Design and Characterization of Hirulogs: A Novel Class of Bivalent Peptide Inhibitors of Thrombin

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ABSTRACT: A novel class of synthetic peptides has been designed that inhibit the thrombin catalytic site and exhibit specificity for the anion-binding exosite (ABE) of α-thrombin. These peptides, called "hirulogs", consist of (i) an active-site specificity sequence with a restricted Arg–Pro scissile bond, (ii) a polymeric linker of glycyl residues from 6 to 18 Å in length, and (iii) an ABE recognition sequence such as that in the hirudin C-terminus. Hirulog-1 [(D-Phe)-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu] inhibits the thrombin-catalyzed hydrolysis of a tripeptide *p*-nitroanilide substrate with K_i = 2.3 nM. In contrast, the synthetic C-terminal hirudin peptide S-Hir_{53-64}, which binds to the thrombin ABE, blocked the fibrinogen clotting activity of the enzyme with K_i = 144 nM but failed to inhibit the hydrolysis of *p*-nitroanilide substrates at concentrations as high as 1 mM. In addition, the pentapeptide (D-Phe)-Pro-Arg-Pro-Gly, which comprises the catalytic-site inhibitor moiety of hirulog-1, was determined to have a K_i for thrombin inhibition >2 μM. Hirulog-1, but not S-Hir_{53-64}, was found to inhibit the incorporation of [^{14}C]diisopropyl fluorophosphate in thrombin. Hirulog-1 appears specific for thrombin as it lacks inhibitory activities toward human factor Xa, human plasmin, and bovine trypsin at inhibitor:enzyme concentrations 3 orders of magnitude higher than those required to inhibit thrombin. The optimal inhibitory activity of hirulog-1 depends upon all three components of its structure. Hirulog-1 inhibited human γ-thrombin and bovine thrombin with K_i values increased 500- and 20-fold, respectively, compared to K_i for human α-thrombin. Also, hirulog-1 inhibition of α-thrombin was reversed in the presence of saturating concentrations of S-Hir_{53-64}. Studies on the optimal length of the oligoglycyl spacer, which forms a molecular "bridge" linking active-site and ABE recognition sequences, showed that at least three to four glycines were necessary for optimal inhibitory activity. Comparison of anticoagulant activities of hirulog-1, hirudin, and S-Hir_{53-64} showed that the synthetic hirulog-1 is 2-fold more potent than hirudin and 100-fold more active than S-Hir_{53-64} in increasing the activated partial thromboplastin time of normal human plasma. Thus, fashioned from studies on hirudin and its fragments, synthetic peptides that bind to both the ABE and catalytic site of thrombin are potent reversible inhibitors of thrombin activities.

The interaction of thrombin with macromolecular substrates, inhibitors, and cofactors involves multiple, discrete structural regions that include the catalytic site, substrate groove, and anion-binding exosite, ABE[1] (Fenton, 1986; Fenton et al., 1988). The involvement of such multiple structures in the association of thrombin with macromolecules may be key in governing specificity of this central bioregulatory enzyme of thrombosis and hemostasis. Hirudin, a natural inhibitor of thrombin, appears to exploit these structural principles in formation of a tight stoichiometric complex with enzyme. The inhibition of thrombin by hirudin has been shown to involve interactions at both a "high-affinity" (K_d = 10^{-8} M), noncatalytic site and a second site at or near the catalytic triad (Stone & Hofsteenge, 1986; Fenton et al., 1990).

We have shown that a Tyr-sulfated dodecapeptide derived from residues 53–64 of hirudin inhibits thrombin-catalyzed hydrolysis of fibrinogen with a potency in plasma reduced by 50-fold from that of the intact inhibitor (Maraganore et al., 1989). However, the sulfated dodecapeptide S-Hir_{53-64} failed to inhibit thrombin-catalyzed hydrolysis of chromogenic substrates at concentrations far exceeding those required to inhibit thrombin fibrinogenolytic activity. Our studies have indicated that the highly anionic C-terminal tail peptide of hirudin is a functional anticoagulant through binding to the ABE and antagonism of fibrinogen recognition by thrombin. In a previous study, we showed that the NH_2 terminus of an undecapeptide, corresponding to residues 54–64 of hirudin, derivatized with dinitrodifluorobenzene specifically modifies Lys-149[2] of human α-thrombin (Bourdon & Maraganore, 1989). Based on a three-dimensional model of the human α-thrombin B-chain (Furie et al., 1982), this finding suggested that the NH_2^+ terminus of hirudin peptides is at least 18–20 Å removed from the β-hydroxyl group of Ser-195. The intramolecular distance between the site on thrombin where the hirudin C-terminal segment binds and the enzyme reactive center has recently been confirmed by examination of the crystallographic structure of the hirudin–thrombin complex.[3]

In the present communication, we document design of a novel, potent class of thrombin-directed inhibitors comprised of a catalytic-site inhibitor moiety, a flexible spacing segment,

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[1] Abbreviations: ABE, anion-binding exosite; S-Hir_{53-64}, *N*-acetylasparaginylglycylaspartylphenylalanylglutamylglutamylisoleucylprolylglutamylglutamyl(OSO_3-tyrosyl)leucine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; APTT, activated partial thromboplastin time; DFP, diisopropyl fluorophosphate.

[2] Numbering is based on alignment of the human thrombin B-chain to chymotrypsin as defined in ref 2.

[3] Dr. Alexander Tulinsky, personal communication.

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Table I: Designation and Sequence of Peptides

designation	sequence
hirulog-1	(D-Phe)-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
hirulog-2	(D-Phe)-Pro-Arg-Pro-$(Gly)_2$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
hirulog-3	(D-Phe)-Pro-Arg-Pro-$(Gly)_6$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
hirulog-4	(D-Phe)-Pro-Arg-Pro-$(Gly)_8$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
hirulog-5	Phe-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
$(Gly)_5$-Hir_{53-64}	$(Gly)_5$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu
S-Hir_{53-64}	ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu

and an ABE recognition moiety. Hirulog peptides were found to inhibit thrombin-catalyzed amidolytic activities in a manner consistent with above design considerations.

Experimental Procedures

Materials. t-Boc amino acids for solid-phase peptide synthesis were from Peninsula Laboratories, Belmont, CA, and included Boc-L-leucine-*o*-divinylbenzene resin, Boc-*o*-2,6-dichlorobenzyl-L-tyrosine, Boc-L-glutamic acid γ-benzyl ester, Boc-L-proline, Boc-L-isoleucine·$^1/_2H_2O$, Boc-L-phenylalanine, Boc-L-aspartic acid β-benzyl ester, Boc-glycine, Boc-L-asparagine, Boc-D-proline, Boc-tosyl-L-arginine, and Boc-D-phenylalanine. Boc-glycylglycine was purchased from Bachem Biosciences, Philadelphia, PA. Spectrozyme TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide), human plasmin, and hirudin were purchased from American Diagnostica, New York, NY. S-2251 (H-D-L-valyl-L-leucyl-L-lysine-*p*-nitroanilide) was purchased from KabiVitrum, Stockholm, Sweden. Bovine α-thrombin was obtained from Sigma, St. Louis, MO, and determined to contain >90% α-thrombin by SDS–PAGE analysis (reducing conditions, 12.5% acrylamide). Human α- and γ-thrombins were purified as reported previously (Fenton et al., 1977; Bing et al., 1977). Human α-thrombin (α309) was at a specific activity of 2678 NIH units/mg, contained 94% of activity as determined in active-site titration measurements, and was 96% pure as determined by SDS–PAGE. Human γ-thrombin (γ78) was determined to have a specific activity < 0.36 NIH unit/mg and to be essentially homogenous as monitored by SDS–PAGE. Human factor Xa was the generous gift of Dr. Fred Ofosu, McMaster University, Hamilton, Ontario, Canada. S-Hir_{53-64} [*N*-acetylasparaginylglycylaspartylphenylalanylglutamylglutamylisoleucylprolylglutamylglutamyl(OSO_3-tyrosyl)leucine] was prepared as described previously (Maraganore et al., 1989). [^{14}C]Diisopropyl fluorophosphate (125 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Pooled, normal human plasma was purchased from George King Biomedical, KS. Calcium chloride (0.03 M) and thromboplastin suspension were from Organon Technicon, Oklahoma City, OK. Solvents for HPLC were purchased from Baker.

Peptide Synthesis and Characterization. Hirulogs-1, -2, -3, -4, and -5, the (D-Phe)-Pro-Arg-Pro-Gly pentapeptide, and $(Gly)_5$-Hir_{53-64} were synthesized by standard solid-phase peptide methodologies employing an Applied Biosystems, Inc (ABI), 430 A Peptide Synthesizer, Foster City, CA. The glycyl linker segments of hirulogs and $(Gly)_5$-Hir_{53-64} were synthesized by using Boc-glycylglycine and manual coupling procedures. Peptides were deprotected and uncoupled from the divinylbenzene resin by treatment with anhydrous HF, *p*-cresol, ethylmethyl sulfide (10:1:1, v/v/v). Peptide thus prepared was lyophilized to dryness and, then, redissolved in 0.1% TFA/water for purification by HPLC as reported previously (Maraganore et al., 1989).

The identity of purified peptide samples was determined by amino acid analysis using ion-exchange chromatography and postcolumn ninhydrin detection with a Beckman System 6300 analyzer. Hydrolysates were prepared by treatment of samples in 6 M HCl, in vacuo, at 110 °C for 24 h (Spackman et al., 1958). The structure of hirulog-1 was confirmed by automated Edman degradation by standard procedures reported previously (Chao et al., 1989) and by fast-atom bombardment mass spectrometry.

Kinetic Studies. Kinetics analyses of thrombin-catalyzed hydrolysis of H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide were performed by using a Cary 219 double-beam spectrophotometer and continuous measurements of absorbance at 405 nm. The kinetics of human α-thrombin-catalyzed peptide hydrolysis were studied with a range of substrate concentrations from 1.1 to 22 μM (0.7–14.3 times K_m). Assays were performed by using a 0.05 M Tris, pH 7.5, buffer containing 0.1 M NaCl (1.0 mL) and a fixed concentration of α-thrombin (3.2 nM) at a controlled temperature of 25 °C. Total volume of reaction mixtures was constant at 1.1 mL. Reactions were initiated by addition of substrate to the cuvette containing thrombin and inhibitor premixed for less than 2 min. Initial rates were calculated at <15% net substrate hydrolysis.

Data from initial rate experiments were used to construct Lineweaver–Burke plots; the relationship of (substrate concentration)$^{-1}$ versus (initial velocity)$^{-1}$ was analyzed by using linear regression. K_m and V_{max} values were calculated accordingly. k_{cat} values were determined by dividing V_{max} by the enzyme concentration.

Inhibition of Thrombin by Hirulogs. Hirulog-1 (5.1–25.4 nM) was added to reaction mixtures containing buffer and human α-thrombin prior to addition of substrate (2.2–22 μM). K_i values were determined by standard procedures using equations for competitive and noncompetitive inhibition. The same procedure was applied to analysis of α-thrombin inhibition by other peptide derivatives (Table I), except use of peptide concentrations from 5 to 10000 nM. The same procedures were used to measure inhibitory activities of hirulog derivatives toward γ-thrombin and bovine thrombin.

Effects of Hirulog-1 on Factor Xa, Plasmin, and Trypsin Catalyzed Enzyme Reactions. The factor Xa (28 nM) and trypsin (6 nM) hydrolysis of Spectrozyme TH (11 μM) was assayed by using a 0.05 M Tris, pH 7.5, buffer containing 0.1 M NaCl at 25 °C as described above for thrombin. Hirulog-1 (100 nM to 125 μM) and enzyme were added to the cuvette containing buffer prior to addition of substrate. Plasmin (48 nM) hydrolysis of S-2251 (9 μM) was monitored in a similar fashion in the presence or absence of hirulog-1 at 40 μM. The enzyme-catalyzed rates of reaction were monitored by continuous spectrophotometric measurements at 405 nm.

Competition by S-Hir_{53-64} of Hirulog-1 Inhibition of Human α-Thrombin. The kinetics of human α-thrombin catalyzed hydrolysis of Spectrozyme TH in the presence of S-Hir_{53-64} (2.6 and 13.0 μM) were studied with a range of substrate concentrations from 2.2 to 22 μM. The inhibition of human α-thrombin by hirulog-1 in the presence or absence of S-Hir_{53-64} (2.6 and 13.0 μM) was measured over the same

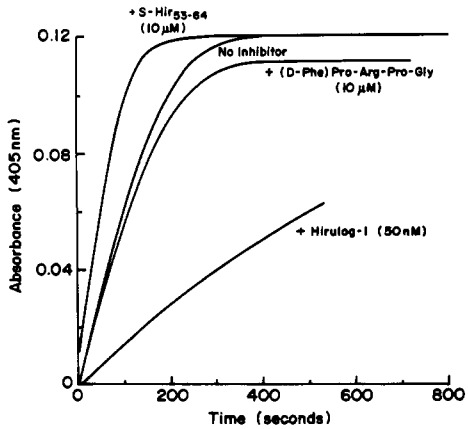

FIGURE 1: Effects of hirulog-1, S-Hir$_{53-64}$, and (D-Phe)-Pro-Arg-Pro-Gly on thrombin amidolytic activity toward a *p*-nitroanilide substrate. Human α-thrombin catalyzed hydrolysis of Spectrozyme TH was studied at an enzyme concentration of 3.2 nM and substrate concentration of 15 μM in a 0.05 M Tris, pH 7.5, buffer containing 0.1 M NaCl at 25 °C. Concentrations of S-Hir$_{53-64}$ and (D-Phe)-Pro-Arg-Pro-Gly were 10 μM and that of hirulog-1 was 50 nM. The progress curves for reactions containing S-Hir$_{53-64}$ or the absence of inhibitor are offset from zero so that lines can be distinguished. Thus, the limiting absorbance in these experiments is, in fact, comparable to that observed when testing the effects of the (D-Phe)-Pro-Arg-Pro-Gly pentapeptide.

range of substrate concentrations with a fixed concentration of inhibitor at 11.2 nM. K_i values for inhibition were calculated as described above.

Inhibition by Hirulog-1 of [^{14}C]DFP Incorporation in Thrombin. Human α-thrombin (600 nM) in a 0.05 M Tris, pH 7.5, buffer containing 0.1 M NaCl was reacted with [^{14}C]DFP (2.0 μCi) in the presence or absence of hirulog-1 (1.8 and 18 μM) or S-Hir$_{53-64}$ (180 and 1800 μM). Hirulog-1 or S-Hir$_{53-64}$ was added to thrombin 5 min prior to addition of radiolabeled DFP. Radiolabeled DFP was used as the propylene glycol solution provided by the manufacturer and added to the enzyme–inhibitor mixture to yield a 270-fold excess of reagent over thrombin. Following a 30-min reaction at 22–23 °C, 0.03 mL of the reaction mixture was removed for SDS–PAGE analysis (reducing conditions, 12% acrylamide). Following electrophoresis, the gel was treated with an autoradiography enhancer (Enlightning, New England Nuclear, Boston, MA), dried, and exposed to X-ray film by using standard fluorographic procedures.

Clotting Assays. Activated partial thromboplastin times (APTT) of pooled, normal human plasma were determined with a Coag-A-Mate XC instrument (Organon Technicon, Oklahoma City, OK) as per the manufacturer's specifications. Plasma (0.1 mL) and inhibitor (0.025 mL) were premixed prior to APTT determination. Final concentrations of hirulog-1, S-Hir$_{53-64}$, and hirudin ranged from 8.4 to 422 ng/mL, 30.8 to 1538 ng/mL, and 2.5 to 32.3 μg/mL, respectively.

RESULTS

Inhibition by Hirulog and D-Phe-Pro-Arg-Pro-Gly of α-Thrombin Amidolytic Activity. Hirulog-1 (Table I) inhibited α-thrombin-catalyzed hydrolysis of a chromogenic substrate, H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide (Spectrozyme TH, American Diagnostica, NY), at nanomolar concentrations (Figure 1) in a concentration-dependent manner. Under the same experimental conditions, micromolar concentrations of the C-terminal, Tyr-sulfated dodecapeptide of hirudin (S-Hir$_{53-64}$) exhibited no measurable inhibition of the thrombin-catalyzed rate. Likewise, in the presence of the (D-Phe)-Pro-Arg-Gly pentapeptide at concentrations as high as 10 μM, no significant inhibition of the enzyme-catalyzed reaction was observed. The (D-Phe)-Pro-Arg-Pro-Gly pentapeptide would be expected to inhibit α-thrombin at concentrations higher than those used here (Bajusz et al., 1978). These data showed clearly that at micromolar concentrations, the individual components of hirulog-1, the ABE recognition and active-site inhibitory moieties, failed by themselves to inhibit the rate of thrombin hydrolysis of the tripeptide *p*-nitroanilide substrate. In contrast, the combination of these components in a single synthetic fragment as in hirulog-1 resulted in potent inhibition of thrombin amidolytic activity at nanomolar concentrations.

Table II: Constants for Hirulog Inhibition of Thrombin-Catalyzed Hydrolysis of H-D-Hexahydrotyrosyl-L-Ala-L-Arg-*p*-nitroanilide[a]

enzyme	inhibitor	additions	K_i (nM)
human α-thrombin	hirulog-1		2.3
human γ-thrombin	hirulog-1		1400
bovine α-thrombin	hirulog-1		42.4
human α-thrombin	hirulog-1	S-Hir$_{53-64}$ (2.6 μM)	19.0
human α-thrombin	hirulog-1	S-Hir$_{53-64}$ (13 μM)	>2000
human α-thrombin	hirulog-2		64.5
human α-thrombin	hirulog-3		3.0
human α-thrombin	hirulog-4		2.6
human α-thrombin	hirulog-5		156.0
human α-thrombin	(D-Phe)ProArg-ProGly		>2000
human α-thrombin	S-Hir$_{53-64}$[b,c]		>2000
human α-thrombin	(Gly)$_5$-Hir$_{53-64}$[c]		>2000

[a] K_i values were determined as reported under Experimental Procedures. Enzyme assays were performed at 25 °C by using a 0.05 M Tris, pH 7.5, buffer containing 0.1 M NaCl. [b] S-Hir$_{53-64}$ has been shown to inhibit thrombin-catalyzed hydrolysis of fibrinogen with K_i = 144 nM (J. Fenton and J. M. Maraganore, unpublished results). [c] S-Hir$_{53-64}$ and (Gly)$_5$-Hir$_{53-64}$ are both observed to accelerate the rate of thrombin hydrolysis of Spectrozyme TH.

At tripeptidyl *p*-nitroanilide substrate concentrations near the K_m for α-thrombin (K_m = 1.5 μM), the inhibitory effects of hirulog-1 were studied in order to determine the class of inhibition and the inhibition constant (K_i). Inhibition by hirulog-1 of anilide hydrolysis by α-thrombin showed a K_i of 2.3 nM (Table II) and was noncompetitive. In the presence of hirulog-1 at 10.1 nM, K_m for thrombin-catalyzed hydrolysis of the tripeptidyl substrate increased from 1.5 to 5.5 μM and k_{cat} decreased from 17.9 to 11.1 s^{-1}. Noncompetitive inhibition by hirulog of the thrombin-catalyzed reaction was observed, in addition, at higher hirulog concentrations than those reported here. It would appear unlikely that inhibitor depletion is responsible for the observed results. It is possible that deviations from simple competitive inhibition are due to a slow dissociation rate for the hirulog–thrombin complex.

The unique ability of hirulog-1 to block thrombin amidolytic function was examined further by testing the effects of hirulog-1 or S-Hir$_{53-64}$ on the modification by [^{14}C]DFP of Ser-195 in the thrombin catalytic site. As shown in Figure 2, concentrations of hirulog-1 in excess of thrombin by 3- and 30-fold blocked the derivatization of thrombin with the active-site-directed reagent to completion. With significantly higher concentrations of S-Hir$_{53-64}$, modification of thrombin by DFP was unaffected. Thus, the inhibitory effects of hirulog-1 toward thrombin-catalyzed hydrolysis of tripeptidyl substrates are consistent with the abilities of this peptide, namely, the NH_2-terminal segment therein, to occupy the thrombin reactive center.

Specificity of Hirulog-1 for Thrombin. The specificity of hirulog-1 for thrombin was investigated by determining the effects of the synthetic peptide toward human factor Xa, human plasmin, and bovine trypsin amidolytic activities

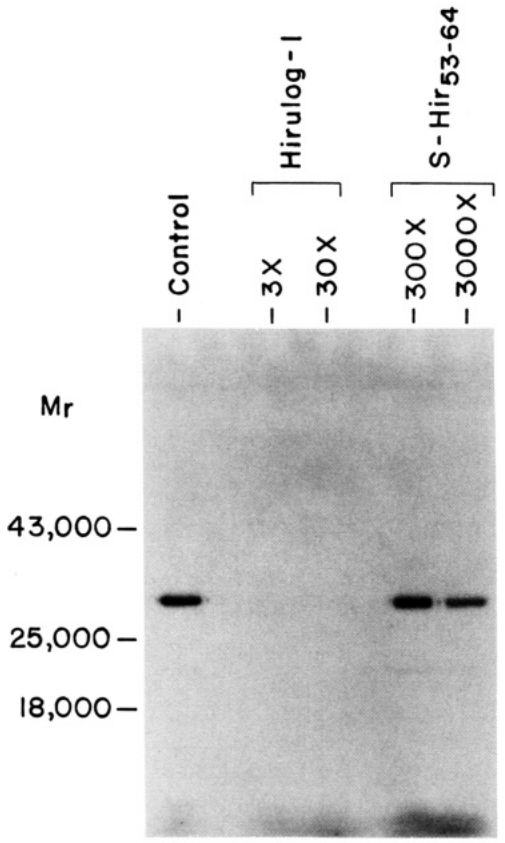

FIGURE 2: Inhibition by hirulog-1 of [^{14}C]DFP incorporation in thrombin. Human α-thrombin was reacted with [^{14}C]DFP in the absence (lane 1) or presence of hirulog-1 (lanes 2 and 3) or S-Hir$_{53-64}$ (lanes 4 and 5). The molar excess of hirulog-1 or S-Hir$_{53-64}$ over thrombin is provided at the top of each lane. Reaction mixtures were subjected to SDS–PAGE (reducing conditions, 12% acrylamide) and fluorography.

(Figure 3). The trypsin- and plasmin-catalyzed hydrolyses of Spectrozyme TH and S-2251, respectively, were not inhibited significantly at concentrations of hirulog-1 exceeding that of enzyme by 3 orders of magnitude. In the case of factor Xa hydrolysis of Spectrozyme TH, a range of hirulog-1 concentrations from 100 nM to 125 μM affected a small, reproducible inhibition of the enzyme-catalyzed rate. This inhibitory effect was independent of hirulog-1 concentration over a 1000-fold range and is consistent with inhibition by hirulog-1 of trace quantities of thrombin as a possible contaminant in the factor Xa preparation.

Role of Anion-Binding Exosite (ABE) Recognition Moiety of Hirulog-1 in Thrombin Inhibitory Activity. To demonstrate a requirement for the ABE recognition moiety (comprised of the dodecapeptide C-terminal sequence of hirudin from residues 53–64) for hirulog-1 inhibition of α-thrombin, the peptide was studied as an inhibitor of γ-thrombin. γ-Thrombin was determined to have catalytic competence toward Spectrozyme TH hydrolysis with K_m = 1.2 μM and k_{cat} = 7.1 s^{-1}. However, the ability of hirulog-1 to inhibit the γ-thrombin-catalyzed reaction was found to be markedly reduced with K_i = 1400 nM (Table II), a value increased some 3 orders of magnitude from that determined for hirulog-1 toward α-thrombin.

The anticoagulant activity of S-Hir$_{53-64}$ has been shown to be 10-fold reduced toward bovine thrombin (Maraganore et al., 1989) perhaps due to the absence of Lys-149 in the ABE of this enzyme (Bourdon & Maraganore, 1989). Likewise, ABE dependent thrombin inhibition by hirulog-1 was found diminished toward bovine enzyme, where K_i = 42.4 mM. As with inhibition of thrombin procoagulant activity by C-terminal hirudin peptides, the activity of hirulog-1 appears dependent on presence of a cationic side chain at position 149 in the exosite of thrombin.

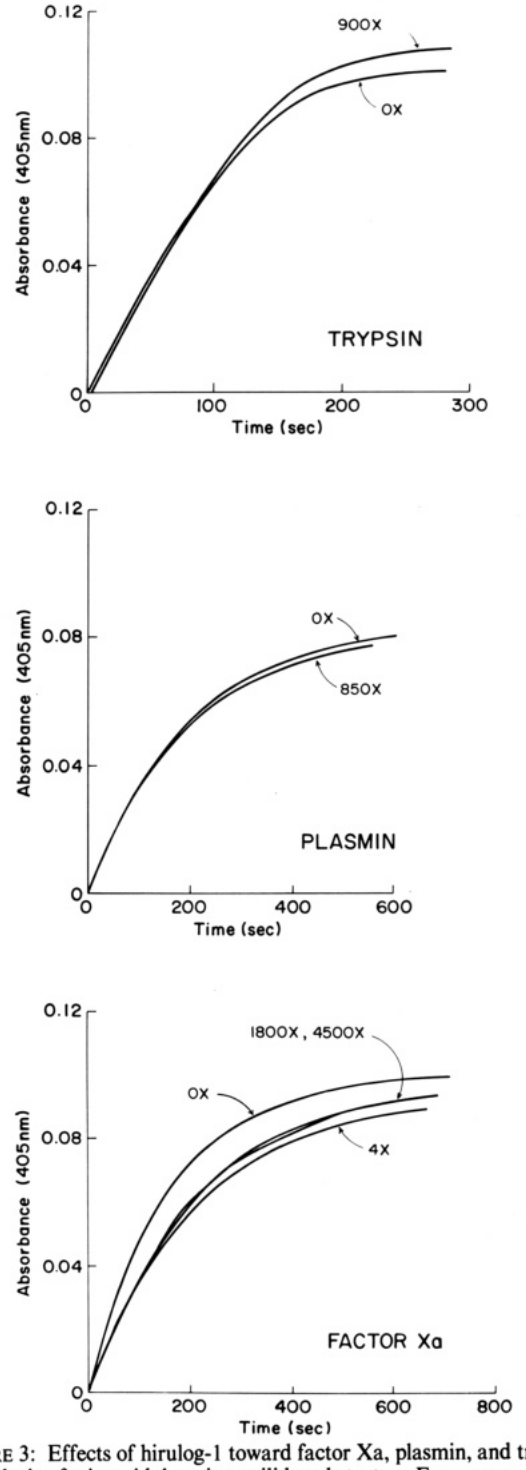

FIGURE 3: Effects of hirulog-1 toward factor Xa, plasmin, and trypsin hydrolysis of tripeptidyl-*p*-nitroanilide substrates. Enzyme reactions were performed at 25 °C in 0.05 M Tris, pH 7.5, and 0.1 M NaCl. The activities of factor Xa and trypsin were measured toward Spectrozyme TH. The plasmin-catalyzed reaction was toward S-2251. The molar concentration of hirulog-1 exceeding that of the enzyme is provided to the right of each curve. For trypsin and plasmin experiments, concentrations of hirulog-1 were 54 and 40 μM, respectively. For factor Xa experiments, the highest hirulog-1 concentration employed was 125 μM.

In the presence of S-Hir$_{53-64}$ at 2.6 and 13.0 μM, the apparent K_i for hirulog-1 inhibition of thrombin was from 2.3 nM to 19 and >2000 nM, respectively (Table II). Thus, occupancy of the ABE with a hirudin peptide, although itself

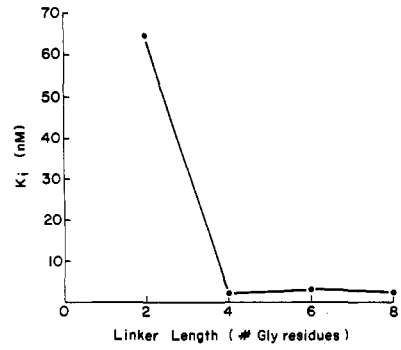

FIGURE 4: Dependence of hirulog activity on glycine linker length. The K_i values for hirulog analogues containing glycine linker segments of two to eight residues were determined toward human α-thrombin catalyzed hydrolysis of Spectrozyme TH as described in the Experimental Procedures section.

unable to block thrombin amidolytic activity, protected thrombin from inhibition by hirulog-1. These studies show further that hirulog activity requires interaction of its ABE recognition moiety with its cognate binding site in thrombin.

Dependence of Hirulog Inhibitory Activity on the Length of the Polymeric Linker. To optimize the length of the glycyl linker segment in hirulog derivatives, synthetic peptides containing two (hirulog-2), six (hirulog-3), and eight (hirulog-4) glycine residues were prepared and compared in kinetic assays to hirulog-1, which contains a four glycyl linker segment. Amino acid sequences of these analogues are provided in Table I. As shown in Figure 4, peptide analogues with spacer lengths of four, six, or eight glycyl residues exhibited maximal thrombin inhibitory activity. These peptides (hirulogs-1, -3, and -4) are found to inhibit the thrombin-catalyzed reaction with K_i values ranging from 2.3 to 3.0 nM. In contrast, hirulog-2, which contains two glycyl residues linking active-site inhibition and ABE recognition sequences, inhibited the hydrolytic reaction with K_i = 64 nM, increased approximately 30-fold from inhibitory constants determined for the longer peptide derivatives. Thus, a minimal glycyl spacer length of three to four residues was found necessary for potent hirulog inhibition of thrombin amidolytic function.

Role of the Catalytic-Site Binding Moiety for Hirulog Activity. The catalytic-site binding moiety of hirulog derivatives presented herein is comprised of the sequence (D-Phe)-Pro-Arg-Pro. In order to demonstrate the essential role of this functionality for hirulog activity, the inhibitory activities were determined for a $(L\text{-Phe})_1$-containing peptide (hirulog-5) and a peptide $(Gly)_5$-Hir_{53-64} lacking the (D-Phe)-Pro-Arg-Pro sequence. As shown in Table II, replacement of D-Phe by L-Phe increased K_i for thrombin inhibition by nearly 2 orders of magnitude. When the (D-Phe)-Pro-Arg-Pro sequence is removed altogether, as in the peptide $(Gly)_5$-Hir_{53-64}, no measureable inhibitory effects were observed at concentrations less than or equal to 10 μM. Thus, hirulog activity is dependent on a catalytic-site binding moiety. In addition to the sequence (D-Phe)-Pro-Arg-Pro, it should be possible to employ additional structures such as nonpeptidic groups or other peptide sequences known to inhibit thrombin activity such as that of the fibrinopeptide fragments (Bettelheim, 1956).

Anticoagulant Activity of Hirulog-1. The anticoagulant activity of hirulog-1 was compared to that of hirudin and S-Hir_{53-64} in assays of the activated partial thromboplastin time (APTT) of pooled, normal human plasma (Figure 5). On a weight basis, hirulog-1 was found to be approximately 2-fold more active than hirudin (hirulog-1 is essentially equivalent in molar potency to hirudin in anticoagulant assays). Increase in the APTT to 150% of the control values was achieved at

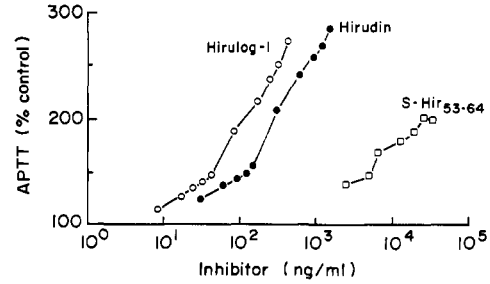

FIGURE 5: Comparison of anticoagulant activities of hirulog-1, hirudin, and S-Hir_{53-64}. APTT assays were performed semiautomatically by using pooled, normal human plasma as described under Experimental Procedures.

56 and 123 ng/mL concentrations of hirulog-1 and hirudin, respectively. Assays with S-Hir_{53-64} showed that this sulfated hirudin peptide was 100- and 50-fold less active as an anticoagulant compared to hirulog-1 and hirudin, respectively. S-Hir_{53-64} concentrations of 4900 ng/mL were required to increase APTT by 150% of control. Also, while S-Hir_{53-64} exhibited a saturable dose response in APTT assays (Maraganore et al., 1989), activities of hirulog-1 and hirudin yielded increases in plasma clotting times past measurable values, i.e., >300 s.

Discussion

Peptides derived from the C-terminus of hirudin have been shown to block fibrinogenolytic activity of human α-thrombin in vitro (Maraganore et al., 1989) and in vivo (Cadroy et al., 1989), to exhibit a 10-fold increase in anticoagulant activity upon sulfation of the tyrosine that corresponds to Tyr-63 of natural hirudin (Maraganore et al., 1989), and to display species specificity toward human versus bovine thrombin (Maraganore et al., 1989). A sulfated hirudin peptide, designated S-Hir_{53-64}, was determined to be 50-fold less active than hirudin as inhibitor of thrombin fibrinogenolytic activity. Moreover, peptides such as S-Hir_{53-64} or those comprising the C-terminal 21 amino acids of hirudin failed to inhibit thrombin amidolytic function (Maraganore et al., 1989; Krstenansky & Mao, 1987). In an earlier paper, we demonstrated that the NH_2 terminus of a hirudin C-terminal undecapeptide, Hir_{54-64}, is proximal to Lys-149 of human thrombin (Bourdon & Maraganore, 1989; Bourdon et al., 1990). The possible role of Lys-149 in the binding of hirudin peptides including S-Hir_{53-64} would explain the ~10-fold reduced binding to bovine thrombin, since this enzyme lacks a cationic amino acid residue at position 149.

Examination of a model for the three-dimensional structure of bovine thrombin (Furie et al., 1982) showed that the amino acid at position 149 is approximately 18–20 Å away from the active-site hydroxyl group of Ser-195. This fact provided the basis for the rational design of novel peptides targeted toward *both* the ABE *and* the catalytic site of thrombin. We have designed a class of peptides, called "hirulogs", where the active-site inhibitory sequence (D-Phe)-Pro-Arg-Pro is attached to a peptide derived from the hirudin sequence (corresponding to residues 53–64 of hirudin) via a "bridge" of glycine residues (Table I). The sequence (D-Phe)-Pro-Arg-Pro was used because it binds tightly to the active site of thrombin. The peptide segment contains the D-phenylalanine residue that is known to bind in a hydrophobic pocket near the thrombin active site (Bajusz et al., 1978; Sonder & Fenton, 1984) and an arginine residue that binds in the proteinase specificity pocket. The COOH-terminal 12 amino acid residues of hirulog peptides derive from studies on C-terminal hirudin

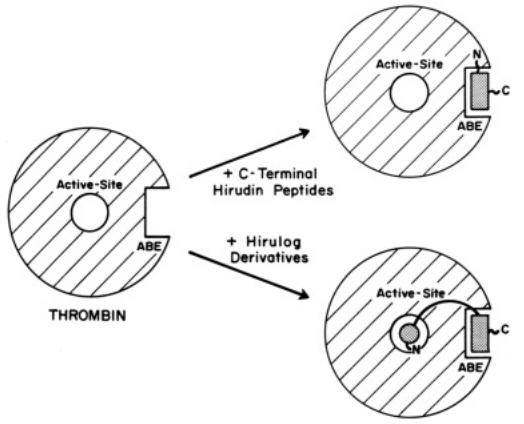

FIGURE 6: Illustrative scheme of thrombin inhibition by hirulog and C-terminal hirudin peptide derivatives.

fragments and are capable of binding to the ABE in thrombin. By themselves, neither the hirudin peptide sequence, S-Hir$_{53-64}$ or unsulfated hirudin peptides (Maraganore et al., 1989), nor the (D-Phe)-Pro-Arg-Pro-Gly moieties inhibit significantly the thrombin-catalyzed hydrolysis of *p*-nitroanilide substrates. On the contrary, S-Hir$_{53-64}$ has been shown to enhance the rate of thrombin cleavage of tripeptidyl *p*-nitroanilide substrates in an uncompetitive fashion (Naski et al., 1990).

In contrast to its separated components (the hirudin-derived peptide and the active-site-inhibitory sequence), hirulog-1, which links these fragments via a four glycine residue spacer, strongly inhibits thrombin activity toward chromogenic substrates. Since hirulog-1 but not S-Hir$_{53-64}$ can block the covalent modification by [^{14}C]DFP of the thrombin reactive center, inhibitory activity of hirulog-1 appears to result from placement of the (D-Phe)-Pro-Arg-Pro moiety within the enzyme active-site pocket. Indeed, a peptide lacking the catalytic-site binding moiety, (Gly)$_5$-Hir$_{53-64}$, showed no measurable inhibition of thrombin activity at concentration as high as 10 μM. In addition to inhibiting the hydrolysis of tripeptidyl substrates, hirulog-1 was found to inhibit the fibrinogenolytic activity of thrombin in plasma assays and, thus, to be an effective anticoagulant. In fact, hirulog-1 was determined to have an anticoagulant activity comparable to that of hirudin and an activity more than 100-fold larger than that of sulfated hirudin peptides.

A key feature for the activities of hirulog peptides is the segment of repeating glycines that links the two components that bind to the thrombin active site and exosite. Peptides containing four to eight glycine residues were found to be significantly more active than one that contains a two-glycine linker. Results obtained with the two-glycine peptide demonstrated that, in fact, a highly constrained linker segment can disrupt thrombin inhibitory activity. These studies have also shown that peptides containing six or eight glycine residue chains do not differ significantly in activity from that containing a four-glycine linker. This unexpected finding can be explained if, in fact, the spacer segment is equally disordered when free in solution as when bound to thrombin. It can thus be inferred that binding to thrombin of active-site and ABE recognition moieties in hirulog peptides is not cooperative, although dependent on a minimal intramolecular spacing.

In the case of hirulog-1, a linker length of four glycines serves minimally to span the distance of ~18 Å from the arginine binding locus at the active site to the point in hirudin peptides where derivatization with dinitrodifluorobenzene allows alkylation of Lys-149 in human thrombin. The inhibition data, therefore, corroborate the results from cross-linking studies, which had indicated that the distance between the NH_2 terminus of hirudin peptides bound to ABE on thrombin and the hydroxyl group of Ser-195 is about 18–20 Å. Results from X-ray crystallographic analysis of PPACK–thrombin (Bode et al., 1989) and hirudin–thrombin[3] complexes support data regarding the distance that separates the ABE and thrombin catalytic site.

Several studies were performed to show that, as with active-site inhibitory and glycyl linker segments, the interaction between the hirudin peptide segment and the thrombin ABE is necessary for hirulog function. Forms of thrombin lacking either an intact exosite or a cationic side chain at position 149 were poor targets for hirulog action. Moreover, hirulog-1 inhibitory activity was reversed fully when the thrombin ABE was saturated with a hirudin peptide. Consistent with our design, therefore, hirulogs interact with thrombin via both the binding of a hirudin peptide segment in ABE and the positioning of an inhibitory substrate analogue, restricted or limited to thrombin cleavage, in the catalytic pocket (Figure 6). While hirudin peptides such as S-Hir$_{53-64}$ inhibit thrombin fibrinogenolytic activity by exosite binding and interference with substrate recognition, hirulog function involves both disruption of substrate recognition and occupancy of the active site. These studies thus document a novel class of proteinase inhibitors, the mechanism of action of which is not limited to specificity for the active-site pocket alone. The bivalent nature of such inhibitors appears to assure increased potency and to ensure high specificity in the process.

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Probing the Coenzyme Specificity of Glyceraldehyde-3-phosphate Dehydrogenases by Site-Directed Mutagenesis†

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ABSTRACT: By combining our knowledge of the crystal structure of the glycolytic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the sequence of the photosynthetic NADP-dependent GAPDH of the chloroplast, two particular amino acid residues were predicted as the principal determinants of differing coenzyme specificity. By use of site-directed mutagenesis, the amino acids Leu 187 and Pro 188 of GAPDH from *Bacillus stearothermophilus* have been replaced with Ala 187 and Ser 188, which occur in the sequence from the chloroplast enzyme. The resulting mutant was shown to be catalytically active not only with its natural coenzyme NAD but also with NADP, thus confirming the initial hypothesis. This approach has not only enabled us to alter the coenzyme specificity by minimal amino acid changes but also revealed factors that control the relative affinity of the enzyme for NAD and NADP.

The NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] (EC 1.2.1.13) from chloroplasts, which is involved in photosynthetic assimilation of CO_2 through the Calvin cycle (McGowan & Gibbs, 1974; O'Brien & Powls, 1976; Pupillo & Fagiani, 1979), has been shown to be closely homologous with the glycolytic enzyme from thermophilic eubacteria (EC 1.2.1.12), from which it has been proposed to have derived (Martin & Cerff, 1986). However, the thermophilic enzyme is strictly NAD-dependent, in contrast to the chloroplast enzyme, which functions with both coenzymes NAD and NADP but with a preference for NADP (Ferri et al., 1978; Cerff, 1978). The dual specificity of the chloroplast enzyme contrasts with that of many other NAD/NADP-dependent dehydrogenases, which have a marked preference for a specific coenzyme (Stryer, 1988).

Analysis of coenzyme binding in several dinucleotide-binding enzymes (Wierenga et al., 1985) showed that there is a structural homology within a $\beta\alpha\beta$ folding unit to which the ADP moiety of the coenzyme binds in a similar fashion. This fingerprint region contains amino acid sequences that are characteristic for NAD or NADP binding (Rice et al., 1984). Recently Scrutton et al. (1990) redesigned the coenzyme specificity of NADP-dependent glutathione reductase by directed mutagenesis and confirmed the set of amino acid residues that are determinants of differing coenzyme specificity.

The three-dimensional structures of both the holo and apo forms of *Bacillus stearothermophilus* GAPDH have been determined at high resolution (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988), and the gene for this enzyme has been cloned, expressed, and sequenced (Branlant et al., 1983, 1989). The coenzyme-binding domain of GAPDH contains an ADP-binding fingerprint typical of an NAD-dependent enzyme. However, the chloroplast enzyme does not show the expected sequence differences for NADP specificity, suggesting that a different mechanism is involved in the molecular recognition of NADP in GAPDH.

We have used the GAPDH structures together with the primary sequences of chloroplast enzymes to explore the molecular basis for coenzyme specificity using molecular modeling. Amino acids have been replaced by site-directed mutagenesis, and our predictions have been tested by measurements of the properties of the mutant enzyme.

Materials and Methods

Molecular modeling was carried out on an Evans & Sutherland graphics system PS300 using the program FRODO (Jones, 1978).

(*a*) *Mutagenesis and Isolation of Mutant Enzyme.* To produce the double mutant, a 34-base oligonucleotide was synthesized (5′ CGCAAATCTTTATGGGAAGCGTCCAAAATTCGTT 3′) with five mismatches as compared to the wild-type sequence (Branlant et al., 1989). The method used to generate the mutated enzyme was that previously described (Mougin et al., 1988; Soukri et al., 1989). The gene was then totally sequenced by the dideoxy method (Sanger et al., 1977) to verify that no other mutation had arisen. Production and purification of the wild-type and mutant enzymes were per-

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[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); G3P, glyceraldehyde 3-phosphate; 1,3-dPG, 1,3-diphosphoglycerate; P_i, inorganic phosphate; NAD and NADH, nicotinamide adenine dinucleotide (oxidized and reduced forms); NADP and NADPH, nicotinamide adenine dinucleotide phosphate (oxidized and reduced forms); 3-CAPAD, 3-(chloroacetyl)pyridine adenine dinucleotide.

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