THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 35, Issue of December 15, pp. 21698-21703, 1990
Printed in U.S.A.

Bifunctional Thrombin Inhibitors Based on the Sequence of Hirudin$^{45-65}$*

(Received for publication, May 7, 1990)

John DiMaio‡, Bernard Gibbs, Debra Munn, Jean Lefebvre, Feng Ni, and Yasuo Konishi

From the Protein Engineering Section, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada

The interaction of α-thrombin with the hirudin (HV1) fragment N^{α}-acetyl desulfo hirudin$^{45-65}$ (P51) was investigated. Kinetic analysis revealed that P51 inhibits the proteolysis of a tripeptidyl substrate with K_i = 0.72 ± 0.13 and 0.11 ± 0.03 μM for bovine and human α-thrombins, respectively. The inhibition was partially competitive, affecting substrate binding to the enzyme-inhibitor complex by a factor α = 2 (bovine) and α = 4 (human) characteristic of hyperbolic inhibitors. P51 also inhibited thrombin-induced fibrin clot formation with IC_{50} values of 0.94 ± 0.20 and 0.058 ± 0.006 μM for bovine and human α-thrombins, respectively. The enhanced antithrombin activity for human thrombin could be attributed to species variations in the putative auxiliary "anion" exosite since N^{α}-acetyl desulfo hirudin$^{55-65}$ displayed the same rank order of potency shift in a clotting assay without inhibiting the amidolytic activity of either enzyme. From these observations, a potent thrombin inhibitor was designed having modified residues corresponding to the P_1 and P_3 recognition sites. N^{α}-Acetyl[D-Phe^{45},Arg^{47}] hirudin$^{45-65}$ (P53) emerged as a pure competitive inhibitor with a K_i = 2.8 ± 0.9 nM and IC_{50} = 4.0 ± 0.8 nM (human α-thrombin) and is designated as a "bifunctional" inhibitor. Its enhanced potency could be explained by a cooperative intramolecular interaction between the COOH-terminal domain of the inhibitor and the auxiliary exosite of thrombin on the one hand, and the modified NH_2-terminal residues with the catalytic site on the other.

Thrombin is an important serine proteinase component of the blood coagulation cascade. Besides initiating blood clotting by cleaving fibrinogen, thrombin activates other hemocoagulant enzymes including factors V, VIII, and XIII and the anticoagulant enzyme protein C (Fenton, 1981). Thrombin is also a potent platelet activator (Fenton *et al.*, 1986) and impairs thrombolysis mediated by tissue plasminogen activator *in vivo* (Fitzgerald and FitzGerald, 1989). Given the diverse functions of this enzyme, its inhibition by potent and specific compounds could provide an invaluable addition to thrombolytic therapy. The most potent natural inhibitor of thrombin known is hirudin, a family of iso-proteins isolated from the glandular secretions of the leech *Hirudo medicinalis* (Markwardt, 1970; Dodt *et al.*, 1986; Trippier, 1988). Hirudin forms a non-covalent stoichiometric complex with thrombin having a dissociation constant in the order of 20 pM to 20 fM for bovine and human thrombins, respectively (Dodt *et al.*, 1990; Stone *et al.*, 1987). It has been shown previously that the specific and tight binding properties of native or recombinant (desulfo) hirudin stem from the cumulative interactions of the inhibitor with multiple but remote regions of the thrombin surface (Noe *et al.*, 1988; Wallace *et al.*, 1989). One of these is the auxillary binding "exosite" domain which may be involved in the specific interaction between thrombin and its endogenous substrate fibrinogen (Wallace *et al.*, 1989), but may also serve to accommodate the COOH-terminal tail of hirudin (Fenton, 1981; Braun *et al.*, 1988; Chang, 1983). Accordingly, the COOH-terminal sequence comprising the residues of hirudin$^{45-65}$ and shorter fragments thereof, incorporate a binding domain that has been reported to inhibit clotting activity without affecting the amidolytic properties of thrombin (Dodt *et al.*, 1990; Owen *et al.*, 1988). The region of hirudin responsible for binding to the catalytic site or the apolar fibrinopeptide groove (Sonder *et al.*, 1984) remains equivocal. Site-directed mutagenesis of basic residues, including Lys^{47} which is a constituent residue within the homologous prothrombin cleavage site, did not alter the inhibitory dissociation constant significantly among the variants (Dodt *et al.*, 1988; Braun *et al.*, 1988). This indicates that the high affinity of hirudin is not necessarily governed by the insertion of a basic amino acid side chain in the S_1 specificity pocket. On the other hand, neutralization of the terminal N^{α}-amino group in hirudin by acetylation or NH_2-terminal extension has been shown to have a deleterious effect, reflected by a large increase in K_i. This result is indicative of a determinant role of NH_2-terminal residues in binding to thrombin (Wallace *et al.*, 1989), although not necessarily to the active site.

The design of small molecular weight inhibitors that utilize accessory binding loci remote from or in conjunction with the catalytic center, similar to the way fibrinogen or hirudin bind to thrombin, constitutes a formidable challenge in protein chemistry. Conceivably, such a "multifunctional" inhibitor could integrate two or more important recognitive elements that are separated by a suitable distance favoring multiple simultaneous interactions which could manifest enhanced potency and specificity (Lipowski, 1987). We envisaged that a bifunctional inhibitor of α-thrombin could be derived based on the sequence homology with desulfo hirudin$^{45-65}$ (HV1,[1] Fig. 1) since this hirudin fragment would be expected to span the distance separating the catalytic site and the fibrinogen exosite in a fully extended conformation. However, it has been reported that although desulfo N^{α}-acetyl hirudin$^{45-65}$ inhibits fibrin clot formation, it does not perturb thrombin amidase activity (Krstenansky *et al.*, 1987; Mao *et al.*, 1988; Maraga-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: HV1, desulfo hirudin variant 1; HPLC, high performance liquid chromatography; Tos, tosyl.

nore *et al.*, 1989). We have reinvestigated this observation and found that desulfo hirudin$^{45-65}$ fragment inhibits not only fibrin clot formation but also the amidolytic activity of both bovine and human α-thrombins toward tripeptidyl substrates. Although the inhibition was weak and partially competitive, an appropriate modification of the NH_2-terminal residues afforded a potent and pure competitive inhibitor. The prototype [D-Phe^{45},Arg^{47}]hirudin$^{45-65}$ behaves as a bifunctional thrombin inhibitor whose enhanced antithrombin properties result from the dual cooperativity of two remote recognition sites that are separated by a 7-amino acid residue spacer.

EXPERIMENTAL PROCEDURES

Materials—The chromogenic and fluorogenic substrates Tos[1]-Gly-Pro-Arg-*p*NA and Tos-Gly-Pro-Arg-AMC were purchased from Boehringer Manheim and Sigma, respectively. Fibrinogen and bovine or human α-thrombins were from Sigma and purity was confirmed by sodium dodecyl sulfate-gel electrophoresis. N^{α}-tert-butyloxycarbonyl protected amino acids for peptide synthesis were obtained from Applied Biosystems. Solvents were glass-distilled and of highest purity.

Peptide Synthesis—The desulfo N^{α}-acetyl hirudin$^{45-65}$ and the synthetic analogs reported in this paper were synthesized by the solid-phase method on an Applied Biosystems 430A Peptide Synthesizer. tert-Butyloxycarbonal-Gln phenylacetamidomethyl resin (Applied Biosystems; 0.64 mmol/g) was used as the solid-phase support. Amino acid coupling was mediated by dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole and deprotection was carried out with 50% trifluoroacetic acid in methylene chloride for 3 min followed by an additional 20-min cycle. Side-chain protecting groups were as follows: Asp(cyclohexyl), Glu(benzyl), His(benzyloxylmethyl), Arg(Tos), Tyr(N^{α}-benzyloxycarbonyl-*p*-bromobenzyl, Ser(benzyl). The fully protected peptide resin was treated with liquid hydrogen fluoride containing anisole and dimethyl sulfide (10% by volume) at −5 °C for 60 min. Excess HF was removed under a stream of nitrogen, and the residual solid was extracted with ether and filtered. The resin was extracted three times with glacial acetic acid and water followed by lyophilization. All the peptides had a desulfated Tyr^{63} residue.

Purification and Analytical Techniques—The lyophilized crude peptides were purified to homogeneity by reverse-phase chromatography on a Vydac octadecyl silica (15 Å) glass column (1.5 × 30 cm, 40 psi) using a linear gradient of a solvent system consisting of (*a*) 500 ml 0.1% trifluoroacetic acid/H_2O and (*b*) 1 liter of 60% acetonitrile/H_2O containing 0.1% trifluoroacetic acid. The collected fractions were analyzed by reverse-phase HPLC on a Varian LC using a Vydac C_{18} analytical column and 215-nm detection. Fractions corresponding to 98% purity or higher were pooled and lyophilized. Peptide content was determined by amino acid analysis on a Beckman model 6300 amino acid analyzer. Samples were dried in a Waters Pico-Tag Work Station. Constant boiling HCl (200 μl) containing 1% phenol was added to the vial and alternatively purged (with dry nitrogen) and evacuated after three purges. Finally, the vial containing the sample was heated at 150 °C for 1 h under vacuum. Mass spectral analyses were carried out on a SCIEX API III spectrometer equipped with an ionspray inlet source.

Amidolytic Assay of Thrombin Activity—Thrombin-catalyzed hydrolysis of Tos-Gly-Pro-Arg-*p*NA was monitored at 405 nm on a Varian-Cary 2000 double-beam spectrophotometer using substrate concentrations of 2.5, 3.5, 5, and 10 μM in a final volume of 1 ml. The hydrolytic reactions were performed at 25 °C in 0.1 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% PEG 6000. The reactions were initiated by addition of the substrate dissolved in 0.1 M Tris-HCl buffer, pH 7.8, to a preincubated solution of enzyme (0.4 nM) and variable concentrations of inhibitor dissolved in the same buffer. Initial velocities were recorded at several inhibitor concentrations and kinetic parameters were determined by fitting the data to a general equation describing enzyme inhibition (Segel, 1975). The data were analyzed using the non-linear regression program RNLIN in the IMSL library (IMSL, 1987) on a microVAX 3500 computer. Dixon and Lineweaver-Burk plots were constructed to qualitatively assign the type of inhibition exhibited by each peptide. Fluorogenic assays were conducted using the same conditions and instrument as above operating in the fluorescence mode in the ratio (λ_{ex} = 383 nm, λ_{em} = 455 nm). Fluorescence intensities were calibrated with 7-amino-4-methyl coumarin solution of known concentration.

Fibrinogen Clotting Assay—Inhibition of fibrinogen clot formation was measured spectrophotometrically at 405 nm on a Varian DMS 90 spectrometer at 37 °C. 300 μl of 0.1% fibrinogen (Sigma) in 0.1 M Tris-HCl, pH 7.8, containing 0.1 M NaCl, 0.1% PEG 6000 and variable concentrations of inhibitor in the same buffer were mixed in polystyrene cuvettes and the reaction was initiated by the addition of the enzyme (human or bovine α-thrombin 0.4 nM) in a total volume of 1 ml. The time from mixing to inflection due to clot formation was recorded for various inhibitor concentrations and IC_{50} values were calculated by log probit analysis. The concentration of the inhibitors in the assays was based on the peptide content.

45 50
Ac-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-Asp-Gly-

55 60
Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-

65
Gln-OH

FIG. 1. **Amino acid sequence of the N^{α}-acetyl desulfo hirudin$^{45-65}$ (HV1) fragment.**

RESULTS

All the peptides used in this study had correct amino acid composition, and mass spectra showed the following pseudomolecular ions in agreement with the calculated molecular weights: N^{α}-acetyl hirudin$^{45-65}$ (P51, 2516.4); N^{α}-acetyl-[D-Phe^{45},Arg^{47}, D-Pro^{48}]hirudin$^{45-65}$ (P52, 2590.4); N^{α}-acetyl-[D-Phe^{45},Arg^{47}]hirudin$^{45-65}$ (P53, 2590.4); N^{α}-acetyl-[Thr^{45}, Arg^{47},D-Pro^{48}]hirudin$^{45-65}$ (P54, 2544.4); N^{α}-acetyl-[D-Phe^{45},Ala^{47}]hirudin$^{45-65}$ (P73, 2549.4). Native desulfo hirudin$^{45-65}$ (P51, Fig. 1) inhibited fibrinogen clotting by both bovine and human α-thrombin in a dose-dependent manner as shown in Fig. 2. The IC_{50} value of 940 ± 200 nM for bovine α-thrombin (Table I) is in good agreement with the reported value for plasma fibrin clot formation for the same fragment (Krstenansky *et al.*, 1987) and three times lower than

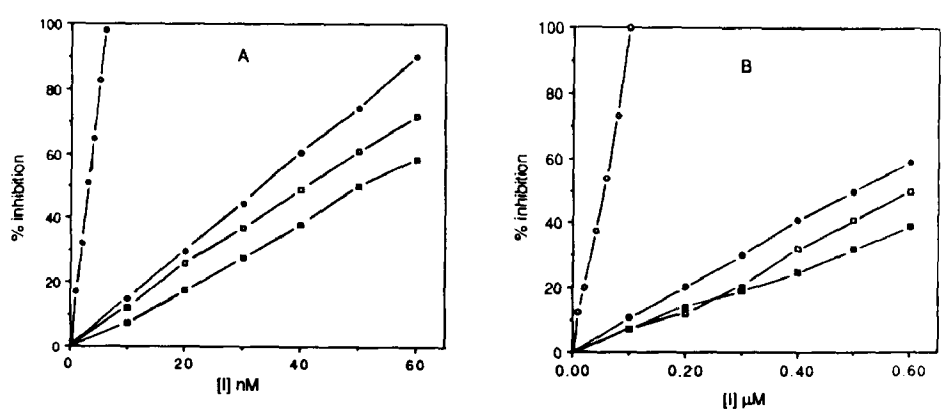


FIG. 2. **Inhibition of human (*A*) and bovine (*B*) α-thrombin-mediated fibrinogen clotting by hirudin$^{45-65}$ (P51) (■), [Thr^{45}, Arg^{47},D-Pro^{48}]hirudin$^{45-65}$ (P54) (□), [D-Phe^{45}, Arg^{47}D-Pro^{48}] hirudin$^{45-65}$ (P52) (●), and [D-Phe^{45},Arg^{47}]hirudin$^{45-65}$ (P53) (○).**

hirudin$^{55-65}$ which has been assigned as the minimum core required for anticoagulant activity (Krstenansky *et al.*, 1988). As shown in Table I, the same peptides were consistently more potent against human α-thrombin than bovine α-thrombin, in agreement with the results obtained with hirudin$^{53-64}$ (Maraganore *et al.*, 1989).

Since one of our objectives was to design a bifunctional inhibitor capable of binding simultaneously to two independent sites, we investigated whether desulfo hirudin$^{45-65}$ could interact with the catalytic center. Fig. 3 illustrates that, contrary to previous reports (Krtstenansky *et al.*, 1987; Mao *et al.*, 1988), P51 does inhibit tripeptidyl substrate hydrolysis mediated by human α-thrombin, at Tos-Gly-Pro-Arg-AMC/*p*NA concentrations ranging between 0.5 and 3 times K_m. K_m was determined experimentally and found to be 6 μM for bovine and 4 μM for human α-thrombin under the present assay conditions. The resulting Dixon plot showed saturating reciprocal velocity with increasing inhibitor concentration (Fig. 3*A*) up to a limiting value beyond which no further inhibition was observed. Taken together, the Dixon and Lineweaver-Burk (Fig. 3*B*) plots for P51 are characteristic of partial competitive inhibition with a mechanism best represented by the enzymatic reaction shown in Scheme 1 where *E*, *S*, and *I* represent enzyme, substrate, and inhibitor, respectively (Segel, 1975). K_s, K_i αK_s and βK_i are the equilibrium constants while k_p and βk_p are the kinetic constants of the hydrolytic reaction. This general enzymatic reaction predicts that if the binding sites for *S* and *I* on the enzyme are not mutually exclusive, then bound substrate or inhibitor may reduce formation of the ternary complex by a factor α (where $1 \leq \alpha \leq \infty$) (Segel, 1975). Evaluation of the kinetic parameters for P51 afforded values of $\alpha = 4$ and $K_i = 0.11 \pm 0.03$ μM (Table II) for human α-thrombin, which satisfy the hyperbolic competitive mechanism exemplified by Scheme 1. The inhibition of Tos-Gly-Pro-Arg-AMC hydrolysis was less effective against the bovine enzyme ($K_i = 0.72 \pm 0.13$ μM, $\alpha = 2$), in agreement with the results of the fibrinogen clotting assay (Table I).

TABLE I

IC_{50} values for the inhibition of fibrin clot formation by modified COOH-terminal fragments of hirudin. Assays were performed as described under "Experimental Procedures"

	Analog[a]	IC_{50} nM[b]	
		Bovine	Human
P24	hirudin$^{55-65}$	3300 ± 500	350 ± 30
P51	hirudin$^{45-65}$	940 ± 190	58 ± 6
P53	[D-Phe^{45}, Arg^{47}]hirudin$^{45-65}$	70 ± 20	4.1 ± 0.8
P73	[D-Phe^{45}, Ala^{47}]hirudin$^{45-65}$	650 ± 150	190 ± 50
P52	[D-Phe^{45}, Arg^{47}, D-Pro^{48}]hirudin$^{45-65}$	340 ± 200	45 ± 7
P54	[Thr^{45}, Arg^{47}, D-Pro^{48}]hirudin$^{45-65}$	640 ± 250	52 ± 5

[a] All peptides are desulfated and N^{α}-acetylated.
[b] Mean of three determinations ± S.E.

Active site-directed thrombin inhibitors have been widely investigated (Mattson *et al.*, 1982; Okamoto *et al.*, 1981; Bajusz *et al.*, 1978; Stuber *et al.*, 1988). Tripeptides of the type *X*-Pro-Arg-*Z* which are based on the sequence of the NH_2-terminal fibrinogen α-chain, have been shown to inhibit thrombin activity. For maximum inhibition, *X* requires a hydrophobic residue such as D-Phe corresponding to the S_3 catalytic subsite while *Z* is a non-hydrolyzable function such as an aldehyde, nitrile, or bulky isopropyl ester. To determine if the inhibition of α-thrombin by P51 was due in part to the interaction of the NH_2-terminal Thr-Pro-Lys-Pro sequence with the catalytic binding site, we introduced the analogous D-Phe-Pro-Arg-Pro substitution into the NH_2-terminal end of the native fragment. The resulting analog [D-Phe^{45}, Arg^{47}] hirudin$^{45-65}$ (P53) displayed potent inhibition of the amidase activity of both bovine ($K_i = 31 \pm 6$ nM) and human ($K_i = 2.8 \pm 0.94$ nM) α-thrombins (Table II). In contrast to native desulfo hirudin$^{45-65}$, P53 was a pure competitive inhibitor (Fig. 4) whose increased affinity for the enzyme was also reflected by enhanced anticoagulant activity (Table I). The competitive inhibition of P53 combined with a 20–40-fold decrease in binding constant compared to P51 indicates that Arg^{47} and D-Phe^{45} are better suited to interact with the S_1 and S_3 α-thrombin subsites of either species.

To investigate the role of the Arg^{47} side chain in P53, this residue was replaced by alanine. The resulting analog [D-Phe^{45},Ala^{47}]hirudin$^{45-47}$ (P73) showed almost the same level of anticoagulant activity as P51, indicative of a significant binding contribution by the Arg^{47} residue present in P53. However, while P73 effectively inhibited the amidolytic activity of the human enzyme ($K_i = 0.041 \pm 0.015$ μM, Table II), bovine α-thrombin was unaffected by this analog.

The Arg^{47}-Pro^{48} bond in P53 is labile and was slowly cleaved by α-thrombin over a period of 24 h as shown in Fig. 5, whereas P51 was proteolytically stable. This result illustrates further that Arg^{47}-Pro^{48} is the scissile bond and the proline residue at P_1' does not confer complete enzymatic resistance. The effect of stereochemistry in Pro^{48} was investigated in an attempt to overcome the proteolytic susceptibility of P53. Table I demonstrates that stereochemical inversion of Pro^{48} resulted in reduced anticoagulant activity (P52 and P54). Furthermore, although [D-Phe^{45},Arg^{47},D-Pro^{48}]hirudin$^{45-65}$

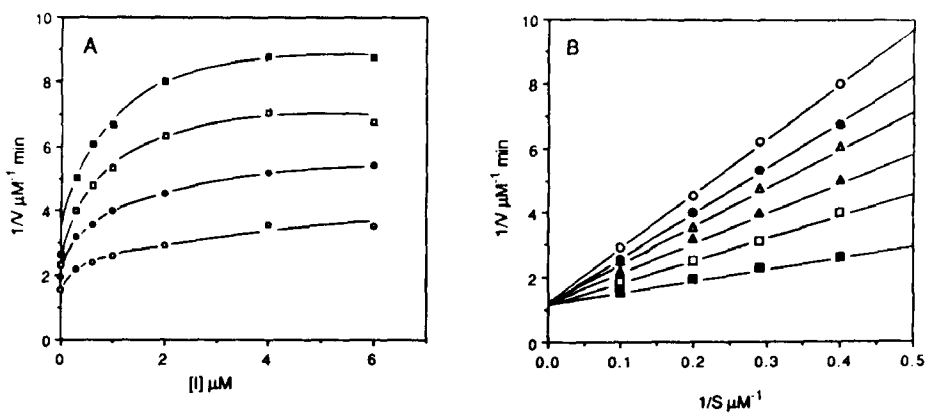
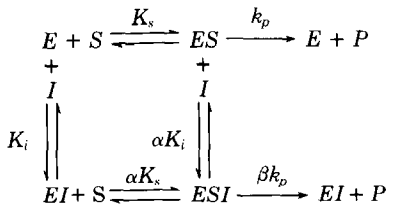


FIG. 3. **Kinetics of the inhibition of human α-thrombin-mediated hydrolysis of Tos-Gly-Pro-Arg-AMC by *N*-acetyl hirudin$^{45-65}$ (P51).** (*A*) Dixon plot; assays were performed as described under "Experimental Procedures" in the presence of 2.5 μM (■), 3.5 μM (□), 5 μM (●), 10 μM (○) substrate. *B*, Lineweaver-Burk plot; inhibitor concentrations were as follows: 0 μM (■), 0.1 μM (□), 0.3 μM (▲), 0.6 μM (△), 1 μM (●) and 2 μM (○).



SCHEME 1

TABLE II

K_i values for the bovine and human α-thrombin-mediated hydrolysis of Tos-Gly-Pro-Arg-AMC by modified hirudin COOH-terminal fragments

α is a factor by which K_s and K_i are modified due to ternary complex formation. Ic_{50} is defined as the concentration of peptide required to cause a 50% delay in clotting time.

	Analog	Bovine		Human	
		K_i μM[b]	α	K_i μM[b]	α
P24	hirudin$^{55-65}$	ni[a]		ni[a]	
P51	hirudin$^{45-65}$	0.72 ± 0.13	2	0.11 ± 0.03	4
P53	[D-Phe^{45}, Arg^{47}]hirudin$^{45-65}$	0.031 ± 0.006	∞	0.0028 ± 0.0009	∞
P73	[D-Phe^{45}, Ala^{47}]hirudin$^{45-65}$	ni[a]		0.041 ± 0.015	3
P52	[D-Phe^{45}, Arg^{47}, D-Pro^{48}]hirudin$^{45-65}$	0.47 ± 0.18	6	0.046 ± 0.003	6
P54	[Thr^{45}, Arg^{47}, D-Pro^{48}]hirudin$^{45-65}$	0.32 ± 0.10	8	0.072 ± 0.032	8

[a] ni, not inhibited.
[b] Mean of three determinations ± S.E.

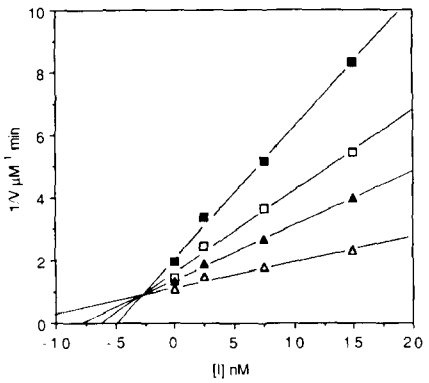


FIG. 4. **Effect of desulfo [D-Phe^{45},Arg^{47}]hirudin$^{45-65}$ (P53) on the amidolytic activity of human α-thrombin.** Assays were performed as described under "Experimental Procedures" in the presence of Tos-Gly-Pro-Arg-AMC, 2.5 μM (■), 3.5 μM (□), 5 μM (▲), 10 μM (△), and variable concentrations of inhibitor.

(P52) was stable to proteolysis, its inhibitory dissociation constant increased over 10-fold, and the inhibition reverted to a hyperbolic competitive type.

DISCUSSION

The high antithrombin activity of hirudin is mediated by cumulative surface interactions with the enzyme which result in tight binding inhibition with an equilibrium dissociation constant of the order of 10^{-10} to 10^{-14} M and correspondingly high anticoagulant activity (Dodt *et al.*, 1986; Stone *et al.*, 1987; Dodt *et al.*, 1990). COOH-terminal hirudin fragments comprising residues 45–65 (Fig. 1) have been reported to delay fibrinogen clotting time but not to inhibit the amidolytic activity of thrombin (Krstenansky *et al.*, 1987; Mao *et al.*, 1988; Maraganore *et al.*, 1989). In this respect, these truncated analogs retain the necessary recognition elements compatible

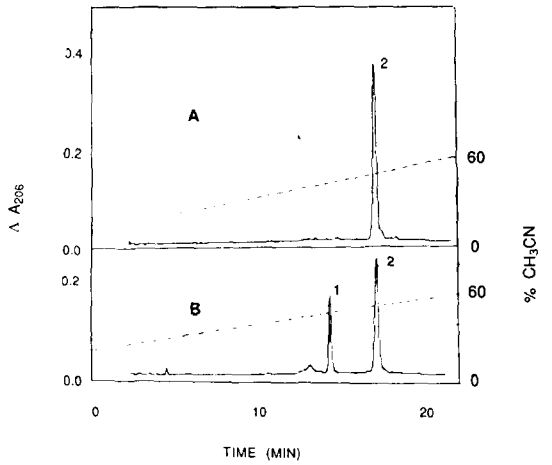


FIG. 5. **HPLC profile of [D-Phe^{45},Arg^{47}]hirudin$^{45-65}$ (P53) and peaks before (*A*) and after 24 h of incubation (*B*) with human α-thrombin.** 72 nmol of human α-thrombin (2.4 μg) and 72 nmol (186 μg) of P53 were mixed and diluted to a final volume of 5 ml. After incubating at 37 °C for 24 h, 100-μl aliquots were removed and loaded on a C_{18} analytical column and chromatographed on a Hewlett Packard model 1090 LC using a linear gradient of A 0.1% trifluoroacetic acid/H_2O, and B, 0.1% trifluoroacetic acid/CH_3CN; 20–80% B in 30 min. Elution of samples was monitored at 206 nm. The separated peaks were collected in 1.5-ml Eppendorf tubes, concentrated and subjected to amino acid analysis as described under "Experimental Procedures." Peak 2 is the intact peptide while peak 1 corresponds to hirudin$^{45-65}$.

with the putative "anion" exosite but cannot interact with the catalytic center. In this paper we report that desulfo hirudin$^{45-65}$ (P51) does inhibit the hydrolysis of the chromogenic or fluorogenic substrate Tos-Gly-Arg-*Z* (*Z* = *p*NA or AMC, respectively) by α-thrombin. The inhibition of either enzyme by P51 was weak (K_i = 0.72 ± 0.13 μM for bovine and 0.11 ± 0.03 μM for human) and partially competitive as shown in Fig. 3, affecting substrate binding to the *EI* complex by a factor α = 4 (human enzyme) as depicted in reaction Scheme 1. The mechanism predicts that the hydrolytic reaction cannot be completely inhibited since substrate does not bind to *EI* as efficiently as to free enzyme, although the hydrolytic kinetic constant k_p remains unchanged (Segel, 1975). Therefore, the saturating reciprocal velocity observed for this inhibitor above concentrations of 1 μM, combined with the use of substrate concentrations significantly higher than K_m of the substrate that were used in previous studies may have contributed to the conflicting conclusion drawn (Chang, 1983) regarding the inability to block thrombin amidase activity.

In contrast to fragment P51, the binding of hirudin is competitive with the binding of *p*-nitroanilide substrates at the active site (Stone and Hofsteenge, 1986). Therefore, the present results are in agreement with previous studies suggesting that the region Thr^{45} to Pro^{48} of intact hirudin is not necessarily the complimentary active-site binding domain, despite its reported analogy with the prothrombin cleavage site (Peterson *et al.*, 1976).

Native desulfo hirudin$^{45-65}$ and its analogs were consistently more effective inhibitors of human than bovine α-thrombin by almost one order of magnitude. This enhanced human antithrombin activity may be ascribed to species variation (Maraganore *et al.*, 1989) in the COOH-terminal exosite since N^{α}-acetyl hirudin$^{55-65}$ showed the same rank of potency shift without affecting the amidolytic activity of either enzyme (Tables I and II). Lys^{149} in human α-thrombin is located in the anion exosite (Bode *et al.*, 1989) and is replaced by Glu^{149} in bovine α-thrombin. Conceivably, the enhanced inhibition of α-thrombin could result, in part, from stabilization through

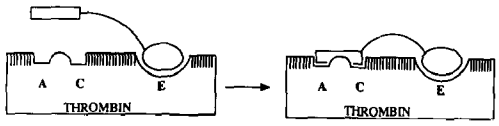


FIG. 6. **A model depicting the dual cooperative binding of the bifunctional inhibitor (P53) to α-thrombin.** The linker (Gin-Ser-His-Asn-Asp-Gly) serves as a suitable spacer for the bridging of an auxillary site (*E*) and the catalytic site (*C*) as well as the adjacent apolar-binding site (*A*).

an additional electrostatic interaction with the COOH-terminal tail of these inhibitors. However, further investigations[2] show that systematic removal of the side chain of each of the acidic residues in the COOH-terminal tail consistently causes a parallel potency drop in both enzymes. Consequently, the potency shift observed for inhibitors interacting with the anion exosite of bovine and human α-thrombin should be ascribed to salient differences other than a single amino acid mutation at residue 149.

The similarity between the hirudin sequence Thr-Pro-Lys-Pro and the prothrombin cleavage site has formed the basis of site-directed mutagenesis to identify the residue that interacts with the S_1 specificity pocket (Braun *et al.*, 1988). Notwithstanding the above similarity, the Thr-Pro-Lys-Pro sequence is also reminiscent of active site-directed inhibitors of the general structure D-Phe-Pro-Arg-*X* where *X* is a non hydrolyzable group. However, the former may lack primary specificity (Lys^{47}, S_1) and secondary recognition (Thr^{45}, S_3) compared to the latter (Arg^{47}, S_1) and (D-Phe^{45}, S_3). Accordingly, although P51 inhibited proteolysis of thrombin substrates, the mechanism of inhibition was partially competitive, increasing K_s to 12 μM (bovine α-thrombin-P51 complex) without affecting k_p. The molecular basis of this mechanism is still uncertain. However, we are undertaking NMR studies to determine whether a subsite distinct from the catalytic binding site may be implicated.

The recent x-ray structure determination (Bode *et al.*, 1989) of the covalent complex between human α-thrombin and D-Phe-Pro-Arg-chloromethylketone reveals that the narrow canyon comprising the active site extends beyond S_2' forming an extended groove that could be the putative fibrinogen or hirudin secondary binding site. This observation combined with the finding that P51 inhibits α-thrombin amidase activity served as the basis for the design of inhibitors that could simultaneously interact more effectively with both sites. A bifunctional inhibitor of this type would be expected to exhibit enhanced activity and specificity resulting from a favored intramolecular interaction depicted in Fig. 6. In this model (Portoghese, 1989) the initial binding of the COOH-terminal core encompassing residues 55–65 with the thrombin exosite could serve as the driving force for the second complimentary interaction within the active-site cleft, effectively bridging two remote binding domains separated by a spacer of suitable length. Indeed, we found that while D-Phe-Pro-Arg-Pro and N^{α}-acetyl desulfo $hirudin^{55-65}$ independently inhibited fibrin clot formation by bovine α-thrombin with IC_{50} values of 250 and 3.5 μM, respectively, their incorporation into a single molecule (P53), separated by a spacer corresponding to hirudin residues 49–54 (Gin-Ser-His-Asn-Asp-Gly), afforded an inhibitor with an $IC_{50} = 70 \pm 20$ nM (bovine α-thrombin) and 4.1 ± 0.8 nM (human α-thrombin). The effect of combining separate IC_{50} doses of $hirudin^{55-65}$ and D-Phe-Pro-Arg-Pro resulted only in a doubling of the fibrinogen clotting time, while the contribution of the spacer was negligible. The synergistic effect observed of P53 in the clotting assay was corroborated by the results of the fluorogenic assay where this analog emerged as a pure competitive inhibitor compared to P51 with K_i values almost 50-fold lower than the latter. Therefore, the enhanced affinity and *in vitro* anticoagulant effect could be attributed directly to a cooperative intramolecular binding mechanism (see above). The D-Phe^{45} residue, as in tripeptidyl inhibitors, is suited to bind within the extended hydrophobic region of the active site whereas the Thr^{45} counterpart in P51 would be expected to be exposed to the solvent (Bode *et al.*, 1989). Furthermore the Arg^{47} side-chain has the characteristics of the S_1 specificity pocket as shown by the large increase in K_i (41 ± 15 nM) compared with P53 (2.8 ± 0.9 nM) when this residue is replaced by alanine (P73). Interestingly, we could not detect any inhibition of the amidolytic activity of bovine α-thrombin with P73, suggesting subtle but important differences in the catalytic site of the two enzymes as well.

P53 is slowly hydrolyzed between Arg^{47}-Pro^{48} despite the presence of an imino acid corresponding to the S_1' subsite. However, although configurational inversion of Pro^{48} to D-Pro^{48} conferred proteolytic resistance, the resulting analog [D-Phe^{45},Arg^{47},D-Pro^{48}]$hirudin^{45-65}$ (P52) exhibited increased K_i and IC_{50} values compared with P53. Further, the configurational inversion restored hyperbolic competitive inhibition analogous to P51, albeit with 10-fold increased potency. This effect was also observed with [Thr^{45},Arg^{47},D-Pro^{48}]$hirudin^{45-65}$ (P54) which lacks the D-Phe^{45} residue. Although it is not immediately apparent why the configurational inversion of the P_1' subsite would manifest hyperbolic inhibition, proline is a strong configurational determinant in proteins, suggesting that the stereochemical change may have affected the mode of binding of the inhibitors in the active site.

Site-directed mutagenesis of the Lys^{47} residue of hirudin to Gln or Glu is not deleterious to hirudin-thrombin interaction (Braun *et al.*, 1988; Degryse *et al.*, 1989) indicating that binding of Lys^{47} to the S_1 primary specificity pocket is not critical for tight binding inhibition. Alternatively, another hirudin domain may be directly involved in binding to the active site (Owen *et al.*, 1988) while Lys^{47} and adjacent residues may obscure the catalytic cleft without direct surface contact with the enzyme. However, the deletion of the NH_2-terminal domain corresponding to residues 1–44 could possibly have exposed new recognitive elements in the NH_2-terminal end of the remaining fragment $hirudin^{45-65}$ that are suitably aligned to interact with the catalytic site.[3] We have demonstrated that the resulting inhibition exemplified by P51 is weak and partially competitive with respect to the thrombin substrate Tos-Gly-Arg-AMC, but the potency increases significantly upon replacing Thr^{45} and Lys^{47} by D-Phe and Arg, analogous to active site-directed tetrapeptide inhibitors. The resulting analog (P53) behaves as a bifunctional inhibitor whose mechanism may be explained by the schematic diagram in Fig. 6. The initial interaction with the exosite **E** which is unique to thrombin may act as a pivot that orients the amidase inhibiting component in the vicinity of the apolar binding site **A** (D-Phe^{45}, S_3) and specificity pocket **C**, (Arg^{47}, S_1) effectively increasing its local concentration compared with an intermolecular mechanism (Portoghese, 1989).

This mechanism and the results reported in this paper were used to design other more potent inhibitors which will be the

[2] J. DiMaio, B. Gibbs, D. Munn, J. Lefebvre, F. Ni, and Y. Konishi, unpublished results.

[3] The x-ray structure of the complex between r-hirudin and human α-thrombin has been solved (Rydel, *et al.*). The complex shows that the side chains of Ile^1 and Tyr^3 occupy the putative apolar-binding site while the side chain of Thr^2 resides near the opening of the S_1 specificity pocket.

subject of a future communication.

Acknowledgments—We are indebted for the useful discussions and expert assistance of Drs. Andy Storer, Robert Menard, Henri Khouri, and Michael Mancini.

REFERENCES

Bajusz, S., Barabas, E., Tolnay, P., Szell, E., and Bagdy, D. (1978) *Int. J. Peptide Protein Res.* **12,** 217–221

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) *EMBO J.* **8,** 3467–3475

Braun, P. J., Dennis, S., Hofsteenge, J., and Stone, S. R. (1988) *Biochemistry* **27,** 6517–6522

Chang, J. Y. (1983) *FEBS Lett.* **164,** 307–313

Degryse, E., Acker, M., Defreyn, G., Bernat, A., Maffrand, J. P., Roitsch, C., and Courtney, M. (1989) *Protein Eng.* **2,** 459–465

Dennis, S., Wallace, A., Hofsteenge, J., and Stone, S. R. (1990) *Eur. J. Biochem.* **188,** 61–66

Dodt, J., Machleidt, N., Seemuller, U., Maschler, R., and Fritz, H. (1986) *Biol. Chem. Hoppe-Seyler* **367,** 803–811

Dodt, J., Köhler, S., Schmitz, T., and Wilhelm, B. (1990) *J. Biol. Chem.* **265,** 713–718

Fenton, J. W., II, (1981) *Ann. N. Y. Acad. Sci.* **370,** 468–485

Fenton, J. W., II, and Bing, D. H. (1986) *Semin. Thromb. Haemostasis* **12,** 200–208

Fenton, J. W., II, Olson, T. A., Zabinski, M. P., and Wilner, G. D. (1988) *Biochemistry* **27,** 7106–7112

Library Reference Manual, 9th ed., version 1.0 (1987) International Mathematical Statistical Library IMSL, Houston

Fitzgerald, D. J., and FitzGerald, G. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7585–7589

Krstenansky, J. L., and Mao, S. J. T. (1987) *FEBS Lett.* **211,** 10–16

Krstenansky, J. L., Owen, T. J., Yates, M. T., and Mao, S. J. T. (1987) *J. Med. Chem.* **30,** 1688–1691

Krstenansky, J. L., Owen, T. J., Yates, M. T., and Mao, S. J. T. (1988) *Biochim. Biophys. Acta* **957,** 53–59

Lipowski, A. W. (1987) *Pol. J. Pharmacol. Pharm.* **39,** 585–596

Mao, S. J. T., Yates, M. T., Owen, T. J., and Krstenansky, J. L. (1988) *Biochemistry* **27,** 8170–8173

Maraganore, J. M., Chao, B., Joseph, M. L., Jablonski, J., and Ramachandran, K. L. (1989) *J. Biol. Chem.* **264,** 8692–8698

Markwardt, F. (1970) *Methods Enzymol* **19,** 924–932

Mattson, C. H., Eriksonn, E., and Nilsson, S. (1982) *Folia Haematol.* **109,** 543–551

Noé, G., Hofsteenge, J., Rovelli, G., and Stone, S. R. (1988) *J. Biol. Chem.* **263,** 11729–11735

Okamoto, S., Hijikata, A., Kikumoto, R., Tonomura, S., Hara, H., Ninomiya, K., Maruyama, A., Sugano, M., and Tamao, Y. (1981) *Biochem. Biophys. Res. Commun.* **101,** 440–446

Owen, T. J., Krstenansky, J. L., Yates, M. J., and Mao, S. J. T. (1988) *J. Med. Chem.* **31,** 1009–1011

Peterson, T. E., Roberts, H. R., Sottrup-Jensen, L., and Magnuson, S. (1976) in *Peptides of the Biological Fluids* (Peeters, H., ed) pp. 145–149, Pergamon Press, Oxford

Portoghese, P. S. (1989) *Trends Pharmacol. Sci.* **10,** 230–235

Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitch, C., and Fenton, J. W., II (1990) *Science* **249,** 277–280

Segel, I. H. (1975) *Enzyme Kinetics,* p. 178, J. Wiley & Sons, New York

Sonder, S. A., and Fenton, J. W., II (1984) *Biochemistry* **23,** 1818–1823

Stone, S. R., and Hofsteenge, J. (1986) *Biochemistry* **25,** 4622–4628

Stone, S. R., Braun, P. J., and Hofsteenge, J. (1987) *Biochemistry* **26,** 4617–4624

Stuber, W., Kosina, H., and Heimburger, N. (1988) *Int. J. Peptide Protein Res.* **31,** 63–70

Trippier, D. (1988) *Folia Haematol.* **115,** 30–35

Wallace, A., Dennis, S., Hofsteenge, J., and Stone, S. R. (1989) *Biochemistry* **28,** 10079–10084