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The structural elements of hirudin which bind to the fibrinogen recognition site of thrombin are exclusively located within its acidic C-terminal tail

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Six lysyl residues of human thrombin (Lys^{B21}, Lys^{B52}, Lys^{B65}, Lys^{B106}, Lys^{B107} and Lys^{B154}) have been previously shown to participate in the binding site of hirudin, a thrombin-specific inhibitor [(1989) J. Biol. Chem. 264, 7141–7146]. In this report, we attempted to delineate the region of hirudin which binds to these basic amino acids of thrombin. Using the N-terminal core domains (*r*-Hir^{1-43} and *r*-Hir^{1-52}) derived from recombinant hirudins and synthetic C-terminal peptides (Hir^{40-65} and Hir^{52-65}) — all fragments form complexes with thrombin — we are able to demonstrate that the structural elements of hirudin which account for the shielding of these 6 lysyl residues are exclusively located within the acidic C-terminal region. Since hirudin C-terminal peptides were shown to bind to a non-catalytic site of thrombin and inhibit its interaction with fibrinogen [(1987) FEBS Lett. 211, 10–16], our data consequently imply that these 6 lysyl residues are constituents of the fibrinogen recognition site of thrombin.

Hirudin; Hirudin C-terminal peptide; Hirudin C-terminal peptide/thrombin interaction

1. INTRODUCTION

Hirudin is a potent thrombin inhibitor isolated from the bloodsucking leech *Hirudo medicinalis* [1,2]. It binds to α-thrombin with exceedingly high affinity [3] and specificity [4] and blocks the clotting activity of the protease. Hirudin is a 65-amino-acid polypeptide [5] which contains 3 disulfide linkages at the N-terminal region [6] and a cluster of acidic residues at the C-terminal region. While the N-terminal domain is highly resistant to enzymatic digestion, the acidic C-terminal segment of hirudin (approximately 14 residues) is freely accessible to endo- and exo-peptidases and its intactness is essential to the activity of the inhibitor [7]. The C-terminal sequence of hirudin also exhibits an intriguing structural homology to both fibrinopeptides A and B. These observations lead to the following hypothesis [7]: (i) hirudin is a tadpole-like molecule with a compact N-terminal head and a hydrophilic C-terminal tail; and (ii) the acidic C-terminal tail of hirudin binds specifically to a site on thrombin which is required for fibrinogen recognition [8,9] and that this binding together with the reactive site (hirudin)–active site (thrombin) interaction account for the tight and specific complex formation of hirudin and thrombin.

Several lines of evidence subsequently confirmed this proposed model. (i) Synthetic C-terminal peptides of hirudin containing the last 10–20 amino acid residues were shown to bind thrombin and impair the clotting activity of the protease [10–12]. However, these peptides did not affect the catalytic site of the enzyme. (ii) Site-directed mutagenesis demonstrated that replacement of acidic amino acids at the C-terminal region of hirudin drastically reduced the binding affinity of the inhibitor [13]. (iii) NMR studies revealed hirudin as a tadpole-shape molecule having its C-terminal tail floating without defined conformation [14,15]. Despite those progresses, the site of thrombin to which the hirudin C-terminal peptides bind remains to be unraveled. Recently, we have shown that 6 lysyl residues of thrombin were protected upon binding of thrombin [16]. The corresponding structural components of hirudin which shield these 6 lysines also remain to be elucidated. This study was therefore undertaken to provide answers to these two relevant questions.

2. EXPERIMENTAL

2.1. *Materials*

Human α-thrombin was purchased from the Center for Diagnostic Products (Boston, USA). Recombinant desulfato hirudin (CGP-39393) has been produced by Ciba-Geigy in collaboration with Plantorgan KG (D-2903 Bad Zwischenahn, FRG) [17]. Hirudin C-

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Abbreviations: S-DABITC, 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyano-2′-sulfonic acid; HPLC, high-performance liquid chromatography

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terminal peptides (Hir^{40-65} and Hir^{52-65}) were synthesized by solid-phase technique using Fmoc strategy starting from a dimethoxy-benzhydryl-protected C-terminal Gln at the alkoxy-benzylalcohol resin. Coupling protocols were as described [18]. Structures of the HPLC-purified peptides were confirmed by FAB-MS. The N-terminal core domain $r\text{-}Hir^{1-43}$ was prepared from limited proteolysis of recombinant hirudin by V8 protease, followed by purification with HPLC [19]. The core domain $r\text{-}Hir^{1-52}$ was prepared as described in [20]. Purities of $r\text{-}Hir^{1-43}$, $r\text{-}Hir^{1-52}$, Hir^{40-65} and Hir^{52-65} were ascertained by quantitative N-terminal analysis [21] and amino acid analysis [22]. The anti-hirudin monoclonal antibody (MAb 4049-83-12) was prepared as described [23]. S-DABITC was synthesized in our laboratory [24]. Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was obtained from Cooper Biochemical.

2.2. *S-DABITC modification of thrombin and hirudin–thrombin complex*

Thrombin (0.5 mg) was first incubated with intact recombinant hirudin (125 μg), $r\text{-}Hir^{1-43}$ (200 μg), $r\text{-}Hir^{1-52}$ (250 μg), Hir^{40-65} (0.5 mg) or Hir^{52-65} (0.5 mg and 25 μg) in 250 μl of 50 mM sodium bicarbonate (pH 8.3) for 5 min. A control sample containing 0.5 mg of thrombin alone was carried out in parallel. Derivatization with S-DABITC was performed as described in [16].

2.3. *Structural analysis of S-DABITC-modified proteins*

Modified thrombin and hirudin/thrombin complexes were first reduced with dithiothreitol in 0.5 M Tris-HCl buffer (pH 8.4, containing 5 M guanidine chloride) and then treated with iodoacetic acid. Thrombin B-chain (259 a.a), hirudin (65 a.a), $r\text{-}Hir^{1-43}$, $r\text{-}Hir^{1-52}$, thrombin A-chain (36 a.a.) and Hir^{52-65} were separated by the chromatographic system described in [16]. The fractions containing thrombin B-chain (I) and A-chain (III) were freeze-dried and digested with trypsin (1:20 by weight to the substrate) in 100 μl of 50 mM ammonium bicarbonate solution for 4 h. The digested samples were directly injected onto HPLC for analysis (fig.1). Purity and structure of S-DABITC-labeled peptides were analyzed by quantitative N-terminal analysis [21] and automatic sequencing [25].

3. RESULTS AND DISCUSSION

3.1. *The compact N-terminal domain of hirudin blocks the catalytic site of thrombin*

Both $r\text{-}Hir^{1-43}$ [19] and $r\text{-}Hir^{1-52}$ [20] form complexes with α-thrombin (with binding constants of 300 nM and 30 nM, respectively) and block the amidolytic activity of the enzyme, clearly indicating that their binding at least involves the shielding of the active site of thrombin. In addition, it is very likely that the affinity of the hirudin N-terminal core domain to thrombin is also enhanced by hydrophobic interaction involving an apolar binding pocket adjacent to the active site of the protease (fig.2) [19,26–28]. There is good evidence which suggests that $r\text{-}Hir^{1-52}$ retains essentially the same conformation as the corresponding segment of the intact hirudin. A monoclonal antibody (MAb 4049-83-12) raised against intact recombinant hirudin recognized a conformation-dependent epitope encompassing Glu^{43} and Lys^{47} [23]. This antibody exhibited 90% cross-reactivity toward both intact hirudin and $r\text{-}Hir^{1-52}$. In addition, the antibody completely neutralized the anticoagulant activity of $r\text{-}Hir^{1-52}$ (data not shown). $r\text{-}Hir^{1-52}$ as well as $r\text{-}Hir^{1-43}$ (data not shown), however, both failed to protect most of the lysyl residues which were shown to be shielded by the intact hirudin (fig.1A–C) [16]. In the free thrombin (fig.1A), approximately 9 lysyl residues of the B-chain were preferentially modified by S-DABITC. These lysyl residues included Lys^{B65} (fraction 1), Lys^{B77} (fraction 2), Lys^{B21} (fraction 3), Lys^{B176} (fraction 4), Lys^{B154} and Lys^{B52} (fraction 5), Lys^{B106} and Lys^{B107} (fraction 6). Upon binding of 1.2 molar of intact hirudin (fig.1B), the modification of 7 lysyl residues (Lys^{B21}, Lys^{B52}, Lys^{B65}, Lys^{B77}, Lys^{B106}, Lys^{B107} and Lys^{B154}) was

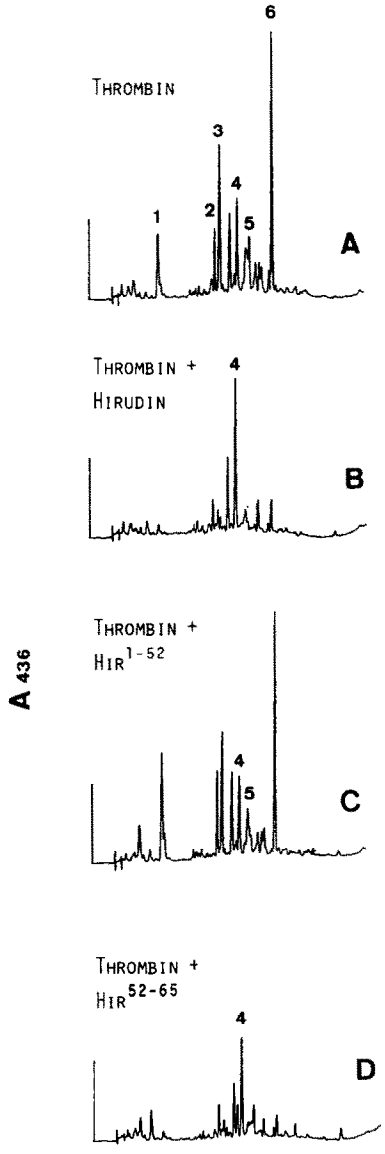

Fig.1. Tryptic peptide maps of S-DABITC-labeled thrombin B-chain. Samples were derived from modified free thrombin (A), hirudin–thrombin complex (B), $r\text{-}Hir^{1-52}$–thrombin complex (C), and Hir^{52-65}–thrombin complex (D). Conditions of tryptic digestion are described in the text. 10 μl containing 3 μg of the digested sample were injected. 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. Detector, 436 nm, 0.02 absorbance units at full scale.

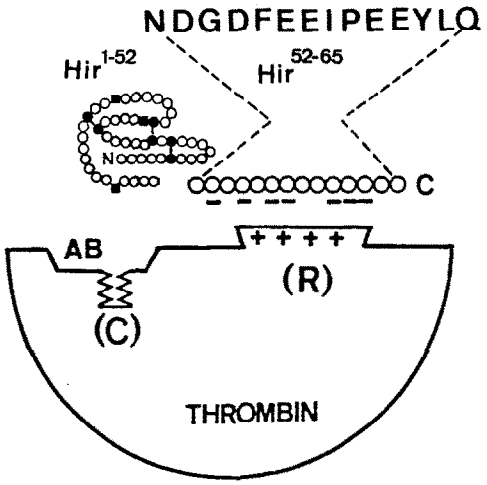

Fig.2. Diagram illustrating the corresponding binding sites of *r*-Hir$^{1-52}$ and Hir$^{52-65}$ to human α-thrombin. The N-terminal compact domain (*r*-Hir$^{1-52}$) binds to the catalytic site (C) and apolar binding site (AB). The C-terminal domain (Hir$^{52-65}$) binds to the recognition site (R). Seven lysyl residues of the thrombin B-chain were identified to be located within the recognition site. They are Lys^{B21}, Lys^{B52}, Lys^{B65}, Lys^{B77}, Lys^{B106}, Lys^{B107} and Lys^{B154}. Negative charges in the Hir$^{52-65}$ (recombinant hirudin is devoid of a sulfate group at Tyr^{63}), 3 disulfide linkages (●——●) and 3 lysine residues (■) in *r*-Hir$^{1-52}$ are also indicated.

drastically reduced [16]. The extent of shielding ranged from 70% to greater than 97%. Binding of *r*-Hir$^{1-52}$, on the other hand, yielded a pattern of labeling very similar to that of free thrombin (fig.1C). The only noticeable difference was observed with fraction 5. This fraction contained multiple peptides which were difficult to separate. Sequence analysis of fraction 5 derived from free thrombin (fig.1A) revealed two major color peptides which corresponded to specific S-DABITC modification at Lys^{B154} and Lys^{B52} (table 1). Analysis of fraction 5 derived from the *r*-Hir$^{1-52}$–thrombin complex (fig.1C) gave only one major color peptide with S-DABITC modification at Lys^{B154}. Thus *r*-Hir$^{1-52}$ binding protected only Lys^{B52} from S-DABITC modification. Since Lys^{B52} was also partially protected by Hir$^{52-65}$, it appears that the binding site of *r*-Hir$^{1-52}$ and Hir$^{52-65}$ may overlap at Lys^{B52}. With the thrombin A-chain, only the α-amino group of the N-terminal Thr was labeled with S-DABITC, and its labeling pattern was not affected by binding of either intact hirudin or *r*-Hir$^{1-52}$. In free thrombin, hirudin–thrombin complex and *r*-Hir$^{1-52}$–thrombin complex, the extent of modification at the N-terminal Thr of the thrombin A-chain was 89%, 90% and 84%, respectively.

Table 1

Amino acid sequences of S-DABITC-labeled peptides

Fraction number	Amino acid sequences
1	I-G-K*-H-S-R (63–68)
2	N-I-E-K*-I-S-M-L-E-K (74–83)
3	K*-S-P-Q-E-L-L-C-G-A-S-L-I-S-D-R (21–36)
4	G-Q-P-S-V-L-Q-V-V-N-L-P-I-V-E-R-P-V-C-K*-D-S-T-R (155–178)
5 (fig.1A)	E-T-W-T-A-N-V-G-K*-G-Q-P-S-V-L-Q-V-V-N-L (146–165)
	W-V-L-T-A-A-H-C-L-L-Y-P-P-W-D-K*-N-F-T-E (37–56)
5 (fig.1C)	E-T-W-T-A-N-V-G-K*-G-Q-P-S-V-L-Q-V-V-N-L (146–165)
6	L-K*-K*-P-V-A-F-S-D-Y-I-H-P-V-C-L-P-D-R (105–123)

Fractions correspond to those numbered in fig.1. S-DABITC-labeled lysines are denoted with (*). Numbers in parentheses indicate sequence positions in the B-chain of human thrombin

3.2. *The acidic C-terminal segment of hirudin (Hir$^{40-65}$ and Hir$^{52-65}$) binds to a protein recognition site of thrombin which is independent of the catalytic site and is comprised of basic amino acid residues*

Synthetic C-terminal peptides of hirudin with sizes ranging from 10 to 21 residues have been shown to bind thrombin, inhibit the thrombin-mediated release of fibrinopeptides and fibrin clot formation [10], as well as increase the activated partial thromboplastin time [12]. However, none of these peptides displayed the ability to inhibit the thrombin activity to hydrolyze the tripeptide substrate. These results strongly suggest that hirudin C-terminal peptides bind to a non-catalytic site of thrombin which is required for thrombin–fibrinogen interaction (recognition). The elucidation of the binding site of hirudin C-terminal peptides on thrombin is therefore of critical importance to our understanding of the mechanism of thrombin specificity at the molecular level. We have shown in fig.1B that intact hirudin protects Lys^{B21}, Lys^{B52}, Lys^{B65}, Lys^{B77}, Lys^{B106}, Lys^{B107} and Lys^{B154} of the B-chain from chemical modification. In the experiments with hirudin C-terminal peptides, we were able to demonstrate that binding of Hir$^{40-65}$ and Hir$^{52-65}$ could as well shield all these lysyl residues (fig.1D). The extent of shielding was dependent upon the molar ratio of Hir$^{52-65}$/thrombin. At a molar ratio of 20 (fig.1D), the percentages of shielding were 96% (Lys^{B65}), 70% (Lys^{B77}), 91% (Lys^{B21}), 80% (Lys^{B154}) and 94% (Lys^{B106} and Lys^{B107}), respectively. At an equal molar ratio, the protections were 62%, 40%, 55%, 42% and 56%, respectively. Based on the protein concentrations used for S-DABITC labeling, the binding affinity of Hir$^{52-65}$ was calculated to be 1.3×10^{-5} M, a figure which is consistent with that reported by Krstenansky and Mao [10].

Some of the lysyl residues shielded by Hir$^{52-65}$ have long been suspected to be located at the fibrinogen recognition site of α-thrombin. Lys^{B65} and Lys^{B77} are very close to an autolytic site which converts α- to β-thrombin [29–31], and Lys^{B154} is located precisely at the autolytic site which converts α- to γ-thrombin

[29,31]. Both β- and γ-thrombins lack clotting activity, but retain essentially intact catalytic activity and specificity against many non-fibrinogen substrates [31]. Antibodies raised against peptides containing residues 62–73 of thrombin B-chain competitively inhibit the binding of hirudin to α-thrombin [32]. Thus it is clear that either shielding or breakdown of this recognition site produces a defective form of thrombin devoid of ability to release the fibrinopeptides from fibrinogen. In addition to lysyl residues, the arginyl residues are most likely also part of the recognition site. If we assume that the recognition site of α-thrombin consists of all the lysyl residues shielded by Hir^{52-65}, plus the 3 arginines located in between Lys^{B65} and Lys^{B77} (Arg^{B68}, Arg^{B70} and Arg^{B73}), then there will be at least 10 Lys/Arg residues participating in this unique site.

Our data unequivocally demonstrate that the structural elements of hirudin which bind to the fibrinogen recognition site of thrombin are exclusively located within the C-terminal segment of the inhibitor. These results and available data [19,20] further confirm the proposal [7] that the N-terminal core domain and the C-terminal tail of hirudin bind to independent sites of thrombin (fig.2) and that the exceedingly high binding affinity of the hirudin–thrombin complex is a consequence of cooperative multiple bindings [31]. One involves the classical reactive/active site interaction in which specificity and affinity are enhanced by a hydrophobic binding pocket adjacent to the active site of thrombin [26–28,33]. The other is the specific interaction between the acidic C-terminal tail of hirudin and the basic recognition site of thrombin, as elucidated in this report.

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