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Structure–function relationships of hirulog peptide interactions with thrombin

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Using hirudin as a model, a novel class of bivalent thrombin inhibitors has been designed and characterized (Maraganore et al. (1990) Biochemistry 29, 7095–7101). These peptides, designated 'hirulogs', interact with both thrombin's catalytic center and its anion-binding exosite for fibrinogen recognition. In order to investigate structure–activity relationships in hirulog peptides, a number of peptide and peptidomimetic derivatives with alterations in catalytic-site binding and anion-binding exosite binding moieties were prepared. Replacements or modifications in the catalytic site and exosite binding moieties were achieved with the consequences of maintaining or improving antithrombin activity. In addition to showing improved affinity for thrombin, some derivatives with K_i's in the sub-nanomolar range showed increased anticoagulant activities. These findings highlight the versatility of hirulog peptides in their bivalent interactions with thrombin.

Thrombin; Inhibitor; Hirulog; Hirudin

1. INTRODUCTION

Hirudin, a 65-amino acid protein originally isolated from the medicinal leech *Hirudo medicinalis*, is a potent natural inhibitor of thrombin [1,2]. Kinetic analysis of the inhibition of thrombin by hirudin [3] revealed two steps in stoichiometric complex formation: first, electrostatic interactions at the anion-binding exosite for fibrinogen recognition [4], and second, interactions at the enzyme catalytic center. X-ray crystallographic analysis of the hirudin:thrombin complex [5] showed that the high affinity interaction ($K_i = 10^{-13}$–10^{-14} M) is achieved by 212 contacts of < 4 Å across the full diameter of the thrombin molecule.

The interactions of thrombin with hirudin have served as a model for the design of new peptide inhibitors designated 'hirulogs' [6]. As with hirudin, hirulog peptides bind to thrombin in a bivalent fashion interacting with both the catalytic site and the anion-binding exosite. However, in the case of hirulog peptides, bivalent interactions are achieved by a short linker segment comprised of glycine residues whose length was optimized to bridge the interatomic distance between the two thrombin functional sites. As a result of these interactions, hirulog peptides inhibit thrombin activities with K_i values in the low nM range.

In the initial design of hirulog peptides [6], the sequence D-Phe-Pro-Arg [7,8] was employed as the active site-inhibitory moiety and a segment homologous to residues 53–64 of the HV2 hirudin variant [9] was used for binding to the anion-binding exosite. In the present report, we have examined the use of additional peptide and non-peptide substituents capable of fulfilling similar cognate, bivalent interactions with thrombin.

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2. MATERIALS AND METHODS

2.1. *Materials*

t-Boc amino acids for solid-phase peptide synthesis were from Chemical Dynamics Corp., South Plainfield, NJ. Protected amino acids from this supplier were: Boc-*O*-(2-bromo-CBZ)-L-tyrosine, Boc-L-glutamic acid γ-benzyl ester, Boc-L-aspartic acid β-benzyl ester, and Boc-*O*-benzyl-L-serine. Boc-L-leucine-*O*-divinylbenzene resin was from Applied Biosystems Inc., Foster City, CA. Boc-L-glutamine-*O*-divinylbenzene, and Boc-γ-benzyl-D-glutamate-*O*-divinylbenzene resins were purchased from Peninsula Laboratories, Belmont, CA. From Bachem Inc., Torrance, CA, were Boc-glycylglycine and Boc-cyclohexyl-L-alanine. *para*-Guanidinobenzoic acid was purchased from Fluka Chemical Corp., Ronkonkoma, NY. Spectrozyme TH (*H*-D-hexahydrotyrosyl-L-alanine-L-arginine-*p*-nitroanilide) was obtained from American Diagnostica, New York, NY. Human α-thrombin was the generous gift of Dr. John W. Fenton, II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY. HPLC solvents were from Baker. Reagents and solvents employed in peptide synthesis were from Applied Biosystems Inc., Foster City, CA.

2.2. *Peptide synthesis*

Hirulog peptides were synthesized by solid-phase procedures, cleaved and extracted from resin, and purified by reverse-phase HPLC as described previously [6]. The structure of peptides was confirmed by amino acid analysis. Hirulog-α2 was prepared by reaction of *p*-guanidinobenzoic acid (4.63 mmol) with 0.46 g of the protected, resin-bound intermediate of the heptadecapeptide (G)$_5$NGDFEEIPEEYL. The structure of hirulog-α2 was confirmed by FAB-MS showing a $M/H^+ = 1901.0$ (theoretical mass = 1899).

2.3. *Tyrosine modification of hirulog-1*

Tyrosine-19 of hirulog-1 was sulfated by a modification [10] of the

method of Nakahara et al. [11]. Sulfated hirulog-1 was purified by reverse-phase HPLC using an Applied Biosystems 150A Liquid Chromatographic system equipped with an Aquapore C_4 RP-300 column (0.46 × 100 mm). The column was equilibrated in 100% solvent A (0.1% TFA/water) and developed with a linear gradient from 0 to 50% solvent B (0.085% TFA/70% acetonitrile) over 45 min at a flow-rate of 1.0 ml/min. The effluent stream was monitored at 214 nm for absorbance and peaks were collected manually. The yield for tyrosine sulfation of hirulog-1 was ~90%.

Diiodo-hirulog-1 was prepared by standard tyrosine iodination methods using chloramine T. The peptide was purified by HPLC on an octasilyl column (Aquapore RP-300, 0.46 × 100 mm) equilibrated with 20% solvent B and developed with a linear gradient from 20 to 65% solvent B over 30 min at a flow-rate of 1.0 ml/min. The effluent stream was monitored at 214 nm. Peaks of UV absorbance were collected manually and, then, lyophilized to dryness.

2.4. *Kinetic studies*

Inhibition by hirulog peptides of thrombin amidolytic activity was performed as reported earlier [6]. Assays were performed using a 0.05 M sodium borate, pH 8.4, buffer containing 0.1 M NaCl at a temperature of 23–25°C. Reactions were initiated by addition of substrate to the cuvette containing thrombin and inhibitor pre-mixed for less than 2 min.

2.5. *Clotting assays*

The anticoagulant activity of hirulog peptides was measured semi-automatically as reported earlier [6]. Final concentrations of hirulog peptides or rec-hirudin (American Diagnostica, New York, NY) were 1.0 μg/ml. Each assay was performed in triplicate.

3. RESULTS AND DISCUSSION

In order to explore the versality of bivalent interactions between hirulog and thrombin, we evaluated the antithrombin activities of several novel derivatives. These derivatives were designed with unique domains for binding to either the active center (hirulogs-αx) or the thrombin exosite (hirulogs-βx). In addition, as tyrosine sulfation was reported previously to significantly enhance the anticoagulant activity in the C-terminal peptide hirugen [10], we examined the consequence of tyrosine sulfation and iodination in hirulog-1. Table I presents a list of peptides prepared for this study, as well as their designation and amino acid sequences.

Hirulog-1 inhibited thrombin-catalyzed hydrolysis of a tripeptidyl chromogenic substrate with K_i = 1.4 nM in a competitive fashion. As observed with C-terminal hirudin fragments, sulfation of tyrosine-19 (corresponding to Tyr-63 in hirudin) led to a significant increase in activity (Table I). Sulfated hirulog-1 (S-hirulog-1) inhibited the thrombin-catalyzed hydrolysis of the tripeptidyl substrate with 3- to 4-fold (K_i = 0.4 nM) greater effectiveness over the unsulfated derivative. The gain in free energy of binding derived from this single modification was 3.1 kJ/mol (Fig. 1), which is somewhat less than the ~6 kJ/mol change in free energy of binding resulting from tyrosine sulfation in hirudin [12]. Nevertheless, this finding showed that the activity of hirulog peptides can be enhanced by increasing their affinity for anion-binding exosite interactions. Together with increased thrombin affinity, sulfated hirulog-1 showed a modestly increased anticoagulant activity in clotting time assays (Fig. 2) as compared to hirulog-1.

As found recently in the X-ray crystallographic structure of the hirugen-thrombin complex [13], tyrosine sulfation increases the binding affinity for complex formation via participation of the sulfate group in an elaborate hydrogen-bonding network including the phenolic OH of Tyr-76 and the backbone amide of Ile-82. Diiodination of tyrosine reduces the pK for its hydroxyl group, allowing the derivatized amino acid to participate as a hydrogen-bond acceptor in the thrombin complex at neutral pH. To determine if Tyr iodination could improve the potency of hirulog peptides in a manner similar to Tyr sulfation, the diiodo-Tyr-19 derivative of hirulog-1 was prepared. In chromogenic substrate assays, diiodo-hirulog-1 showed a K_i = 0.3 nM, i.e. a similar increase in antithrombin activity as observed with tyrosine sulfation.

Interactions at the anion-binding exosite were also modified by employing divergent sequences from two hirudin-related isoforms, hirullin [14] and hirudin PA [15], as well as a higher affinity fragment designed by Krstenansky and co-workers [16] called MDL-28,050. As Table I shows, these exosite recognition fragments could be integrated within the framework of a hirulog peptide to maintain inhibitory activities toward thrombin hydrolysis of a tripeptidyl substrate. Of these

Table I

Designation and amino acid sequences of hirulog peptides, and kinetic constants for human α-thrombin inhibition

Peptide	Sequence:	K_i (nM)
Hirulog-1	(D-Phe)ProArgPro(Gly)$_4$AsnGlyAspPheGluGluIleProGluGluTyrLeu	1.4
S-Hirulog-1	(D-Phe)ProArgPro(Gly)$_4$AsnGlyAspPheGluGluIleProGluGlu(SO_3-Tyr)Leu	0.4
dI-Hirulog-1	(D-Phe)ProArgPro(Gly)$_4$AsnGlyAspPheGluGluIleProGluGlu(I_2-Tyr)Leu	0.3
Hirulog-α1	acGlyAspPheLeuAlaGluGlyGlyGlyValArgPro(Gly)$_4$AsnGlyAspPheGluGluIleProGluGluTyrLeu	3.2
Hirulog-α2	GBA(Gly)$_5$AsnGlyAspPheGluGluIleProGluGluTyrLeu	7000
Hirulog-β1	(D-Phe)ProArgPro(Gly)$_4$AspGlyAspPheGluGluSerLeuAspAspIleAspGln	1.1
Hirulog-β2	(D-Phe)ProArgPro(Gly)$_4$AspGlyAspPheGluProIleProGluGluTyrLeuGln	5.4
Hirulog-β3	(D-Phe)ProArgPro(Gly)$_4$AspGlyAspTyrGluProIleProGluGlu-Cha(D-Glu)	0.3

K_i values were determined as reported in section 2. Assays were performed at 23–25°C with a 0.05 M sodium borate, pH 8.4, buffer containing 0.1 M NaCl.

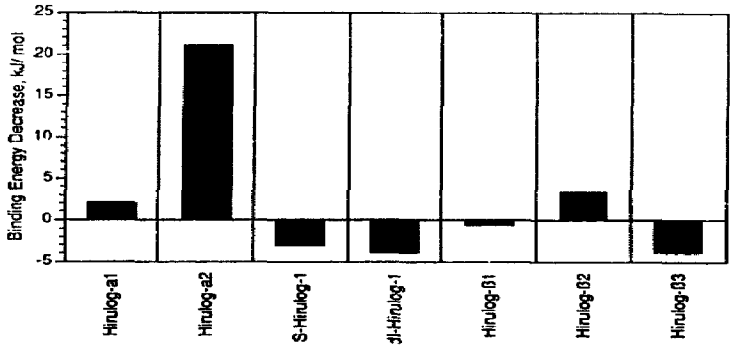


Fig. 1. Binding energy decrease for hirulog peptides as compared with hirulog-1. The inhibitory activities of hirulog peptides toward thrombin-catalyzed hydrolysis of chromogenic substrates were measured as described in section 2 and previously [6]. Free energy of binding values were calculated using the determined K_i values for thrombin in amidolytic assays.

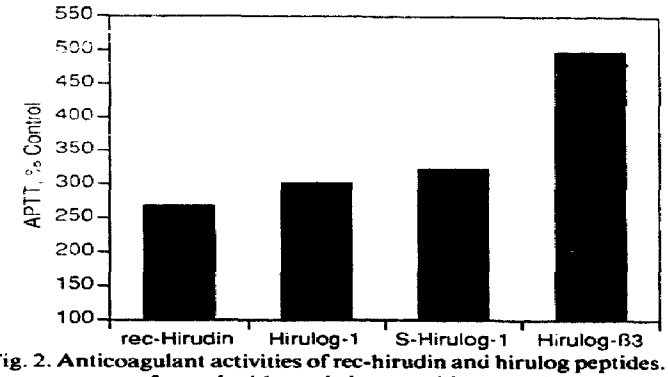


Fig. 2. Anticoagulant activities of rec-hirudin and hirulog peptides. All assays were performed with pooled, normal human plasma with rec-hirudin or hirulog peptide concentrations of 1.0 μg/ml. Data are expressed as mean (SEM < 10%).

new hirulog derivatives, hirulog-β3 showed the greatest improvement in activity (K_i = 0.3 nM), a finding consistent with its enhanced exosite affinity. As with tyrosine sulfation in hirulog-1, the increased activity of hirulog-β3 was also observed in coagulation assays (Fig. 2). However, while sulfated hirulog-1 and hirulog-β3 showed similar K_i's for thrombin in amidolytic assays, the latter was unexpectantly more effective in clotting time assays. At 1.0 μg/ml, hirulog-β3 prolonged the APTT to 498% control, an effect which exceeded significantly that for hirulog-1, sulfated hirulog-1, and rec-hirudin. A discontinuity between antithrombin and anticoagulant activities had been observed previously for other hirulog derivatives [17].

Hirulog peptides described previously [6] employed the sequence D-Phe-Pro-Arg-Pro as the active site-binding moiety. In the present study, we found that the function of this moiety can be achieved with a divergent peptide sequence or, in fact, a non-peptidic substitution. The fibrinopeptide A (FPA) fragment arises from thrombin's cleavage of the Aα-chain of fibrinogen, and is known to bind near or at the active center of thrombin [18]. A segment of FPA corresponding to residues 6–16 was used to replace the sequence D-Phe-Pro-Arg in the synthesis of hirulog-α1. As with hirulog-1, the α1 derivative inhibited thrombin-catalyzed hydrolysis of a chromogenic substrate with a K_i in the low nM range (Table I). The similarities in thrombin inhibition by hirulog-1 and -α1 would indicate that the D-Phe-Pro-Arg tripeptide and the FPA undecapeptide themselves bind to the active center with comparable affinity and, perhaps, similar loci.

Since highly divergent sequences could be used as active site-binding moieties in hirulog peptides, the possibility of replacing this segment with a non-peptidic substituent was considered in design of hirulog-α2. The peptidomimetic function employed was *p*-guanidinobenzoic acid (GBA), based on the observations that benzamidine and benzamidine-related compounds can inhibit thrombin amidolytic activity by binding to the S_1 or 'primary' specificity pocket [19]. Further, by analogy with the structure of the thrombin complex with D-Phe-Pro-Arg-CH_2Cl [20], the GBA functionality would be expected to occupy the position of the P_1 (nomenclature of Schechter and Berger [21]) arginine side chain in the S_1 pocket. Using carbodiimide chemistry, GBA was coupled in essentially stoichiometric yield to the resin-bound, protected intermediate of a heptadecapeptide (Gly_5-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu). The resulting GBA-peptide inhibited thrombin hydrolysis of a tripeptidyl substrate with K_i = 7 μM. In contrast, the unmodified heptadecapeptide did not inhibit thrombin amidolytic activity at concentrations exceeding 50 μM [6]. Thus, hirulog-α2 maintains bivalent interactions with thrombin as with other hirulog peptides, but with active site interactions limited to the S_1 pocket. The affinity of hirulog-α2 for active site inhibition differed substantially from that of hirulog-1, where a loss in binding energy of 21.1 kJ/mol was observed (Fig. 1). This reduced affinity can be explained, if not anticipated, by the absence of interactions at P_2 and P_3 sites in the active center of thrombin.

The present findings highlight the versatility of hirulog peptide interactions with thrombin. A number of divergent sequences can be employed for binding to the anion-binding exosite, including those derived from hirudin isoforms. Interactions at the active site can be achieved using both peptidic and non-peptidic functionalities. By increasing the affinity for anion-binding exosite interactions, the antirthrombin and anticoagulant activities of hirulog can be improved. These studies show that a wide range of peptide and non-peptide moieties can fulfill bivalent thrombin interactions in hirulog peptides.

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