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The C-terminal binding domain of hirullin P18

Antithrombin activity and comparison to hirudin peptides

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Hirullin P18 is a 61-amino acid hirudin-related protein having potent antithrombin activity. Similar to hirudin, it contains a highly acidic C-terminus, but has a significantly different sequence from any other known hirudin variant. The present study demonstrates that the C-terminal fragment acetyl-hirullin $P18_{40-61}$ possesses an antithrombin potency similar to that of acetyl-desulfatohirudin$_{45-65}$. Additionally, like the hirudin fragment analog, it inhibits fibrin-clot formation by binding to a non-catalytic site on thrombin. Sequential shortening of the hirullin P18 C-terminal fragment demonstrates the critical nature of Phe^{51}, which corresponds to the important Phe^{56} residue of hirudin. Although the sequences of hirullin $P18_{54-61}$ and hirudin$_{59-65}$ have substantial differences, the C-terminal functional domain represented by hirullin $P18_{50-61}$ appears to be comparable to hirudin$_{55-65}$ in terms of its functional role in antithrombin activity.

Hirullin P18; Hirudin; Synthetic peptide; Thrombin

1. INTRODUCTION

Hirullin P18 is a 61 amino acid protein isolated from the leech, *Hirudinaria manillensis*, having potent antithrombin activity ($K_i = 7.8$ pM) [1]. Although the protein appears to be related to hirudin, based on its sequence, it contains less homology to hirudin (variant 1) than any of the other previously reported hirudin variants [1,2]. Hirullin P18 contains considerable differences in its C-terminal region, which includes the loss of a conserved sulfated tyrosine residue (Fig. 1). These differences are significant because the C-terminus of hirudin represents a highly conserved domain which binds to thrombin at a non-catalytic site [3,4]. This domain accounts for a significant portion of hirudin's overall binding energy to thrombin. Therefore, in order to examine the importance of this variant domain and compare it to hirudin's C-terminal functional domain, C-terminal fragment peptides of hirullin P18 were synthesized and studied for their ability to inhibit thrombin mediated fibrin-clot in human plasma. The effect of these hirullin fragments on thrombin's ability to cleave the small chromogenic substrate S-2238 was also tested in order to further confirm that these peptides bind to a non-catalytic site on thrombin as do the hirudin C-terminal peptides [3,5,6].

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Abbreviations: AAA, amino acid analysis; Boc, *t*-butoxycarbonyl; Bzl, benzyl; Chx, cyclohexyl; 2-ClZ, 2-chlorobenzyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; FAB-MS, fast-atom bombardment mass spectrometry; HOBT, 1-hydroxybenzotriazole; HP18, N^{α}-acetylhirullin $P18_{40-61}$; HPLC, high performance liquid chromatography; Hyp, 4-hydroxy-L-proline; Pip, L-pipecolic acid; pNA, *p*-nitroanilide; Tos, tosyl

2. EXPERIMENTAL

2.1. *Peptide synthesis*

Peptides were synthesized on a 0.1 mmol scale by solid-phase methods starting with 0.5 mmol Boc-Gln-PAM resin (0.64 mmol/g; Applied Biosystems, Foster City, CA) using an Applied Biosystems model 430-A peptide synthesizer. As the synthesis proceeded, approximately 0.1 mmol theory of resin was removed at the appropriate points to yield the peptide fragment analogs **1-5**. All N^{α}-Boc protected amino acids were double coupled as their symmetrical anhydrides, first in dimethylformamide (DMF) then in dichloromethane (DCM), using protocols supplied by the manufacturer. Arginine and glutamine were coupled using *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT). The side chain protection was as follows: Arg(Tos), Asp(Chx), Glu(Bzl), Lys(2-ClZ), Ser(Bzl) and Thr(Bzl). The peptides (0.1 mmol, theory) were cleaved from the resin support and deprotected in liquid HF containing 5% anisole at -5°C for 40 min. After removal of the HF in vacuo the peptide was extracted from the resin with 30% aqueous acetic acid then 25% aqueous acetonitrile. Purification was performed by preparative HPLC on a Dynamax C_{18} column (41.4 × 250 mm: Rainin Instruments) using acetonitrile in 0.1% aqueous trifluoroacetic acid as the eluant. The purity and identity of the peptides was assessed by analytical PHLC, amino acid analysis (AAA) and fast-atom bombardment mass spectrometry (FAB-MS).

2.2. *Fibrin-clot inhibition*

The assay was performed in a manner similar to that previously described [3,5,6]. The assay buffer (pH 7.4) from which all solutions were made consists of 0.12 M sodium chloride, 0.01 M sodium phosphate, 0.01% sodium azide and 0.1% bovine serum albumin. Bovine thrombin (Sigma, St. Louis, MO) was titrated to a concentra-

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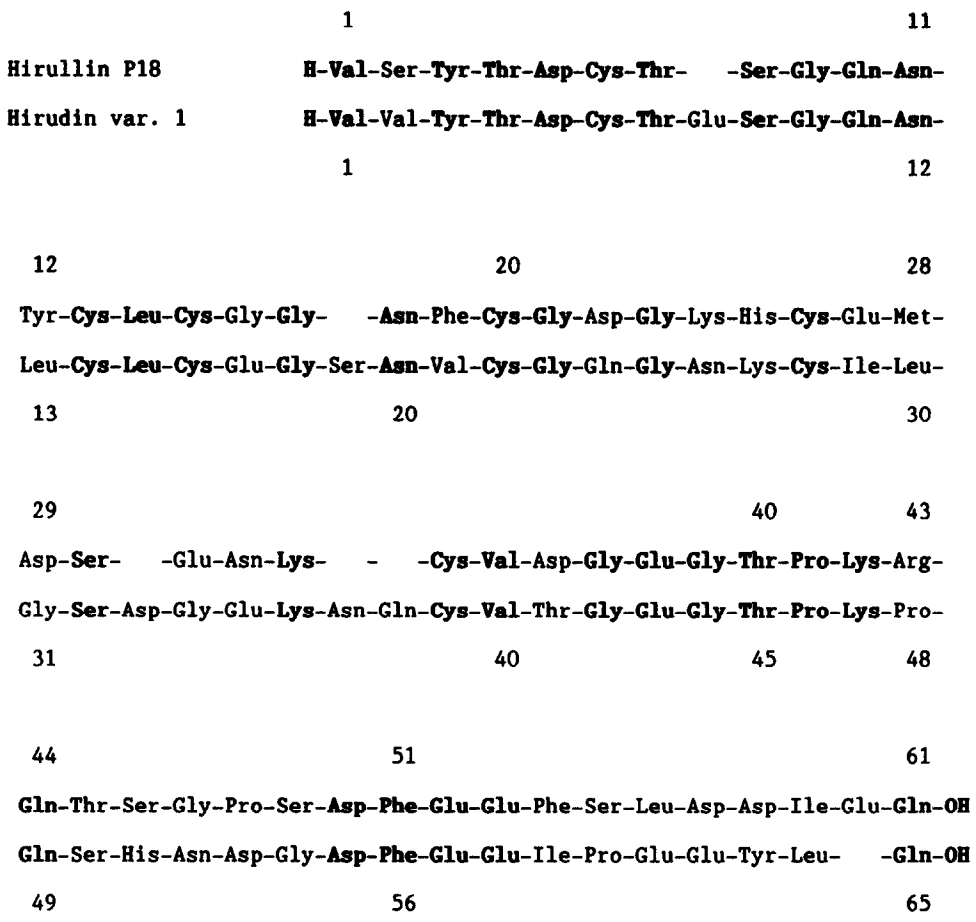

Fig. 1. Sequences of hirullin P18 and hirudin [1]. Homologous regions are highlighted by boldface print.

tion so that fibrin clot formation could be monitored by a microtiter plate reader (Bio-Tek EL 309) within 60 min at 380 nm. This thrombin solution (50 μl) was added to the wells of a 96-well microtiter plate containing 50 μl of a solution of the peptide being tested. After 1 min agitation and an additional 10 min incubation at 24°C, 100 μl of diluted human plasma (1:10) in 0.1% EDTA were added and agitated for 20 s. The turbidity of the solutions in the wells was monitored by the reader at 5 min intervals. The IC_{50} was calculated as the concentration of peptide which led to half of the turbidity observed in a control sample containing no inhibitory peptides.

2.3. *S-2238 cleavage*

The ability of human α-thrombin (the kind gift of Dr. John W. Fenton II, New York State Department of Health, Albany, New York) to cleave the tripeptide chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNA) was examined in the presence and absence of both N^{α}-acetylhirullin $P18_{40-61}$ (1) and hirudin from leeches (Sigma, St. Louis, MO). The stock solutions were as follows: thrombin, 0.0276 mg/ml in 10 mM Tris buffer pH 8; S-2238, 2 mM in water; acetylhirullin $P18_{40-61}$, 1.95 mM in 10 mM Tris buffer pH 8; hirudin, 5 ATU/ml 10 mM Tris buffer pH 8. The assays were carried out in 1 cm^3 cuvettes using a Beckman DU-7 spectrophotometer monitoring absorbance at 405 nm at 20 s intervals. The samples were prepared by adding 25 μl of the S-2238 solution to the cuvettes followed by the appropriate amounts of the hirullin and/or hirudin solutions and buffer to bring the total volume at this point to 490 μl. The samples were mixed and the spectrophotometer calibrated using these solutions. The thrombin solutin (10 μl) was then added, the cuvettes mixed and the absorbance at 405 nm was monitored for 20 min. The results of these experiments are in Figs 2 and 3.

3. RESULTS AND DISCUSSION

N^{α}-Acetyl-hirullin $P18_{41-62}$, **1** had an IC_{50} for the inhibition of thrombin-induced fibrin-clot formation of 3 μM, which is virtually the same as that of N^{α}-acetyl-desulfatohirudin$_{45-65}$, **6** [3]. Progressive truncation of the hirullin peptide, as exemplified by compounds **2–5**, resulted in a gradual loss of potency until Phe^{51} had been removed, which yielded an very weakly active peptide. This is directly comparable to what is seen with the C-terminus of hirudin, compounds **7–10**, where the removal of Phe^{56} results in the loss of antithrombin activity [5]. Phe^{56} of hirudin binds into a lipophilic pocket on the surface of thrombin and is involved in an interaction with Phe^{34} of thrombin [4]. Phe^{51} of hirullin P18 may interact with thrombin in a similar fashion particularly since it is flanked by Asp and Glu residues which are also involved in specific thrombin interactions in the hirudin-thrombin complex [4].

Like the hirudin C-terminal fragment peptides [3,5,6], hirullin $P18_{40-61}$ does not block thrombin's ability to cleave the small chromogenic substrate S-2238 demonstrating that it also blocks fibrin-clot formation by binding to a non-catalytic site on thrombin (Fig. 2).

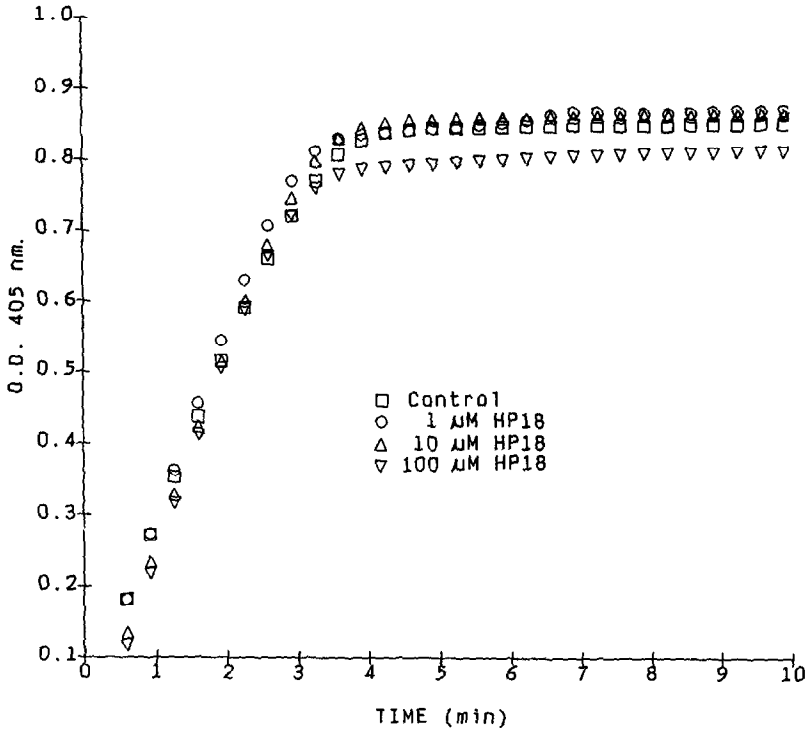

Fig. 2. The cleavage of S-2238 by human α-thrombin alone (control) and in the presence of 1, 10 and 100 μM N^{α}-acetyl-hirullin $P18_{40-61}$ (1; HP18). There is no inhibition of S-2238 cleavage at concentrations of HP18 that can inhibit fibrin-clot formation (Table I) and hirudin's ability to block S-2238 cleavage by thrombin.

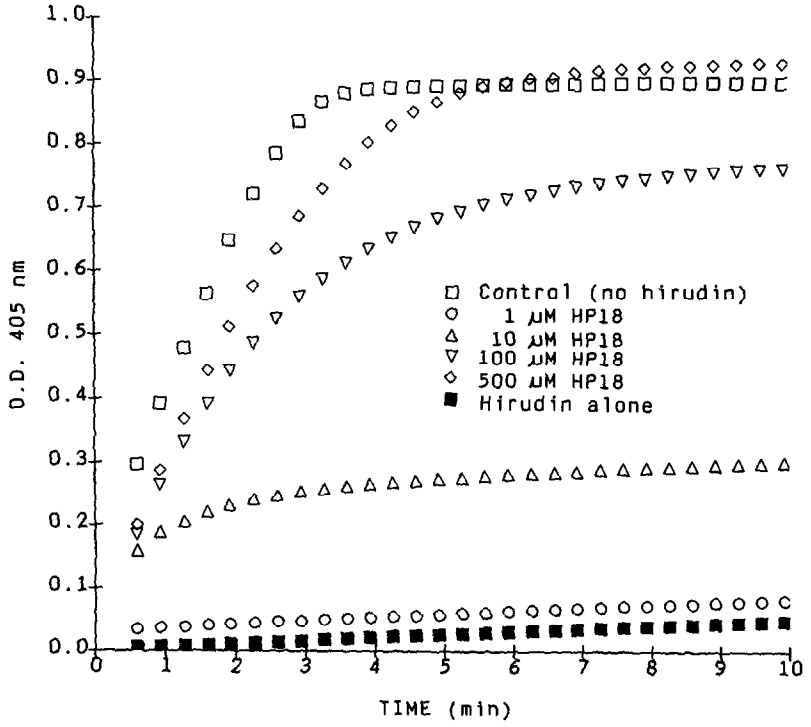

Fig. 3. The effect of 1, 10, 100 and 500 μM N^{α}-acetyl-hirullin $P18_{40-61}$ (1; HP18) on the ability of hirudin (Hirudin alone) to block the cleavage of S-2238 by human α-thrombin (control = no hirudin or HP18). HP18 can block hirudin binding to thrombin but does not block S-2238 cleavage and thereby prevents hirudin's blockade of S-2238 cleavage by thrombin.

Also the hirullin P18 peptide can block hirudin's ability to inhibit thrombin-induced S-2238 cleavage (Fig. 3) as has been demonstrated for the hirudin peptides [6]. The experiment, whose results are depicted in Fig. 3, was constructed in the following way. Human α-thrombin was added to a cuvette containing S-2238, hirudin and N^{α}-acetyl-hirullin $P18_{40-61}$. The subsequent cleavage of S-2238 by the thrombin was followed by measuring the absorbance at 405 nm. With no hirudin or hirullin P18 peptide present the S-2238 was cleaved within 4 min (open boxes). A concentration of hirudin was chosen which, in the absence of the hirullin P18 peptide, gave a nearly total blockade of the S-2238 cleavage (filled boxes). The hirullin P18 peptide was able to compete in a dose-dependent fashion with hirudin for binding to thrombin as evidenced by the level of S-2238 cleavage permitted by the hirullin P18 peptide-protected thrombin. Over time hirudin was eventually able to fully occupy the thrombin present as the thrombin-hirullin P18 peptide complex in solution dissociated, This occurs because of hirudin's extremely tight, but non-covalent, binding to thrombin which results in a virtually irreversible complex. Therefore, eventually, the hirudin-thrombin complex dominates and the S-2238 cleavage rate reflects this event. This can be seen most clearly from the 10 and 100 μM hirullin P18 peptide curves of Fig. 3, where the initial rates of S-2238 cleavage are not much different from the 'no hirudin' control sample, but over time the rate slows to that of the 'hirudin alone' control.

While it is likely that hirullin $P18_{50-61}$ and $hirudin_{55-65}$ bind to the same site on thrombin, the 54–59 region of hirullin P18, which corresponds to $hirudin_{59-64}$, should bind to thrombin in a different conformation. The ability of these hirullin P18 fragments to bind to thrombin is not readily explained by the structure activity relationships of the comparable hirudin peptides [6]. There are three notable differences between hirullin P18 and hirudin in this domain which support differing modes of interaction: (i) Pro^{60} of hirudin is replaced by Ser^{55}. In hirudin this residue plays a critical conformational role [6] and in the crystal structure of the hirudin-thrombin complex, it is in a lipophilic environment packed against Tyr^{76} and Ile^{82} [4]. Replacement by Ser would not be predicted by the structure-activity studies of hirudin C-terminal peptides to be favorable for thrombin interaction even though 4-hydroxy-proline is an acceptable Pro substitution [6]. NMR studies of the complex between hirudin C-terminal peptide fragments and thrombin demonstrate that the Pro^{60} side chain resides on the lipophilic face of an amphipathic structure in close proximity to the side chains of Ile^{59} and Tyr^{63} as evidenced by the observation of transfer NOEs [7]. With no alteration of conformation, Ser at this position results in the unfavorable placement of a hydrophilic group on the lipophilic face. (ii) There is no Tyr residue in hirullin P18 for sulfation, which although not necessary for activity, can contribute a significant amount to the potency. In view of the thrombin-bound hirudin conformation [4], the Asp^{58} residue of hirullin P18 would not be of sufficient length to interact with the basic thrombin residues which have been proposed to interact with a hirudin sulfated Tyr residue. (iii) The lipophilic side chains Ile^{59}, Pro^{60} and Leu^{64} of hirudin pack neatly in a lipophilic cleft on thrombin's surface [4]. Phe^{54}, Leu^{56} and Ile^{59} of hirullin P18 would require a significantly different backbone conformation in order to pack these lipophilic residues into the same cleft. If this region of hirullin P18 bound in the same conformation as hirudin, the Leu^{56} residue would be fully exposed to aqueous phase as is Glu^{61} of hirudin. Again the NMR studies of the complex of hirudin C-terminal fragment peptides and thrombin emphasize the prominent feature of an amphipathic structure [7]. Such an arrangement is favorable for macromolecular interaction since it allows for optimum removal of the lipophilic residues from the aqueous solution upon their interaction with the binding site. Hirullin P18's C-terminal region would require a different backbone conformation to remove Leu^{56} from the polar face yielding a fully amphipathic structure. (iv) It can also be noted that

Table I

Inhibition of thrombin-induced fibrin-clot formation by synthetic peptides

No.	Sequence	Compound	IC_{50} (μM)
1	Ac-TPKRQTSGPSDFEEFSLDDIEQ	Ac-hirullin $P18_{40-61}$	2.7
2	SDFEEFSLDDIEQ	hirullin $P18_{49-61}$	4.2
3	DFEEFSLDDIEQ	hirullin $P18_{50-61}$	9.3
4	FEEFSLDDIEQ	hirullin $P18_{51-61}$	23
5	EEFSLDDIEQ	hirullin $P18_{52-61}$	410
6	Ac-TPKPQSHNDGDFEEIPEEYLQ	Ac-$hirudin_{45-65}$	1.6
7	GDFEEIPEEYLQ	$hirudin_{54-65}$	3.7
8	DFEEIPEEYLQ	$hirudin_{55-65}$	7.1
9	FEEIPEEYLQ	$hirudin_{56-65}$	34
10	EEIPEEYLQ	$hirudin_{57-65}$	>270

Standard one-letter code is used for the amino acid sequences; Ac, acetyl.

acetylhirullin $P18_{40-61}$ does not appear to cause the slight acceleration of S-2238 cleavage by thrombin that is seen with the hirudin peptides (Fig. 2) [6]. This might suggest some subtle differences in their modes of interaction with thrombin.

In conclusion, hirullin P18 represents a unique variant of hirudin which has a significantly different C-terminal functional domain that binds thrombin with similar affinity to that of hirudin's C-terminal functional domain. Similarities in a portion of the domain, a comparable potency profile for the peptide fragments, the inability to block thrombin-induced S-2238 cleavage, and the ability to inhibit hirudin blockade of S-2238 cleavage by thrombin all suggest that despite differences, which would necessitate a different conformational mode of binding, this domain probably binds the same site on thrombin as $hirudin_{55-65}$. Physical studies are underway to further characterize the interaction of hirullin P18's C-terminal domain with thrombin. Hirullin P18 represents a new approach for the examination of thrombin's selectity for ligands and offers potential therapeutic agents based on the protein or its functional domains.

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