different mechanisms (Krstenansky and Mao, 1987; Mao et al., 1988; Chang et al., 1990a, 1990b; Dennis et al., 1990; Dodt et al., 1990). The carboxyl-terminal fragment may be readily produced by solid-phase synthesis (Krstenansky et al., 1987; Marascano et al., 1988; Ni et al., 1990). The amino-terminal core fragment, however, is preferably prepared from recombinant hirudin. Dennis et al. (1990) prepared the core fragment (Hser5-Hir1-52) from a recombinant hirudin variant (Me55-Hir1-65). The mutant protein was treated with cyano bromide in acidic solution to generate Hir1-52 (with homoserine at its carboxyl terminus) and Hir5-65. Dodt et al. (1990) constructed a different hirudin variant, Ala48-Hir1-52 and generated Hir1-77 following digestion with endoprotease Lys-C. Chang et al. (1990a) obtained Hir43 by limited proteolysis of wild-type recombinant hirudin with V8 protease. The shorter versions of the amino-terminal domain (Hir1-48 and Hir1-43), however, were shown to be inferior to Hir1-52 in terms of binding affinity to the active site of thrombin.

In this report, we describe methods for the production of three hirudin core fragments, Hir1-56, Hir1-52, and Hir1-49 from wild-type recombinant hirudin (hirudin variant 1). We also investigated the stability and thrombin inhibitory mechanism of these core fragments. One major goal of this study is to tailor a derivative of hirudin which is resistant to digestive enzymes.

EXPERIMENTAL PROCEDURES

Materials—Recombinant desulfated hirudin (CGP 39939) was produced by Ciba-Geigy in collaboration with Platorgan KG (Federal Republic of Germany). Hirudin core fragment Hir1-52 was prepared as described (Chang et al., 1989a). Endoprotease Asp-N, carboxypeptidases A and B, Chromozym TH, Chromozym TRY, Chromozym X, Chromozym U, Chromozym PL, and Factor Xa were purchased from Boehringer Mannheim. Suc-Ala-Ala-Phe-pNA (substrate for chymotrypsin), salmon calcitonin, secretin, and sulfated Hir44-65 were from Bachem. Hir43-65 was kindly provided by H. Rink (Ciba-Geigy). Human a-thrombin was purchased from the Center for Diagnostic Products. Standard fibrinopeptides, urokinase, plasmin, a-cyano-4-hydroxy-cinnamoyl-trypsin (C-7762), and trypsin were obtained from Sigma. Pepsin (no. 7192) was from Merck. Elastase (no. 45125) and carboxypeptidase Y were products of Fluka and Millipore respectively. 4-NN-dimethylaminoazobenzene-4'-isothiocyanato-2'-sulfonic acid (S-DABITC)1

1 The abbreviations used are: S-DABITC, 4-dimethylaminoazobenzene-4'-isothiocyanato-2'-sulfonic acid; S-Hir44-65, (Try44)-sulfated-Hir44-65, HPLC, high performance liquid chromatography.
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was prepared in our laboratory (Chang, 1989a).

Enzymatic Digestion of Intact Recombinant Hirudin—Hirudin—Hir D40 (6 mg/ml) was incubated with various endoproteinases at room temperature. Digestions with Asp-N, chymotrypsin, elastase, thermolysin, and carboxypeptidase A were carried out in 50 mM ammonium bicarbonate, pH 8.0. Aliquots (2 µl) were withdrawn at various time intervals and acidified with 10 µl of 1% trifluoroacetic acid to terminate the reaction. The samples were performed in 0.01 N HCl. Thus, control-digested samples (2 µl) were mixed with 10 µl of 50 mM ammonium bicarbonate and freeze-dried. The extent of cleavage was analyzed by HPLC peptide mapping, by quantitative amino-terminal analysis, and in the case of carboxypeptidase A by amino acid analysis. The locations of cleavage sites were determined by three cycles of sequenc-
ing (Fig. 2).

HPLC Purification of Hirudin Fragments—Hirudin core fragments and Hir D42 were purified by reverse-phase HPLC using conditions described in the legend of Fig. 3.

Structural Analysis of Hirudin Fragments—Amino acid composition was determined by the dimethylaminoazobenzene sulfonil chloro-ride precipitation derivatization method (Knecht and Chang, 1986). One-step quantitative amino-terminal analysis was performed by the DABITC method (Chang, 1988). Quantitative amino-terminal se-
quencing was done by the DABITC/phenyl isothiocyanate method (Chang, 1988b, 1988) using a specific protocol designed to obtain quantitative recoveries of Ser and Thr derivatives (Chang, 1985).

Analysis of the Stability of Hirudin Core Fragments—Hirudin core fragments were digested in 25 µl of buffer or solutions as specified in Fig. 6. The treated samples were diluted with 200 µl of 67 mM Tris-HCl buffer, and their anticoagulant activities were measured by Coagulometer as described in the following section. Core fragments dissolved in Tris-HCl buffer without heating were used as control samples (their anticoagulant activities were taken as 100%). For analysis of stability, the core fragments (5 µg) were incubated with 1 µg of various enzymes in 20 µl of ammonium bicarbonate solution, acetate buffer (pH 5.4, for carboxypeptidase Y) or 0.01 N HCl (pepsin). Incubation was performed at 37°C for 7 h. The samples were diluted with 200 µl of Tris-HCl buffer and analyzed for their surviving anticoagulant activities, or freeze-dried and evaluated by quantitative amino-terminal analysis, sequencing, and amino acid analysis. For each core fragment, two kinds of control samples were processed in parallel, one was core fragment alone without addition of enzyme, the other was enzyme alone without hirudin core fragment. All control samples with enzyme alone were found to have no effect on the coagulation activity of thrombin.

Anti-amidolytic and Anticoagulant Assays—Anti-amidolytic activ-
ties of the core fragments toward thrombin, urokinase, plasmin, trypsin, factor Xa, and chymotrypsin were measured by their ability to inhibit the target enzyme from digesting p-nitroanilide based sub-
strates. The reaction was carried out at 23°C in 67 mM Tris-HCl buffer, pH 8.0, containing 153 mM NaCl and 0.1% polyethylene glycol 6000. The enzyme was followed at 405 nm for a period of 2 min. The concentration of chromogenic substrate was 200 µM. The concentration of enzyme was adjusted between 2.5 and 25 nm.

The anticoagulant activity of hirudin derivatives was measured with a Coagulometer KCI (from Amelung GmbH, F.R.G.). The assay was carried out at 23°C in Tris-HCl buffer (the same as in the chromopyrim assay) in a total volume of 500 µl. Thrombin (150 nM) was incubated with various concentrations of hirudin core fragments in the Tris-HCl buffer for 2 min. Recording of the clotting time was started by mixing 100 µl of the incubated sample with 400 µl of fibrinogen solution (2 mg/ml). Thus, the final concentrations were 30 µM thrombin and 1.6 mg/ml for fibrinogen. Under these conditions, the clotting time for the control sample (without added hirudin fragment) was 18 ± 1 s. The concentrations of hirudin fragments were adjusted such that the clotting time was between 20 and 200 s.

S-DABITC Modification of the Core Fragment—Thrombin (40 µM) was incubated with an equal concentration of the hirudin derivative for 5 min in 250 µl of sodium bicarbonate (50 mM, pH 8.3). The sample was mixed with 250 µl of S-DABITC solution (2 mM in the same sodium bicarbonate buffer) and left at room temperature for 5 h. Removal of the excess reagent, denatura-
tion of the complex and isolation of the labeled thrombin B-chain and S-DABITC, were performed as described (Chang, 1989b; Chang et al., 1990b). Modified thrombin B-chain was digested with trypsin (enzyme/substrate, 1:20, by weight) in 50 mM ammonium bicarbonate and then analyzed by HPLC using the conditions de-
scribed in the legend of Fig. 8. The ratio of the peak response (extent of modification) between the samples obtained from free thrombin (Fig. 8A) and those obtained from the hirudin fragment-thrombin complex (Fig. 8, B and C) were used to calculate the extent of protection and enhancement (Fig. 9).

Effect of S-Hir D40, on Thrombin Cleavage of Fibrinogen (Release of Fibrinopeptides A and B)—Fibrinogen (2 mg/ml) was incubated with thrombin (0.25 nm) at 22°C in 300 µl of 50 mM ammonium bicarbonate. The reaction was performed both in the absence (control) and presence of S-Hir D40 (5 and 50 µM). At various time intervals, the samples were withdrawn, immediately mixed with an equal volume of 1% trifluoroacetic acid, and heated at 70°C for 10 min. The acidified samples were directly used for HPLC fibrinopeptide analysis using the following conditions. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was 10% B to 50% B in 20 min, held at 50% B for 20 to 30 min and then returned to 10% B in 2 min. The column was Vydac C-18 (5 µm) for peptide and protein analysis. Column temperature was 23°C. Peptides were detected at 214 nm. Under these conditions fibrinopeptide A, fibrinopeptide B, and S-Hir D40 were eluted at 15.6, 16.8, and 17.3 min, respectively.

Effect of S-Hir D40 on Thrombin Cleavage of Non-fibrinogen Poly-
peptide Substrates—Polypeptides (0.5 µM) were digested with thrombin (200 nm) in the absence and presence of S-Hir D40 (5 and 50 µM) in 50 mM ammonium bicarbonate. Aliquots (40 µl) containing 1 ng of the substrates were removed at various time intervals and freeze-dried overnight. New amino termini generated by thrombin cleavage were quantitatively determined by the DABITC method (Chang, 1986).

RESULTS

Production of Hirudin Amino-terminal Core Fragments—Hirudin core fragments were produced from intact recombinant hirudin by extensive cleavage at the hinge region of its two functional domains (Fig. 1). Five endoproteinases were used. They were Asp-N, pepsin, chymotrypsin, elastase, and thermolysin. The extent of cleavage (time course analysis) and the cleavage sites of each enzyme were first examined by quantitative amino-terminal analysis and three cycles of quantitative sequencing prior to fragment purification. As an example, chymotryptic cleavage is shown in Fig. 2.

Endoproteinase Asp-N has been known to cleave peptide bonds at the amino-terminal end of aspartic acid and cysteic acid (Drapeau, 1980). Recombinant hirudin contains four Xaa-Asp bonds (Dodd et al., 1984). Two are located within the amino-terminal core domain (Thr4-Asp9 and Ser32-Asp33) and the other two are in the carboxyl-terminal domain (Asp65-Tyr66 and Asp78-Gly83). Although it was expected that Asp-N might preferentially cleave the two Xaa-Asp bonds located in the carboxyl-terminal domain (Chang, 1983a), the results nonetheless turned out to be surprisingly specific. Only Asp 65-Asp 78 was selectively and quantitatively cleaved. Even under the most rigorous digestion conditions used in this study no detectable cleavage of the other three Xaa-Asp bonds was found. Amino acid sequencing of the 24-h sample (substrate/ enzyme weight ratio of 100) revealed only two sequences at roughly equal molar ratio, Val1-Val-Tyr2- and Asp32-Gly34 (Fig. 1). The two new sequences were: 1) Val1-Val-Tyr2-Asp32-Gly34 and 2) Val1-Val-Tyr2-Asp32-Gly34. These were found to be identical to the amino termini of the two fragments obtained from free thrombin.

![Fig. 1. Selective cleavage sites in recombinant hirudin with core hirudin core fragments (Hir D40, Hir D42, Hir D44, and Hir D45) and Hir D42 and Hir D45. By V8 protease (1) By chymo-
trypticin, elastase, and thermolysin (2) By Asp-N (3) By pepsin (4). By carboxypeptidase A (5). Natural hirudin differs from recombinant hirudin by substitution at Tyr2. Three lysines (●) and three disulfide linkages (●—●) are denoted.](image-url)
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Fig. 2. Quantitative amino-terminal sequencing (three cycles) by the DABITC/phenylisothiocyanate method for characterization of chymotryptic cleavage sites in recombinant hirudin. A mixture of 10 pmol of each DABTH amino acid standard is shown in the bottom panel. The results indicate that selective cleavage occurred at Gln9-Ser60 and Tyr63-Leu64. Gln62 was mostly washed out at the second cycle.

Fig. 3. HPLC separation of Hir1-60, Hir1-62, and Hir62-65. The column was Vydac C-18 for peptide and portains, 5 μm. Column temperature was 23 °C. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was 10% B to 48% B in 30 min. Hir3-25, Hir51-55, and Hir56-65 were eluted at 13.95, 21.15, and 22.35 min, respectively. Top panel, 30-min digestion, hirudin/Asp-N weight ratio of 2500. Bottom panel, 24-h digestion, hirudin/Asp-N weight ratio of 2500.

Asp62-. Phe was not detected at the second cycle which indicated that Glu64-Asp65 was not digested (for sequence, see Fig 1), and no Thr and Glu was found at the third cycle, which confirmed that Thr4 Asp5 and Ser32-Asp33 remained intact. HPLC analysis showed similarly clear-cut results (Fig. 3). Only two new peaks appeared following the digestion of Hir1-60. Amino acid composition analysis and sequencing established that these two new peaks represented the amino-terminal core fragment (Hir1-52) and the carboxyl-terminal fragment (Hir53-65), respectively. Furthermore, quantitative amino-terminal analysis of the fraction containing Hir1-52 gave only Val (~99.5%). No trace of Asp was detected.

Pepsin preferentially hydrolyzes peptide bonds on the amino- or carboxyl-terminal sides of Phe, Tyr, Leu, Glu, Cys (for review, see Kasper, 1975). However, pepsin hydrolysis of peptide bonds also occurred on the carboxyl side of all l-amino acids except Pro. With hirudin, cleavage occurred selectively at Phe36-Glu57 and Glu62-Tyr63, generating three fragments Hir1-56, Hir57-62, and Hir63-65 which were separated by HPLC (Fig. 4, top panel). A minor cleavage at Asp52-Gly54 was also observed, producing Hir1-53 with a yield of approximately 10% of Hir1-56. It is relevant to mention that Hir1-56
was not further converted to Hir1-53 even after prolonged (24 h) digestion with a high concentration of pepsin. This suggested that any cleavage of Asp52-Gly54 must precede breakdown of Phe66-Glu77. There was no evidence that cleavage occurred within the core domain of hirudin.

Chymotrypsin, elastase, and thermolysin generally exhibit broad specificity toward protein substrates (Kasper, 1975). With hirudin their cleavages were all exclusively in the carboxy-terminal domain (Fig. 1). Chymotrypsin and thermolysin selectively attacked Gln49-Ser60 and Tyr43-Leu44, elastase cut Gln46-Ser50 Hir41-Asn52, Glu77-Glu82 (minor), Glu41-Glu62 (minor), and Tyr44-Leu45. All three proteinases produced the core fragment containing residues 1-49. The yields, based on 2.5 mg of starting material, were 81% (chymotrypsin), 57% (thermolysin), and 67% (elastase), respectively.

Hir1-56, Hir1-52, and Hir1-49 were readily isolated in pure form using HPLC conditions described in Figs. 3 and 4. Since the core fragments differ in size from the remains of the carboxy-terminal domain, they could also be purified by gel filtration (on Sephadex G-50). Both isolation techniques, if coupled with the development of immobilized proteinases, should allow automated large scale production of the hirudin core fragments.

Preparation of Hir1-49—Hir1-49 was formed by carboxypeptidase A digestion of Hir1-46. Based on amino acid analysis of time course-digested samples, the release of the last three amino acids was instant, quantitative, and selective at room temperature at enzyme/substrate weight ratios ranging from 1:100 to 20:100. Unlike cleavage by carboxypeptidase Y (Chang, 1983a), digestion by carboxypeptidase A came to a complete stop at Glu52 even following overnight incubation. Hir1-49 was purified by HPLC using the same conditions as described in Fig. 3 (eluted at 17.72 min). Hir1-49 is not considered a core fragment because it still contains a substantial portion of its carboxy-terminal domain (residues 54-62). It was prepared for the purpose of investi gating the functional role of the last three amino acids in the hirudin-thrombin interaction.

The Digestion Patterns of the Hirudin Carboxy-terminal Domain by Proteinases Were Identical Regardless of Whether the Digestion Was Carried Out Using Isolated Carboxy-terminal Fragment or Intact Hirudin—To examine whether the carboxy-terminal domain of hirudin would behave differently toward proteinases when detached from the core fragment, a synthetic peptide containing residues 40-65 of hirudin (Hir40-65) was subjected to digestion by pepsin, chymotrypsin, elastase, and carboxypeptidase A using the conditions applied for stability analysis of core fragments. The digested samples were analyzed by amino-terminal analysis, sequencing, and amino acid analysis. The results showed that the cleavage sites of Hir40-65 were identical to those of intact hirudin. Pepsin specifically cleaved Phe66-Glu77 and Glu41-Tyr44, chymotrypsin digested Gln46-Ser50 and Tyr43-Leu44, elastase cut Gln46-Ser50, His41-Asn52, and Try44-Leu45, and only the last three carboxy-terminal amino acids of Hir40-65 were selectively removed by carboxypeptidase A.

Structural Characterization of Hirudin Core Fragments—The structures of hirudin core fragments and Hir1-49 were confirmed by amino-terminal sequencing and amino acid composition analysis (Table I). The core fragments, however, were resistant to digestion by carboxypeptidase A, B, and Y. Only the carboxy-terminal Phe of Hir1-49 was selectively and quantitatively released by carboxypeptidase A (see stability of core fragments).

Stability of Hirudin Amino-terminal Core Fragments—The stability of hirudin core fragments was tested at various extreme conditions (Fig. 5). The molecules were stable at high temperature (95 °C) in Tris-HCl buffer, pH 8.0, and in acidic, pH 1.47, or alkaline, pH 12.6, solutions. The anticoagulant activity of the core fragments also remained essentially intact with simultaneous treatment at low pH (1.47) and high temperature (70 °C). One condition which inactivated the core fragments was a combination of high pH and high temperature (70 °C). Stability was measured by the surviving anticoagulant activity. The activity of the control sample (pH 8.0, 23 °C) was taken as 100%.

Analysis was performed by the dimethylaminoazobenzene sulfonyl chloride method. Approximately 1 μg of peptide was hydrolyzed and derivatized, and 50 ng was injected for analysis.

Table I

<table>
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<tr>
<th>Hir1-49</th>
<th>Hir1-52</th>
<th>Hir1-55</th>
<th>Hir1-46</th>
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<td>Asp</td>
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</tr>
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<td>4.09 (4)</td>
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</tr>
<tr>
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<tr>
<td>1/4Cys</td>
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<td>0.89 (1)</td>
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<tr>
<td>Tyr</td>
<td>0.87 (1)</td>
<td>0.96 (1)</td>
<td>0.86 (1)</td>
<td>1.00 (1)</td>
</tr>
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</table>

*The numbers given in the parenthesis are expected values.
**The lower than expected value of Val was due to incomplete hydrolysis of the Val-Val sequence.

![Fig. 5. Stability of hirudin core fragments under extreme pH and temperature. Column 1. pH 8.0, 95 °C for 10 min. Column 2. pH 8.0, 95 °C for 30 min. Column 3. pH 1.47, 23 °C for 2 h. Column 4. pH 1.47, 70 °C for 15 min. Column 5. pH 12.6, 23 °C for 2 h. Column 6. pH 12.6, 70 °C for 15 min. Stability was measured by the surviving anticoagulant activity. The activity of the control sample (pH 8.0, 23 °C) was taken as 100%.](image-url)
**Anticoagulant Activity and Specificity of Hirudin Core Fragments**—Hirudin core fragments bind to the active site of thrombin and block its proteolytic activity toward both fibrinogen and non-fibrinogen substrates. The dissociation constants of hirudin core fragment-thrombin binding were 19 nM for Hir\(^{1-56}\), 35 nM for Hir\(^{1-52}\), and 72 nM for Hir\(^{4-49}\). This binding is also highly specific. Based on various assays with synthetic substrates, the dissociation constants for Hir\(^{4-49}\) binding to trypsin, chymotrypsin, urokinase, plasmin, and Factor Xa were minimally greater than 10 µM.

Hirudin core fragments are effective anticoagulants at nanomolar concentration. The most potent core fragment was Hir\(^{1-56}\). On a molar basis, it is about 2-, 4-, and 30-fold more potent than Hir\(^{1-52}\), Hir\(^{1-49}\), and Hir\(^{1-43}\) respectively (Fig. 7).

Mechanism of the Core Fragment-Thrombin Interaction—The mechanism of core fragment to thrombin binding was investigated by mapping the lysyl residues which participate in the combining site. This information was obtained by the S-DABITC technique (Chang, 1989a and 1989b) which measures the accessibility of lysines to S-DABITC modification upon formation of the complex. Using this strategy, it was previously shown that the N-terminus of Hir\(^{54-65}\) binds to the fibrinogen recognition site instead of the catalytic site of thrombin. The anticoagulant activities of the core fragments are, however, notably less potent than that of intact hirudin. Hir\(^{5-52}\) and Hir\(^{4-49}\) bind to thrombin with dissociation constants in the low picomolar range. At nanomolar concentrations, both rendered the same fibrinogen system practically unclottable.

**Fig. 6. The pathways which lead the conversion of intact hirudin (Hir\(^{1-65}\)) to its core fragments.** By pepsin (1). By Asp-N (2). By chymotrypsin and elastase (3). By V8 protease (4).

However, V8 protease is isolated from *Staphylococcus aureus* cells (Houmard and Drapeau, 1972) and there is at present no evidence that enzymes with similar specificity exist in mammals. It is notable that all three core fragments were completely resistant to pepsin, the principal digestive enzyme of the stomach. The core fragments also withstood carboxypeptidase A, B, and Y. The only core fragment sensitive to carboxypeptidase was Hir\(^{1-56}\) which was converted to Hir\(^{1-50}\) by carboxypeptidase A. Hir\(^{1-56}\) had an anticoagulant activity indistinguishable from that of Hir\(^{1-52}\).

**Fig. 7. Anticoagulant activity of hirudin amino-terminal core fragments.** The anticoagulant activity of a hirudin carboxyl-terminal fragment, sulfated Hir\(^{54-65}\), was included for comparison. Concentration of fibrinogen was 1.6 mg/ml. Thrombin was maintained at 30 nM. The control sample (without inhibitor) gave a clotting time of 18.5 ± 1.0 s.
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Thrombin + Hir'-62

Fig. 8. Peptide maps of S-DABITC-labeled thrombin B-chain. Samples were derived from labeled free thrombin (A), Hir'-62-thrombin complex (B), and Hir'-52-thrombin complex (C). Conditions for sample preparation are described in the text. Chromatographic conditions are as follows. The column was Vydac C-18, 5 µm; temperature 22 °C; solvent A was 17.5 mM sodium acetate, pH 5.0; solvent B was acetonitrile; gradient was 20-50%B (linear) in 40 min. Peptides were detected at 436 nm.

FIG. 9. Effects of binding of hirudin derivatives on the accessibility of Lys63, Lys66, Lys77, Lys106, and Lys109 in thrombin. These 5 lysines are situated within the fibrinogen recognition site of thrombin. The accessibility was evaluated by comparing the extent of their chemical modifications in the absence and presence of hirudin derivatives. Binding of Hir1-66 and Hir1-65 protected these 5 lysines from S-DABITC modification. By contrast, binding of the core fragments enhanced the modification of Lys66.

TABLE III
Accessibility of the amino-terminal valine (Val1) of hirudin core fragments in the absence and presence of thrombin

<table>
<thead>
<tr>
<th>Core fragment</th>
<th>Accessibility of the a-amino group of Val1 (%)</th>
<th>Accessibility of the a-amino group of Val1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core fragment alone</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Complexed with thrombin</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>Hir1-48</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>Hir1-52</td>
<td>86</td>
<td>92</td>
</tr>
<tr>
<td>Hir1-56</td>
<td>86</td>
<td>92</td>
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conformational change which leads to increased exposure of its fibrinogen recognition site.

Comparison of the Thrombin Binding Affinity of the Core Fragments with Hir1-62 and Hir1-65—By comparing the thrombin inhibitory potency of Hir1-62 and Hir1-65 with various hirudin core fragments, it can be concluded that practically every region within the last 22 carboxyl-terminal amino acids of hirudin performs crucial functions. Starting with Hir1-43, each step of expansion of its carboxyl-terminal sequence results in additional enhancement of thrombin binding affinity. The effects are shown in Fig. 11. It is, however, the extension from Hir1-56 to Hir1-62 which causes the most dramatic change, with a nearly 400-fold decrease of the thrombin dissociation constant. This remarkable enhancement is apparently attributed to the additional binding of Hir1-62 to a site of thrombin which comprises Lys63, Lys66, Lys77, Lys106, and Lys109 of its B-chain (see Fig. 10).

Effect of S-Hir64-65 on the Proteolytic Activity of Thrombin Toward Fibrinogen and Non-fibrinogen Substrates—In the process of converting fibrinogen to fibrin, thrombin selectively cleaves two Arg-Gly bonds of fibrinogen and releases fibrinopeptides A and B. This proteolytic activity is drastically inhibited by S-Hir64-65 (Fig. 12A). In the absence of S-
Hirudin Amino-terminal Core Fragment Preparation and Properties

FIG. 10. Delineation of the structural elements of hirudin which shield Lys1, Lys3, Lys17, Lys18, and Lys197 of thrombin in the hirudin-thrombin complex. The results were deduced from comparison of the binding patterns of HII\(^{36}\) and HII\(^{45}\) to thrombin (see Fig. 9). The drawings are taken from Chang, J.-Y. (1986). AB, C, and R stand for apolar binding site, catalytic site, and fibrinogen recognition site of thrombin. For detailed structure of hirudin-thrombin complex, see Rydel et al. (1990). In the hirudin-thrombin complex, the amino-terminal core domain of hirudin binds to the catalytic and apolar binding sites of thrombin, whereas the carboxyl-terminal domain binds to the fibrinogen recognition site (anion-binding exosite) of the enzyme.

**DISCUSSION**

It has been proposed that hirudin contains two functional domains (Chang, 1983a, 1986; Markwardt, 1985) that bind to different sites in thrombin (Fenton, 1981). The carboxyl-terminal domain of hirudin has been predicted (Chang, 1983a) and shown to bind to the fibrinogen recognition site (a non-catalytic site) of thrombin to inhibit the enzyme's interaction with fibrinogen (Krstenansky and Mao, 1987; Krstenansky et al., 1987; Mao et al., 1988; Maraganore et al., 1989; Ni et al., 1990). By chemical analysis, at least 7 lysyl residues of the thrombin B-chain were found to be part of this fibrinogen recognition site (Chang, 1988b; Bourdon et al., 1990). Recently, the amino-terminal core fragments of hirudin were isolated and demonstrated to bind to the active site of thrombin (Chang et al., 1990a; Dennis et al., 1990; Dodt et al., 1990). These findings were subsequently verified by x-ray analysis of the hirudin-thrombin complex (Rydel et al., 1990; Gruetter et al., 1990). In the crystal structure of thrombin (Bode et al., 1989), there exists a long groove which extends from the active site and is coated by clusters of positively charged side chains. This groove is complementary to the extended carboxyl-terminal segment of hirudin (Rydel et al., 1990; Gruet-
ter et al., 1990). The 5 lysyl residues of thrombin (Lys$^{32}$, Lys$^{66}$, Lys$^{72}$, Lys$^{85}$, and Lys$^{105}$) contribute to the accessibility to S-DABITC modification of which is reduced upon binding of intact recombinant hirudin (Chang, 1989b) and hirudin carboxyl peptide (Chang et al., 1990b) are situated along this groove.

Our data and those of others (Dennis et al., 1990) also reveal that the two functional domains of hirudin bind to thrombin in a cooperative manner. Binding of the carboxyl-terminal peptide alone slightly activates the active site of thrombin and enhances its cleavage of non-fibrinogen substrates (Fig. 12). Binding of the amino-terminal core fragments to the active site of thrombin causes increased exposure of a lysyl residue (Lys$^{66}$) located within the fibrinogen recognition site.

The unique thrombin-binding mechanism and specificity of the two functional fragments of hirudin suggest that they are potentially useful anticoagulants. In this report, methods for the production of three hirudin amino-terminal core fragments (Hir$^{-49}$, Hir$^{55}$, and Hir$^{1-56}$) are described. Although the core fragments are less potent than intact hirudin in terms of thrombin inhibitory activity, they are still anticoagulants of high efficacy, and they bind to thrombin with considerable affinity and specificity. The binding of the core fragments to thrombin is, however, distinguished from that of intact hirudin. As a putative drug for controlling the activity of thrombin, a hirudin core fragment may offer some potential advantages over intact hirudin (Hir$^{1-56}$). (a) There are indications (Fenton, 1986, 1987) that numerous non-enzymatic (hormonal) activities of thrombin are regulated by inhibitors like hirudin blocking this exosite. (b) Unlike intact hirudin which contains a carboxyl-terminal part that is highly susceptible to cleavage by proteinases (Chang, 1983a), the core fragments are much more resistant to enzymatic digestion. This implies that in vivo core fragments might exhibit a more predictable and constant efficacy than Hir$^{1-56}$. The extreme stability of the core fragments against pepsin and various digestive enzymes further suggests that they might be applicable as oral anticoagulants. (c) There is evidence that antibodies raised against intact hirudin recognize epitopes predominantly in the carboxyl-terminal domain (Grabher, 1989; Schlaeppi et al., 1990). This means that the core domain may be even less immunogenic than the intact inhibitor.

The availability of large quantities of the hirudin core fragments will now permit us to examine these potential advantages.

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REFERENCES