Deciphering the Structural Elements of Hirudin C-Terminal Peptide That Bind to the Fibrinogen Recognition Site of α-Thrombin

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ABSTRACT: The C-terminal peptide of a hirudin acts as an anticoagulant by binding specifically to a noncatalytic (fibrinogen recognition) site of thrombin. This binding has been shown to shield five spatially distant lysines of the thrombin B-chain (Lys^{21}, Lys^{65}, Lys^{77}, Lys^{106}, and Lys^{107}). It was also demonstrated that modification of the sequence of the hirudin C-terminal peptide invariably diminished its anticoagulant activity. The major object of this study is to investigate how the decreased activity of the modified hirudin C-terminal peptide is reflected by the change of its binding properties to these five lysines of thrombin. A synthetic peptide representing the last 12 C-terminal amino acids of hirudin (Hir^{54-65}) was (1) truncated from both its N-terminal and its C-terminal ends, or (2) substituted with Gly along residues 57–62, or (3) chemically modified to add (sulfation at Tyr^{63}) or abolish (Asp and Glu modification with carbodiimide/glycinamide) its negatively charged side chains. The binding characteristics of these peptides to thrombin were investigated by chemical methods, and their corresponding anticoagulant activities were studied. Our results demonstrated the following: (1) the anticoagulant activities of hirudin C-terminal peptides were quantitatively related to their abilities to shield the five identified lysines of thrombin. The most potent peptide was sulfated Hir^{54-65} (S-Hir^{54-65}) with an average binding affinity to the five lysines of 120 nM. A heptapeptide (Hir^{54-60}) also displayed anticoagulant activity and thrombin binding ability at micromolar concentrations. (2) All active hirudin C-terminal peptides regardless of their sizes and potencies were shown to be capable of shielding the five lysines of thrombin. The results are discussed in relation to the recently elucidated X-ray model of the hirudin/thrombin complex. Furthermore, the stability of S-Hir^{54-65} and its relative anticoagulant potency to the N-terminal core fragment of hirudin were examined.

Hirudin is a thrombin-specific inhibitor isolated from leech *Hirudo medicinalis* (Markwardt & Walsmann, 1958; 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 & Hofsteenge, 1986). Hirudin is a 65 amino acid polypeptide (Dodt et al., 1984, 1985) and contains 2 functional domains (Chang, 1983a; Dennis et al., 1990; Dodt et al., 1990; Gruetter et al., 1990; Naski et al., 1990; Rydel et al., 1990). The C-terminal sequence of hirudin has been predicted (Chang, 1983a) and was shown to bind to the fibrinogen recognition site (anion binding exosite) of thrombin to inhibit the enzyme's interaction with fibrinogen (Krstenansky & Mao, 1987). Subsequent studies identified the last 10 C-terminal amino acids as the minimally required structure (Mao et al., 1988) and several hydrophobic residues and acidic residues which were important to maintain the function of hirudin C-terminal peptides (Krstenansky et al., 1987; Braun et al., 1988; Ni et al., 1990). Maraganore et al. (1989) further demonstrated that sulfation at Tyr^{63} increased the anticoagulant activity of Hir^{53-64}. Chang et al. (1990b) showed that the structural elements of hirudin which bind to the fibrinogen recognition site of thrombin were exclusively located within the last 14 C-terminal amino acids of hirudin. At least six lysines within the thrombin B-chain have been shown to be located within or near the binding site of the hirudin C-terminal peptide (Chang et al., 1990b; Bourdon et al., 1990). Most recently, potent hirudin derivatives (hirulogs) designed by linking the hirudin C-terminal peptide and a thrombin active-site sequence have been synthesized (Maraganore et al., 1990; DiMaio et al., 1990).

Synthetic C-terminal peptides of hirudin represent a novel class of anticoagulant because of its unique inhibitory mechanism (Krstenansky et al., 1988; Maraganore et al., 1989; Naski et al., 1990). Potentially, these peptides may find applications beyond the indication of anticoagulation. α-Thrombin is a multifunctional enzyme which displays enzymatic as well as nonenzymatic activities [for reviews, see Fenton (1986, 1987)]. There are suggestions that most of the nonenzymatic activities of thrombin are regulated by an anion binding exosite and are blocked upon binding of hirudin, presumably through interaction with the the inhibitor's C-terminal region. In this study, several modified hirudin C-terminal peptides were prepared. The major object was to study the molecular mechanism of the interaction of hirudin C-terminal peptides and thrombin.

Experimental Procedures

Materials

Human α-thrombin was supplied by the Center for Diagnostic Products (Boston, MA) with a specific activity of 3241 NIH units/mg. 4-(*N*,*N*-Dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid (S-DABITC)[1] was prepared in our laboratory (Chang, 1989a). Hirudin C-terminal peptides Hir^{54-65} and S-Hir^{54-65} (Tyr^{63}-sulfated Hir^{54-65}) were purchased from Bachem (Bubendorf, Switzerland). Hir^{56-65} and Hir^{57-65} were prepared from Hir^{54-65} through stepwise Edman degradation using the protocol described in Chang (1983b), except the first coupling reaction was omitted. Hir^{54-62}, Hir^{54-60}, Hir^{54-58}, [Gly^{57}]Hir^{56-65}, [Gly^{57}, Gly^{58}]Hir^{56-65}, [Gly^{62}]Hir^{56-65}, [Gly^{61},Gly^{62}]Hir^{56-65}, [Gly^{59}]Hir^{56-65}, and [Gly^{60}]Hir^{56-65} were synthesized by Neosystem S.A. (Strasbourg, France). Complete modification of the carboxyl groups of Hir^{54-65} was

[1] Abbreviations: S-DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene-2′-sulfonic acid; DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanatoazobenzene; HPLC, high-performance liquid chromatography.

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performed as described in Carraway and Koshland (1972), and the modified sample (2 mg) was desalted by passing it through a PD-10 column (Pharmacia) preequilibrated in 50 mM ammonium bicarbonate. Recombinant hirudin (CGP 39393) was produced by Ciba-Geigy in collaboration with Plantorgan AG (Germany) (Meyhack et al., 1987). Hirudin was denatured either by oxidation with performic acid or by reduction–carboxymethylation. Hirudin N-terminal core fragments Hir^{1-43} and Hir^{1-52} were produced by enzymatic cleavage of intact recombinant hirudin (Chang, 1990; Chang et al., 1990a). Fibrinogen was from KabiVitrum (Sweden). Stock solutions (2.5 nmol/μL in H_2O) were prepared for all peptides, and their concentrations were determined by amino acid analysis.

Methods

Characterization of Hirudin C-Terminal Peptides. Purity and identity of peptides were evaluated by (a) HPLC profile, (b) quantitative N-terminal analysis using the (dimethylamino)isothiocyanatoazobenzene method (Chang, 1988), (c) sequence analysis using the (dimethylamino)isothiocyanatoazobenzene/phenylisothiocyanate method (Chang, 1983b), and (d) amino acid analysis using the (dimethylamino)azobenzenesulfonyl chloride precolumn derivatization method (Knecht & Chang, 1986).

S-DABITC Modification of the Hirudin C-Terminal Peptide/Thrombin Complex. Hirudin C-terminal peptides (20–800 μM) were incubated with thrombin (20 μM) in 250 μL of sodium bicarbonate solution (50 mM, pH 8.3) for 10 min. The samples were then mixed with an equal volume of S-DABITC solution (2 mM in the same buffer). S-DABITC labeling was performed at room temperature for 5 h. The subsequent desalting, reduction–carboxylmethylation, and isolation of labeled thrombin B-chain were carried out as described in Chang (1989b). Modified thrombin B-chain was digested by trypsin (enzyme:substrate ratio 1:20 by weight) in 50 mM ammonium bicarbonate solution and analyzed by HPLC using the conditions described in the legend of Figure 2. The ratio of the peak response (extent of modification) between the samples obtained from thrombin alone (Figure 2A) and those obtained from hirudin C-terminal peptide/thrombin complexes (e.g., Figure 2B–D) were used to calculate the percentage of protections (Figures 3 and 4). The thrombin dissociation constant of a given peptide was then calculated by applying the equation:

$$\frac{(A - x)(1 - x)B^2}{xB}$$

where A is the molar ratio of hirudin peptide/thrombin, B is the concentration of thrombin which was kept at 20 μM throughout the study, and x is the average of the percentages of protection of the five lysyl residues (see Figures 3 and 4). If the extent of protection of an individual lysine is applied, the equation will allow calculation of the dissociation constant of a ligand at the amino acid level.

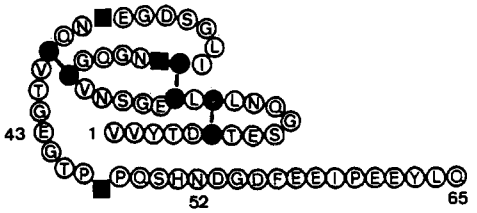
Anticoagulant Assay. The anticoagulant activity was measured by a coagulometer Model KCl manufactured by Heinrich Amelung (Germany). The assay was carried out at 23 °C in Tris-HCl buffer [67 mM, pH 8.0, containing 133 mM NaCl, and 0.13% poly(ethylene glycol)] with a total volume of 500 μL. Thrombin (150 nM) was incubated with various amounts of hirudin C-terminal peptides in the Tris-HCl buffer for 2 min. The clotting time was recorded by mixing 100 μL of the incubated sample with 400 μL of fibrinogen solution (2 mg/mL in the same Tris-HCl buffer). The final concentrations were 30 nM for thrombin and 1.6 mg/mL for fibrinogen. The concentrations of hirudin peptides were adjusted so that the clotting times were recorded between 30 and 200 s. It is relevant to mention that reproducibility of this coagulometer (which is typically ±2–3%) hinges upon strict control of the temperature of coagulation and the freshness of the fibrinogen solution.

Analysis of the Stability of S-Hir^{54-65}. Samples of S-Hir^{54-65} (2.5 nmol) were each dissolved in 25 μL of solutions and incubated under conditions as specified in Table I. The samples were dried in a Speedvac, diluted with 200 μL of the Tris-HCl buffer (see Anticoagulation Assay), and used directly for measurement of anticoagulant activity. For analysis of enzymatic stability, S-Hir^{54-65} (2.5 nmol) was incubated with 1 μg of enzymes in selected solutions (0.01 N HCl for pepsin; 50 mM ammonium bicarbonate, pH 8.0, for chymotrypsin, elastase, and carboxypeptidase A). The treated S-Hir^{54-65} was analyzed for its surviving anticoagulant activity by a coagulometer and its structure by sequencing and amino acid analysis. Control samples containing enzyme alone were found to have no effect on the coagulation activity of thrombin.

Results

Characterization of Synthetic C-Terminal Peptides and Derivatives of Hirudin. By use of HPLC profiling, quantitative N-terminal analysis, and amino acid composition as criteria, all peptides used in this study had purity exceeding 95%. The extent of sulfation of S-Hir^{54-65} at h-Tyr^{63}, which could not be quantitated by amino acid analysis following total hydrolysis, was examined by carboxypeptidase Y digestion (Chang, 1983a) and was found to be 94%. Amino acid analysis revealed that following carbodiimide/glycinamide modification, 6.2 mol of Gly/mol of Hir^{54-65} was incorporated (peptide XIV). The possibility that oxidized (or reduced–carboxymethylated) hirudin might be contaminated by a trace amount of intact hirudin was ruled out based on the observation that even at a concentration of 50 μM, both denatured hirudins exhibited no antiamidolytic activity.

Binding of Hirudin C-Terminal Peptides to Thrombin. Binding capacity of hirudin C-terminal peptides (Figure 1) to the fibrinogen recognition site of thrombin was evaluated by their abilities to protect five lysyl residues (Lys^{21}, Lys^{65}, Lys^{77}, Lys^{106}, and Lys^{107}) within the B-chain of thrombin from chemical modification by S-DABITC. These five lysyl residues were previously shown to reside within a noncatalytic site of thrombin which is required for fibrinogen recognition (Chang, 1989b; Chang et al., 1990b). The data are shown in Figures 2–4. It was found that the binding affinities of Hir^{40-65}, Hir^{52-65}, Hir^{53-65}, and Hir^{54-65} were indistinguishable (unpublished observations). These data are consistent with those reported by Mao et al. (1988), who showed that the first significant decrease of anticoagulant activity was associated with the truncation of h-Gly^{54} and h-Asp^{55}. The optimal size of an active hirudin C-terminal peptide thus resides at the last 12 C-terminal residues of hirudin (Hir^{54-65}). Each and every amino acid residing within this sequence appeared to be crucial. The dissociation constant of the Hir^{54-65}/thrombin complex was about 13 μM. Removal of h-Gly^{54} and h-Asp^{55} (Hir^{56-65}) increased the dissociation constant to 33 μM. Further deletion of h-Phe^{56} (Hir^{57-65}, peptide IV) weakened the binding affinity by a 64-fold increase of the dissociation constant (to about 825 μM). Truncation from the C-terminal end (peptides V–VII) also caused an incremental loss of the binding affinity, and following deletion of the last seven C-terminal amino acids (Hir^{54-58}), the binding affinity was no longer detectable. Substitutions of acidic or hydrophobic residues of Hir^{54-65} by Gly (peptides VIII, IX, X, XI, XII, and XIII) all led to the



S-Hir$^{54\text{-}65}$	G D F E E I P E E Y L Q (SO_3H)	(I)
Hir$^{54\text{-}65}$	G D F E E I P E E Y L Q	(II)
Hir$^{56\text{-}65}$	F E E I P E E Y L Q	(III)
Hir$^{57\text{-}65}$	E E I P E E Y L Q	(IV)
Hir$^{54\text{-}62}$	G D F E E I P E E	(V)
Hir$^{54\text{-}60}$	G D F E E I P	(VI)
Hir$^{54\text{-}58}$	G D F E E	(VII)
[G^{57}] Hir$^{56\text{-}65}$	F G E I P E E Y L Q	(VIII)
[G^{57},G^{58}] Hir$^{56\text{-}65}$	F G G I P E E Y L Q	(IX)
[G^{62}] Hir$^{56\text{-}65}$	F E E I P E G Y L Q	(X)
[G^{61},G^{62}] Hir$^{56\text{-}65}$	F E E I P G G Y L Q	(XI)
[G^{59}] Hir$^{56\text{-}65}$	F E E G P E E Y L Q	(XII)
[G^{60}] Hir$^{56\text{-}65}$	F E E I G E E Y L Q	(XIII)
Hir$^{54\text{-}65}$ (★)	G D F E E I P E E Y L Q★	(XIV)

FIGURE 1: Amino acid sequences of Hir$^{54\text{-}65}$ and its derivatives. Peptide XIV derived from Hir$^{54\text{-}65}$ (II) by modification with carbodiimide/glycinamide. The asterisk indicates a carboxyl side chain coupled with glycinamide.

impairment of its ability to bind thrombin, but it was the Gly substitution at h-Ile^{59} that showed the most drastic effect. Complete modification of the carboxyl groups of Hir$^{54\text{-}65}$ (XIV) increased the dissociation constant from 13 μM to greater than 8 mM.

The only modification which enhanced the thrombin binding affinity of Hir$^{54\text{-}65}$ was sulfation at h-Try^{63}. On the basis of the extent of protection obtained from the complex containing equal moles of S-Hir$^{54\text{-}65}$ and thrombin, sulfation enhanced thrombin binding with an approximately 100-fold decrease of the dissociation constant.

The results also revealed that there was no preferential or selective shielding among the five lysines of thrombin by various sizes of hirudin C-terminal peptides (Figures 3 and 4). Implications of this finding are put forward under Discussion.

Anticoagulant Activity of Hirudin C-Terminal Peptides. The efficacy of hirudin C-terminal peptides to inhibit the formation of thrombin-induced fibrin clot is presented in Figures 5–7. The relative anticoagulant activities of Hir$^{54\text{-}65}$ and its derivatives (Figure 5) are well correlated to their capacity to shield the fibrinogen recognition site of thrombin. The most potent hirudin C-terminal peptide, sulfated Hir$^{54\text{-}65}$ (S-Hir$^{54\text{-}65}$), had an anticoagulant activity comparable to that of a hirudin amino-terminal core fragment, Hir$^{1\text{-}43}$ (Chang et al., 1990a). On a molar basis, S-Hir$^{54\text{-}65}$ was roughly 25-fold more potent than Hir$^{54\text{-}65}$ (Figure 6). Hir$^{54\text{-}65}$ in turn was 8 times more effective than Hir$^{56\text{-}65}$ (Figure 5). The remaining hirudin C-terminal peptides displayed only insignificant anticoagulant activity at a micromolar range (Figure 5). Hir$^{54\text{-}58}$ (VII) and [Gly^{59}]Hir$^{54\text{-}65}$ (XII) exhibited no detectable activity even at a concentration of 800 μM.

Extension of Hir$^{54\text{-}65}$ at the N-terminal end to Hir$^{45\text{-}65}$

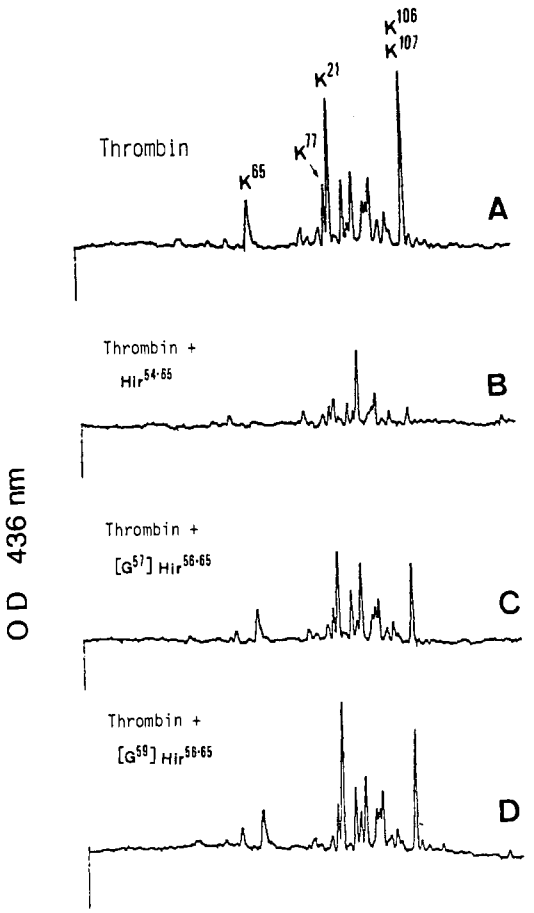


FIGURE 2: Tryptic peptide mapping of S-DABITC-modified thrombin B-chain. Samples were derived from S-DABITC modification of (A) thrombin alone (control sample), (B) Hir$^{54\text{-}65}$ thrombin complex (molar ratio 20:1, 400 μM/20 μM), (C) [Gly^{57}]Hir$^{56\text{-}65}$/thrombin complex (molar ratio, 20:1), and (D) [Gly^{59}]Hir$^{56\text{-}65}$/thrombin complex (molar ratio, 20:1). Modification of five lysyl residues (Lys^{21}, Lys^{65}, Lys^{77}, Lys^{106}, and Lys^{107}) within the thrombin B-chain was protected upon binding of hirudin C-terminal peptide, and the extent of protection was used to measure the binding affinity of hirudin C-terminal peptide/thrombin complex. Conditions of tryptic digestion are described in the text. Ten microliters containing 4 μg of the digested sample was injected. Chromatographic conditions are as follows: the column was Vydac C-18, 5 μM; temperature was 23 °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; flow rate was 1 mL/min.

(Krstenansky & Mao, 1987) or Hir$^{40\text{-}65}$ (data not shown) neither increased nor decreased its activity. We have indeed found that even the attachment of the entire N-terminal core domain (in denatured form) to Hir$^{54\text{-}65}$ produced no effect. Denatured Hir$^{1\text{-}65}$ (both oxidized and reduced–carboxymethylated) and Hir$^{54\text{-}65}$ displayed indistinguishable potency in preventing the formation of fibrin clot (Figure 7). It is important to mention that denatured Hir$^{1\text{-}65}$ did not at all bind to the active-site region of thrombin. At concentrations of 25 and 50 μM, no measurable antiamidolytic activity was observed for both oxidized and carboxymethylated Hir$^{1\text{-}65}$.

Stability of S-Hir$^{54\text{-}65}$. S-Hir$^{54\text{-}65}$ was stable at 95 °C, in acidic (pH 1.47) as well as in alkaline (pH 12.6) solutions (Table I). It was also stable under the combination of high temperature (70 °C) and extreme pH. h-Tyr^{63} was desulfated at 70 °C in 25‰ and 50% trifluoroacetic acid, converting S-Hir$^{54\text{-}65}$ to Hir$^{54\text{-}65}$, which had a much lower potency of anticoagulation.

S-Hir$^{54\text{-}65}$ was promptly inactivated in the acidic solution by pepsin as a result of cleavages at h-Phe^{56}-Glu^{57} and h-Glu^{62}–Tyr^{63} (sulfated). Inactivation was also effected by

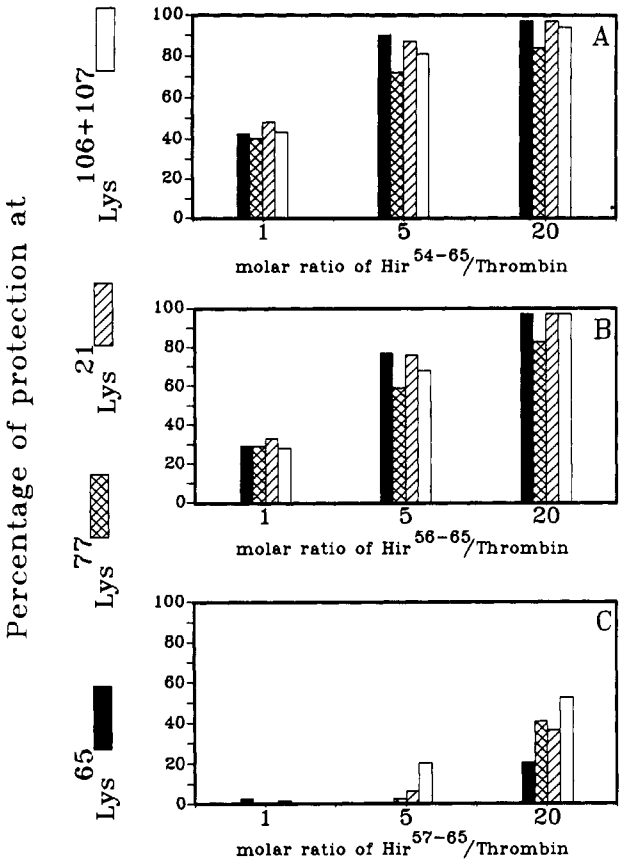


FIGURE 3: Shielding of the fibrinogen recognition site of thrombin by Hir^{54-65}, Hir^{56-65}, and Hir^{57-65}. Analyses were carried out with peptide/thrombin molar ratios of 1, 5, and 20. The extent of shielding is presented by the peptide's ability to protect five lysyl residues (Lys^{21}, Lys^{65}, Lys^{77}, Lys^{106}, and Lys^{107}) of thrombin B-chain from S-DABITC modification. Percentage of protection of each lysine was calculated from the peptide mapping as shown in Figure 2.

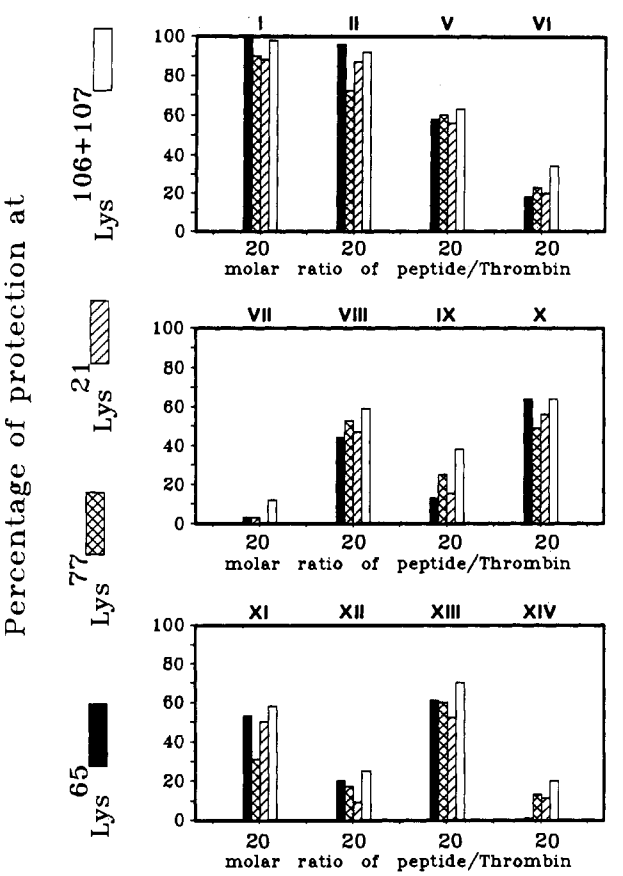


FIGURE 4: Shielding of the fibrinogen recognition site of thrombin by hirudin C-terminal peptides. Analyses were performed on the peptide/thrombin complex with a molar ratio of 20:1 (400 μM/20 μM). The results should be allowed to have ±5% deviation.

carboxypeptidase A and carboxypeptidase B which selectively removed the last two and one C-terminal amino acids from S-Hir^{54-65}, respectively (Table I). S-Hir^{54-65} was completely resistant to chymotrypsin. Elastase had the same effect as carboxypeptidase B which specifically deleted h-Gln^{65} and converted S-Hir^{54-65} to S-Hir^{54-64}.

Discussion

Binding of Hirudin C-Terminal Peptides to Thrombin. The three-dimensional structures of hirudin (Folker et al., 1989; Haruyama & Wuethrich, 1989), thrombin (Bode et al., 1989), and the hirudin/thrombin complex (Gruetter et al., 1990; Rydel et al., 1990) have been recently elucidated. The specific binding of hirudin C-terminal domain to thrombin involves both ionic and hydrophobic interactions. The drastic effects of deleting h-Phe^{56} (Hir^{56-65} to Hir^{57-65}) (Figure 3) and replacing h-Ile^{59} (peptides III–XII) (Figure 5) are obvious from the structure of the complex. Both residues are engaged in compact hydrophobic interactions with thrombin (Rydel et al., 1990). h-Phe^{56} interacts with Phe^{19} and Leu^{26} of the thrombin B-chain,[2] whereas h-Ile^{59} of hirudin is close to Leu^{60} and Ile^{78} of thrombin.

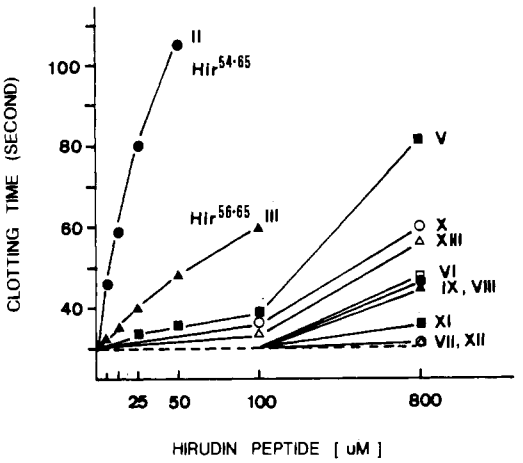


FIGURE 5: Anticoagulant activity of hirudin C-terminal peptides. The measurement was carried out with a coagulometer. The clotting time of the control sample (thrombin alone) was 30 ± 1.4 s (average of 12 recordings) as indicated by a dashed line. The concentration of fibrinogen was 1.6 mg/mL. The thrombin concentration was 50 nM. The temperature was 23 °C.

In the X-ray model of thrombin (Bode et al., 1989), Lys^{21}, Lys^{65}, Lys^{77}, Lys^{106}, and Lys^{107} of the B-chain[2] form a planar pentagon-shape cluster with a diameter of about 15 Å. The C-terminal sequence of hirudin binds to this region (Rydel et al., 1990). Lys^{65} is somewhat buried, but this does not preclude it from reacting with S-DABITC, because the reactivity of a lysine side chain depends upon its accessibility as well as the pK_a value. Lys^{77}, Lys^{106}, and Lys^{107} of thrombin are close to h-Tyr^{63} of hirudin, and one of these three lysines could be involved in ion pairing if h-Tyr^{63} is sulfated. The fact that

[2] Numbering is based on the sequence of thrombin alone. No insertions are introduced. Human α-thrombin contains an A-chain (36 residues) and a B-chain (259 residues). The five lysines which are shielded by the hirudin C-terminal peptide are all located within the B-chain. Correlations to *the numbering system based on the topological similarity with chymotrypsinogen* (Bode et al., 1989) are given as follows: Lys^{21} = Lys^{36}; Lys^{65} = *Lys^{70}*; Lys^{77} = *Lys^{81}*; Lys^{106} = *Lys^{109}*; Lys^{107} = *Lys^{110}*; Phe^{19} = *Phe^{34}*; Leu^{26} = *Leu^{40}*; Leu^{60} = *Leu^{65}*; Ile^{78} = *Ile^{82}*. The letter "h" preceding the three-letter code of a residue designates hirudin.

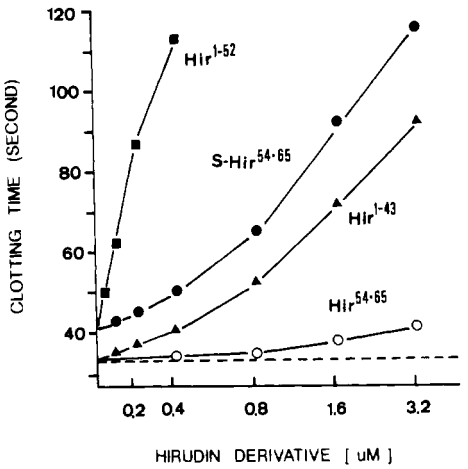


FIGURE 6: Anticoagulant activity of Hir^{54-65} and its sulfated derivative (S-Hir^{54-65}). The clotting times in the presence of two hirudin N-terminal core fragments (Hir^{1-43} and Hir^{1-52}) were included for comparison. The clotting time of the control sample in this set of experiments was 33 ± 1.6 s (average of eight measurements). Other conditions were the same as described in Figure 5.

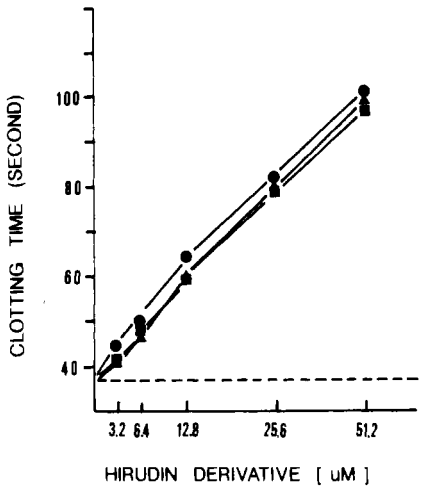


FIGURE 7: Anticoagulant activity of Hir^{54-65} and denatured Hir^{1-65} (both oxidized and reduced–carboxymethylated). Hir^{54-65} (●); oxidized Hir^{1-65} (▲); reduced–carboxylmethylated Hir^{1-65} (■).

unsulfated peptide is still able to protect Lys^{77}, Lys^{106}, and Lys^{107} suggests that sulfated and unsulfated hirudin C-terminal peptides bind to thrombin through a similar fashion but with different affinities. Lys^{21} of thrombin forms a salt bridge with the carboxylate of h-Gln^{65} of hirudin. This binding model could well explain the shielding of the five lysines by both intact hirudin (Chang, 1989b) and hirudin C-terminal peptide containing the last three amino acids, such as S-Hir^{54-65} or Hir^{52-65} (Chang et al., 1990b). Deletion of h-Gln^{65}, h-Leu^{64}, and h-Tyr^{63}, on the other hand, should leave sufficient space to allow reaction of Lys^{106}, Lys^{107}, or Lys^{21} with S-DABITC. Our results nonetheless reveal that the five lysines are about equally protected by hirudin C-terminal peptide lacking the last three amino acids (Hir^{54-62}, peptide V, see Figure 4). This finding is consistent with the result obtained from the analysis using hirudin N-terminal core fragments containing various lengths of the C-terminal tail. In this separate study (Chang, 1990), it was demonstrated that the structural elements of hirudin which protect these five lysines are exclusively located within Hir^{57-62}. Indeed, an even smaller peptide, Hir^{54-60} (peptide VI), still possesses the ability to shield the five lysines (Figure 4). This result is unlikely to be an artifact since the potency of Hir^{54-60}, however weak as compared to S-Hir^{54-65}, has been

Table I: Structure and Surviving Anticoagulant Activity of S-Hir^{54-65} following Treatment with Extreme pH, Temperature, and Proteinases

condition of treatment[a]	structure [b]	% surviving[c] activity
pH 8.0, 95 °C, 10 min		101
pH 8.0, 95 °C, 30 min		100
pH 1.47, 23 °C, 2 h		103
pH 1.47, 70 °C, 15 min		99
pH 12.6, 23 °C, 2 h		104
pH 12.6, 70 °C, 15 min		98
25% TFA, 70 °C, 15 min	desulfation of Tyr^{63}	35
50% TFA, 70 °C, 15 min	desulfation of Tyr^{63}	14
CPA, 45 min	Gln^{65} (100%), Leu^{64} (96%) deleted	3.5
CPA, 5 h	Gln^{65} (100%), Leu^{64} (100%) deleted	2.7
CPB, 45 min	Gln^{65} (71%) deleted	71
CPB, 5 h	Gln^{65} (93%) deleted	54
pepsin, 45 min	Phe^{56}–Glu^{57}, Glu^{62}–Tyr^{63} quantitatively cleaved	<2
elastase, 5 h	Leu^{64}–Gln^{65} (85%) cleaved	66
chymotrypsin, 5 h	no detectable cleavage	>98

[a] Incubations were carried out in Tris-HCl buffer (pH 8.0), 0.1 N HCl (pH 1.47), and 0.1 N NaOH (pH 12.6) or in a trifluoroacetic acid/water mixture. The conditions for the enzymatic incubation were described in the text. CPA and CPB stand for carboxypeptidases A and B, respectively. [b] The structures were evaluated by quantitative N-terminal analysis, sequencing, and amino acid analysis. In the case of evaluating the extent of desulfation, the samples were digested by carboxypeptidase Y followed by amino acid analysis. [c] The clotting time of thrombin alone was taken as 0% activity. The clotting time of thrombin in the presence of untreated S-Hir^{54-65} was taken as 100% activity.

reproducibly displayed by its anticoagulant activity (Figure 5) as well as its thrombin binding ability. One likely explanation is that upon binding of hirudin C-terminal peptide, a subtle conformational change of thrombin (Mao et al., 1988; Rydel et al., 1990) alters the microenvironment of these lysines and thus indirectly protects them from S-DABITC modification. Alternatively, the protection could be a consequence of electrostatic effects exerted by the carboxylate of Hir^{54-60}, or the presence of h-Pro^{60} of the bound Hir^{54-60} causes steric hindrance to the accessibility of Lys^{21}, Lys^{106}, and Lys^{107} by the bulky S-DABITC. Additional speculations are available. Hir^{54-60} might bind to a site of thrombin which is different from what was observed in the hirudin/thrombin complex. In this model, Hir^{54-60} could slide along the groove of thrombin which accommodates the hirudin C-terminal domain (Bode et al., 1989), and h-Phe^{56} of Hir^{54-60} interacts with hydrophobic residues of thrombin which were occupied by h-Ile^{59} in the hirudin/thrombin complex. Alternatively, the groove of thrombin might be occupied by more than one Hir^{54-60} molecule (see discussion below).

Anticoagulant Activity of Hirudin C-Terminal Peptides. The anticoagulant activities of hirudin C-terminal peptides are found to be quantitatively associated with their abilities to bind the fibrinogen recognition site of thrombin as exemplified by the five lysines mentioned above. Our results also confirm that the optimal size of an active C-terminal peptide resides in residues 54–65 of the inhibitor (Mao et al., 1988) and suggest that the borderline which divides the functional C-terminal domain and the N-terminal domain of hirudin could be drawn at either h-Asn^{52}–Asp^{53} or h-Asp^{53}–Gly^{54}. This finding is further supported by the X-ray structure of the hirudin/thrombin complex (Rydel et al., 1990; Gruetter et al., 1990). The conclusion is also compatible with the results obtained from the characterization of the hirudin N-terminal domain which showed that Hir^{1-52} and Hir^{1-53} were more potent than

the shorter versions of the core fragment (Chang, 1990; Dennis et al., 1990; Dodt et al., 1990). Within Hir^{54-65}, just about every residue was found to be critical to maintain the activity of the peptide, and despite the fact that some residues are more sensitive than others, there is no clear boundary to which complete inactivation of the peptide could be related. For example, the deletion of h-Gln^{65} alone from S-Hir^{54-65} causes a 50% decrease of its specific anticoagulant activity (Table I), while a heptapeptide (Hir^{54-60}) still displays thrombin binding affinity and anticoagulant activity. Examination of amino acid sequences of the C-terminal domain of hirudin variants (Harvey et al., 1986; Steiner, 1988; Scharf et al., 1989) and an active peptide derived from heparin cofactor II (Hortin et al., 1989) reveals that a common feature of their structures is the repeating units of hydrophobic and acidic amino acids. This implies that the potency of the hirudin C-terminal peptide might be a consequence of cooperative binding of these repeating units to thrombin. The hypothesis could explain the binding characteristic of Hir^{54-60} discussed above. In this case, the five spatially separated lysines of thrombin are simultaneously bound to more than one Hir^{54-60} molecule.

Finally, this report also provides some useful information regarding the stability of the hirudin C-terminal peptide (Table I). S-Hir^{54-65} is resistant to various extreme conditions but could not sustain the assault of digestive enzymes (Table I). This suggests that S-Hir^{54-65} in its naked form is not suitable as an oral anticoagulant. In further attempts to design a enzyme-resistant version of the hirudin C-terminal peptide, one should therefore bear in mind that pepsin-susceptible bonds (h-Phe^{56}–Glu^{57} and h-Glu^{62}–Tyr^{63}) involve some of the most pivotal residues of S-Hir^{54-65}.

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