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Thrombin-specific inhibition by and slow cleavage of hirulog-1

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Hirulog-1 {D-Phe-Pro-Arg-Pro-$[Gly]_4$-desulphohirudin-(53–64) (HV1)} was designed to bind by its first four and last 12 residues to the α-thrombin catalytic site and anion-binding exosite for fibrin(ogen) recognition respectively, with a $[Gly]_4$ bridge and an Arg–Pro bond at the scissional position. Human α-, γ- and ζ-thrombins, as well as bovine trypsin, readily hydrolyse Spectrozyme-TH (D-hexahydrotyrosyl-Ala-Arg *p*-nitroanilide) at pH 7.4 and approx. 23 °C. Both α- and ζ-thrombins, which have high fibrinogen-clotting activities (> 3000 kunits/g), were inhibited with this substrate by hirulog-1 [$K_i = 2.56 \pm 0.35$ nM ($n = 3$) and 1.84 ± 0.15 nM ($n = 3$) respectively] and slowly cleaved the inhibitor [$k = 0.326 \pm 0.082$ min^{-1} ($n = 12$) and 0.362 ± 0.056 min^{-1} ($n = 18$) respectively], whereas γ-thrombin, which has essentially no clotting activity (approx. 4 kunits/g), and trypsin were not inhibited with > 1000-fold molar excess of hirulog-1. Similar inhibition parameters were also obtained for hirulog-1 incubated with α-thrombin or ζ-thrombin at approx. 23 °C and by measuring thrombin activity with fibrinogen in the clotting assay at 37 °C. Cleavage of the Arg-3–Pro-4 bond in hirulog-1 by either α- or ζ-thrombin was shown by identical cleavage products of either thrombin on h.p.l.c. and by sequence analysis of the α-thrombin products. These data demonstrate that hirulog-1 is a specific inhibitor of thrombin forms with high fibrinogen-procoagulant activities and that its Arg-3–Pro-4 bond is slowly cleaved by these thrombin forms.

INTRODUCTION

Procoagulant α-thrombin (EC 3.4.21.5) is the activation product of prothrombin in blood and has central bioregulatory functions in thrombosis/haemostasis, various disease states and wound healing (Fenton, 1981, 1986, 1988; Fenton *et al.*, 1991). Other forms, such as β-thrombin and γ-thrombin, which arise by autoproteolytic or tryptic fragmentation, are thermodynamically less stable than α-thrombin, and unlike it they essentially lack fibrinogen-clotting activity, but retain certain proteolytic capabilities (Bing *et al.*, 1977; Fenton *et al.*, 1977*a,b*, 1979; Chang *et al.*, 1979; Lewis *et al.*, 1987). These forms are of questionable physiological importance, since physiological mechanisms for their generation have yet to be identified. In contrast, neutrophil elastase and cathepsin G cleave the B-chain of α-thrombin at Ala-150–Asn-151 and Tyr-148–Thr-149 bonds to generate ϵ- and ζ-thrombins respectively, and these forms could arise in aging thrombi on neutrophil activation (Brower *et al.*, 1987; Brezniak *et al.*, 1990). Like α-thrombin, ζ-thrombin retains high fibrinogen-clotting activity (Brezniak *et al.*, 1990) and has similar inhibition constants (K_i) with recombinant hirudin variants (Witting *et al.*, 1991), but is more readily denatured, like γ-thrombin (Brezniak *et al.*, 1990).

Independently of its catalytic site and adjacent region (the traditional active site of serine proteinases), α-thrombin has an anion-binding exosite for fibrin(ogen) recognition and is blocked by hirudin (Fenton *et al.*, 1988). In contrast with hirudin, which blocks both clotting and tripeptide *p*-nitroanilide substrate activities, peptides corresponding to the highly negatively charged *C*-terminal approx. 20% of hirudin inhibit the former but not the latter activities (Krstenansky & Mao, 1987; Mao *et al.*, 1988; Fenton *et al.*, 1989; Maraganore *et al.*, 1989). From these findings and the marked difference between hirudin binding with α- versus γ-thrombin (Fenton *et al.*, 1979), the *C*-terminal tail of hirudin was concluded to bind at the exosite for fibrin(ogen) recognition, and the largely hydrophobic head to interact with apolar regions next to the catalytic site (Fenton, 1986, 1988, 1989; Fenton & Bing, 1986). Indeed, recently detemined crystallographic structures of human α-thrombin complexes with recombinant hirudin variants have confirmed such bivalent or bridge binding (Grutter *et al.*, 1990; Rydel *et al.*, 1990). In addition, α-thrombin also appears to have another anion-binding exosite for heparin binding (Church *et al.*, 1989) and perhaps other substances (Fenton *et al.*, 1991).

Before there was any knowledge of the crystallographic structures of human α-thrombin (Bode *et al.*, 1989) and its complexes with recombinant hirudins (Grutter *et al.*, 1990; Rydel *et al.*, 1990), hirulog-1 {D-Phe-Pro-Arg-Pro-$[Gly]_4$-desulphohirudin-(53–64) (HV1)} was designed so that the first four and last 12 residues would interact with regions adjacent to the catalytic site and the anion-binding exosite for fibrin(ogen) recognition of α-thrombin respectively (Maraganore *et al.*, 1990). The D-Phe-Pro-Arg ligand was chosen because of its high affinity for the catalytic site and adjacent regions (Bajusz *et al.*, 1978; Kettner & Shaw, 1978; Witting *et al.*, 1988), and a proline residue was subsequently added to form an imide bond thought to prevent cleavage (Bagdy *et al.*, 1976; Fenton & Bing, 1986). The length of the flexible $[Gly]_4$ spacer was initially based on affinity-labelling experiments with hirudin-(53–64) that indicated a distance of approx. 1.8 nm (18 Å) from the catalytic site to the exosite (Bourdon *et al.*, 1990) and was subsequently determined by varying the number of glycine residues from two to eight (Maraganore *et al.*, 1990). Because of the nanomolar K_i value of hirulog-1 with α-thrombin, sulphonation of Tyr-19 (correspond-

Abbreviations used: hirulog-1, D-Phe-Pro-Arg-Pro-$[Gly]_4$-desulphohirudin-(53–64) (HV1); Spectrozyme-TH, D-hexahydrotyrosyl-Ala-Arg *p*-nitroanilide.

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ing to Tyr-63 in hirudin) was unnecessary and desulphohirudin-(53–64) was used as the second ligand. Subsequent to our initial report (Maraganore *et al.*, 1990), we found that hirulog-1 is a slow-turnover inhibitor of α- and ζ-thrombins (Fenton *et al.*, 1990). Similar hirudin derivatives have been reported by others (Di Maio *et al.*, 1990), and non-cleavable analogues have been reported by both groups of investigators (Di Maio *et al.*, 1991; Kline *et al.*, 1991). The present paper reports detailed kinetics for cleavage of the Arg-3–Pro-4 bond in hirulog-1 by human α- and ζ-thrombins. Neither γ-thrombin nor trypsin was inhibited, demonstrating that hirulog-1 is specific for thrombin forms with high clotting activities.

EXPERIMENTAL

Chemicals and reagents

Hirulog-1 was prepared and characterized as previously described (Maraganore *et al.*, 1990). Spectrozyme-TH (D-hexahydrotyrosyl-Ala-Arg *p*-nitroanilide) was a gift from Dr. Richard Hart, American Diagnostic, Greenwich, CT, U.S.A. *p*-Nitrophenyl *p*′-guanidinobenzoate was purchased from ICN Pharmaceuticals, Cleveland, OH, U.S.A., [^{14}C]di-isopropyl phosphorofluoridate was from New England Nuclear, Boston, MA, U.S.A., poly(ethylene glycol) 6000 was from EM Science, Cherry Hill, NY, U.S.A., imidazole was from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., and Hepes, Tris and NaCl were from Research Organics, Cleveland, OH, U.S.A.

Freeze-dried 95% clottable bovine fibrinogen was obtained from Miles Laboratory, Elkart, IN, U.S.A., and type XIII Tos-Phe-CH_2Cl-treated bovine trypsin was from Sigma Chemical Co., St. Louis, MO, U.S.A. Human plasma fraction III was a gift from Dr. Henry S. Kingdon, Baxter Hyland Division, Hayward, CA, U.S.A. All other materials were from sources given in publications cited below.

Thrombin preparations

Human α-thrombin was prepared from fraction III paste as previously described (Fenton *et al.*, 1977*a*,*b*). It was converted into γ- or ζ-thrombin by controlled passage through trypsin- or chymotrypsin-immobilized Sepharose 4B respectively (Bing *et al.*, 1977; Fenton *et al.*, 1977*b*; Brezniak *et al.*, 1990). An absorption coefficient of 1.75 litre·g^{-1}·cm^{-1} was used for all thrombin forms in 1.0 M-acetic acid, and an M_r of 36500 was assumed for these forms. Preparations were evaluated (Table 1) and stored at −70 °C in 0.75 M-NaCl until used (Fenton *et al.*, 1977*a*,*b*).

Spectrozyme-TH amidolytic activities

A Varian 210 dual-beam u.v.-visible recording spectrophotometer was used where reactions were carried out in 1.0 cm-pathlength polystyrene semi-micro cuvettes (Sarstedt, Princeton, NJ, U.S.A.) at approx. 23 °C. Enzyme concentrations were based on active-site titrations with *p*-nitrophenyl *p*′-guanidinobenzoate (Table 1), whereas Spectrozyme-TH concentrations were determined from the 342 nm isosbestic absorption coefficient of 9920 M^{-1}·cm^{-1} for it and *p*-nitroanilide at a 1.0 nm bandwidth (Witting *et al.*, 1991). Reactants were dissolved and subsequently diluted in 150 mM-NaCl/10 mM-Hepes/10 mM-Tris buffer, pH 7.4, containing 2.5 g of poly(ethylene glycol) 6000/l. Spectrozyme-TH hydrolysis was monitored by recording the 405 nm absorbance change/s for *p*-nitroanilide generation. Initial rates were obtained by extrapolating the recorded slopes to time zero.

The procedure used to determine the K_m is as follows: 200 μl of the thrombin solution was added to 800 μl of the preceding buffer solution and mixed by drawing in and out about three times with a pipetting device while the cuvette remained in the spectrophotometer. The recorder was then activated to monitor the absorbance at 405 nm for 5 min. Steady-state velocities were obtained for five concentrations of Spectrozyme-TH ranging from 1 to 6 μM and then fitted by non-linear regression to eqn. (1):

$$v_s = V_{max.}\, S(S+K_m)^{-1} \tag{1}$$

where S is the initial substrate concentration, v_s is the initial steady-state velocity, $V_{max.}$ is the limiting maximal velocity and K_m is the dissociation constant of the complex. The least-squares analysis of the non-linear curve-fitting program of Minsq (Micromath Scientific Software, Salt Lake City, UT, U.S.A.) was employed to determine K_m and $V_{max.}$ (Table 2).

To determine K_i values, the above procedure was used except that enzyme (Table 3) was incubated with various amounts of hirulog-1 for various times at approx. 23 °C before assay. Because of the high-affinity binding of hirulog-1 to thrombin, an allowance becomes necessary to correct for the reduction in inhibitor concentration that occurs on formation of the enzyme–inhibitor (EI) complex (Morrison, 1969, 1982; Henderson, 1972; Stone & Hofsteenge, 1986). Therefore the variation of the steady-state velocity (v_s) with total inhibitor (I_t) is expressed in eqn. (2) (Morrison, 1969; Henderson, 1972):

$$v_s = 0.5\, v_0\, E_t[4K_i\, E_t + (K_i + I_t - E_t)^2]^{\frac{1}{2}} - (K_i + I_t - E_t) \tag{2}$$

Here v_0 is the velocity observed in the absence of the inhibitor, E_t is the total enzyme concentration, and K_i' is the apparent inhibition constant, which is related to K_i by eqn. (3):

$$K_i = K_i'[1+(S/K_m)]^{-1} \tag{3}$$

During determination of the K_i of hirulog-1, the concentrations of Spectrozyme-TH, α-thrombin and ζ-thrombin were kept constant at 18 μM, 5.51 nM and 5.23 nM respectively, and the concentration of hirulog-1 was varied from 0 to 95 nM. The K_i of hirulog-1 for both thrombin forms was determined by obtaining steady-state velocities at zero incubation time with six concentrations of hirulog-1. These velocities were fitted by non-linear regression to eqn. (2) with Minsq. K_i' was subsequently converted into K_i by using eqn. (3). The K_i values from three different runs were averaged. For the determination of the rate constant, k, for the destruction of hirulog-1, measurements were obtained every 5 min at all five concentrations until all the inhibitor was inactivated.

In the determination of k for hirulog-1 consumption from progress curves, the velocity (v_s) of substrate hydrolysis was proportional to the enzyme–substrate complex (ES) concentration, whereas in the absence of inhibitor the velocity (v_0) was proportional to the total enzyme (E_t). The inhibition fraction (i) was EI/E_t or $1-(ES/E_t)$ or $1-(v_s/v_0)$. From this, the I_t/E_t ratio was obtained by eqn. 4 (Webb, 1963):

$$\frac{I_t}{E_t} = i + \frac{K_i'}{E_t}\left(\frac{i}{1-i}\right) \tag{4}$$

Because of an assay dilution factor (D) of 4- and 5-fold in clotting (see below) and amidolytic assays (see above) respectively, the total enzyme concentration (E_0) in the incubation mixtures is DE_t and the total inhibitor concentration (I_0) in the mixture is DI_t. Thus, eqn. (4) becomes eqn. (5):

$$\frac{I_0}{E_0} = i + \frac{K_i}{E_0}\left(\frac{i}{1-i}\right) \tag{5}$$

This allows the calculation of k for the destruction of the inhibitor according to eqn. (6) (Goldstein, 1944):

$$k = \left[\left(\frac{1}{1-i_0} + \ln\left(\frac{i_0}{1-i_0}\right) - E_1' \cdot \ln i_0\right) - \left(\frac{1}{1-i} + \ln\left(\frac{i}{1-i}\right) - E_1' \cdot \ln i\right)\right](E_1' t)^{-1} \quad (6)$$

Here i_0 is i at time zero, E_1' is E_0/K_i, and t is the time of duration of the enzyme–inhibitor reaction. Least-squares analysis provided by the Minsq software program was used to obtain v_s/v_0 and k values.

Fibrinogen-clotting activities

Clotting times were determined with a fibrometer equipped with a 0.40 ml probe and a constant-temperature block at 37 °C. Fibrinogen was reconstituted at 8.0 g/l in 150 mM-NaCl buffered with 10 mM-imidazole at pH 7.4, whereas the thrombins and/or hirulog-1 were diluted in 150 mM-NaCl/30 mM-$CaCl_2$/10 mM-imidazole buffer, pH 7.4, containing 8.8 g of poly(ethylene glycol) 6000/l (Fenton & Fasco, 1974). Concentrations of 8.29 nM and 8.64 nM for α-thrombin and ζ-thrombin respectively were incubated with seven concentrations of 0 nM- to 95 nM-hirulog-1 at approx. 23 °C for various times. To assay, 100 μl of the incubated mixture was added to 200 μl of the preceding dilution buffer that had been prewarmed to 37 °C in a fibrometer cup. Clotting was then initiated by adding 100 μl of fibrinogen solution with the fibrometer pipette, which activated the timer. Control samples (without hirulog-1) were also assayed twice every 5 min in between each hirulog-1 run, and this procedure was repeated three times for both α-thrombin and ζ-thrombin. Hirulog-1 samples were assayed immediately on mixing ($t = 0$) and then twice every 5 min until the control clotting time was obtained.

Although the clotting assay measures an end point, clotting times are an inverse velocity function and are linearly related to the reciprocal thrombin concentration in clotting units (CU) (Fenton *et al.*, 1986). From this relationship, the ratio of the inhibited thrombin activity (CU_s) to the control activity (CU_0) of thrombin alone is equal to the corresponding velocity ratio (v_s/v_0) and K_i was determined by eqns. (2) and (3), where 7.2 μM was used for the K_m for fibrinogen Aα cleavage by thrombin (Higgins *et al.*, 1983), assuming that this cleavage is rate-limiting (Fenton *et al.*, 1989). The inhibition (i) fraction is $1-(CU_s/CU_0)$. Values for k were determined from eqns. (4), (5) and (6).

Thrombin cleavage of hirulog-1

Hirulog-1 at 232 μM was incubated overnight at approx. 23 °C with 1.8 μM-α- or ζ-thrombin in 100 mM-NaCl/50 mM-Na_3BO_3, pH 8.4. These incubation mixtures were then chromatographed by reverse-phase h.p.l.c. on an Aquapore C_8 RP-300 column (0.46 mm × 100 mm) (Rainin, Emeryville, CA, U.S.A.) equilibrated with 0.1% (v/v) trifluoroacetic acid in water and developed with a linear gradient of 0–35% (v/v) acetonitrile in water over 45 min at 1.0 ml/min. The effluent was monitored at 214 nm, and peaks were collected manually. Products from α-thrombin incubation were identified by automated Edman degradation as described elsewhere (Maraganore *et al.*, 1990).

RESULTS AND DISCUSSION

The thrombin preparations used met specifications for high purity and enzyme activities (Table 1). These preparations contained more than 90% of their respective forms and titrated with p-nitrophenyl p'-guanidinobenzoate to more than 80% active enzyme. Both α-thrombin and ζ-thrombin possessed specific clotting activities of more than 3000 kCU/g, whereas γ-thrombin had 800-fold less clotting activity. Since trypsin cleaves three residues beyond the Aα-cleavage site for α-thrombin in fibrinogen (Lewis *et al.*, 1987) and lacks true clotting activity, this activity was not examined, but the trypsin preparation was 94% active on p-nitrophenyl p-guanidinobenzoate titration.

Like α-, γ- and ζ-thrombins, trypsin cleaves Spectrozyme-TH and exhibits a specificity constant ($k_{cat.}/K_m$) more similar to γ-thrombin than to α- or ζ-thrombin (Table 2). Although within ± 2 S.E.M., ζ-thrombin has the lowest K_m of the four preparations, indicating a slightly greater affinity, which is consistent with its higher p-nitrophenyl p'-guanidinobenzoate titration value (Table 1). Furthermore, ζ-thrombin binds somewhat more tightly to Amberlite CG-50 resin (cross-linked polymethylacrylic acid) (Brezniak *et al.*, 1990) and slightly more strongly to recombinant hirudin HV1 and HV2K47 variants than does α-thrombin (Witting *et al.*, 1991). Whereas both α-thrombin and ζ-thrombin were inhibited by hirulog-1 in both Spectrozyme-TH amidolytic and clotting assays, neither γ-thrombin nor trypsin was inhibited in the amidolytic assay with more than 1000-fold molar excess of hirulog-1 (Table 3). This finding thus demonstrates that clotting activity and inhibition by hirulog-1 are correlated. Such a

Table 1. Evaluation of human thrombin and bovine trypsin preparations

Preparations were analysed as described elsewhere (Fenton *et al.*, 1977*a*,*b*; Brezniak *et al.*, 1900).

Enzyme preparation	Enzyme form (%)			Enzyme activities	
	α	β	γ (ζ)*	p-Nitrophenyl p'-guanidinobenzoate titration (% active)	Fibrinogen clotting (kCU/g)†
α-Thrombin (no. 320)	92.27	7.53	0.01	95.9 ± 3.0 ($n = 6$)	3335 ± 102 ($n = 5$)
γ-Thrombin (no. 72)	0.01	0.00	99.00	83.0 ± 4.7 ($n = 4$)	3.94 ± 0.30 ($n = 5$)
ζ-Thrombin (no. 16)	5.40	2.70	91.90	100 ± 3.9 ($n = 6$)	3227 ± 135 ($n = 6$)
Trypsin	–	–	–	94.1 ± 3.3 ($n = 4$)	–

* Both γ- and ζ-thrombins labelled with [^{14}C]di-isopropyl phosphorofluoridate co-migrate on electrophoresis in 0.1% SDS/10% cross-linked polyacrylamide gels.

† Clotting activities are expressed in U.S. NIH-equivalent kiloclotting units/g of protein.

Table 2. Kinetic parameters for Spectrozyme-TH with human thrombins and bovine trypsin

Preparations are described in Table 1. Spectrophotometric measurements at 405 nm were determined with 18 μM-Spectrozyme-TH in 150 nM-NaCl buffered with 10 mM-Hepes/10 mM-Tris buffer, pH 7.4, containing 2.5 g of poly(ethylene glycol) 6000/l at approx. 23 °C (see the text). Enzyme concentrations are from *p*-nitrophenyl *p*′-guanidinobenzoate titrations in Table 1.

Enzyme preparation	Concn. of enzyme (nM)	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($\mu M^{-1} \cdot s^{-1}$)
α-Thrombin	5.51	1.87±0.37 (*n* = 8)	27.7±3.3 (*n* = 8)	14.8
γ-Thrombin	5.56	2.39±0.92 (*n* = 3)	24.0±4.9 (*n* = 3)	10.0
ζ-Thrombin	5.23	1.10±0.37 (*n* = 3)	17.0±3.1 (*n* = 3)	15.4
Trypsin	6.27	3.47±0.73 (*n* = 4)	24.1±4.2 (*n* = 4)	7.0

Table 3. Hirulog-1 kinetic parameters determined by Spectrozyme-TH amidolytic and fibrinogen clotting activities with human thrombin and bovine trypsin preparation

Values for K_i were determined at time zero by the tight-binding method; *k* values were based on inhibitor consumption. Incubations were carried out at approx. 23 °C as in Fig. 1. Enzyme preparations are those in Table 1. Amidolytic activities were measured with Spectrozyme-TH in 150 nM-NaCl buffered with 10 mM-Hepes/10 mM-Tris buffer, pH 7.3, containing 2.5 g of poly(ethylene glycol) 6000/l at approx. 23 °C (see the text). Clotting activities were measured with 2.0 g of fibrinogen/l in 150 nM-NaCl buffered with 10 mM-imidazole buffer, pH 7.4, containing 30 mM-$CaCl_2$ and 6.6 g of poly(ethylene glycol) 6000/l at 37 °C. Parameters were determined from I_{50} values (see the text). Enzyme concentrations were based on *p*-nitrophenyl *p*′-guanidinobenzoate titrations.

	Spectrozyme-TH			Fibrinogen		
Enzyme preparation	Concn. of enzyme (nM)	K_i (nM)	*k* (min^{-1})	Concn. of enzyme (nM)	K_i (nM)	*k* (min^{-1})
α-Thrombin	5.51	2.56±0.35 (*n* = 3)	0.326±0.082 (*n* = 12)	8.21	1.31±0.37 (*n* = 3)	0.311±0.023 (*n* = 10)
γ-Thrombin	5.56	No inhibition with 7.85 μM-hirulog-1	(*n* = 3)			
ζ-Thrombin	5.23	1.84±0.15 (*n* = 3)	0.362±0.056 (*n* = 18)	8.64	1.88±0.58 (*n* = 3)	0.403±0.054 (*n* = 9)
Trypsin	6.27	No inhibition with 7.85 μM-hirulog-1	(*n* = 3)			

correlation requires that both the catalytic site and adjacent regions and the fibrin(ogen) recognition exosite be involved, which is after all the design of hirulog-1 (see the Introduction).

While carrying out detailed kinetic experiments with hirulog-1 by tight-binding analysis, we found that, unlike with hirudin (Witting *et al.*, 1991), the potency of hirulog-1 depended on the length of incubation time with α-thrombin. This time-dependency was observed for incubations with either α- or ζ-thrombin assayed by amidolytic and clotting assays (Table 3 and Fig. 1). These incubations were carried out at approx. 23 °C because both thrombins exhibit similar stabilities at this temperature, in contrast with 37 °C, where ζ-thrombin is less stable than α-thrombin (Brezniak *et al.*, 1990). The observed loss of inhibitory activities thus suggested that hirulog-1 was being consumed by degradation (e.g. enzyme cleavage) or by loss (e.g. vessel wall adsorption, phase partitioning). The most likely explanation of the cleavage of the Arg-3–Pro-4 bond was verified by incubating hirulog-1 with either α- or ζ-thrombin overnight at pH 8.4 and approx. 23 °C. Both incubation mixtures gave identical products on h.p.l.c. being eluted with 9.8 or 9.8% and with 26.5 or 26.7% acetonitrile for α- or ζ-thrombin incubations respectively. For the α-thrombin products, the first was identified as the *N*-terminal fragment by three Edman degradation cycles and the second as the *C*-terminal fragment by 17 cycles, thus establishing the single cleavage at the Arg-3–Pro-4 bond in hirulog-1.

Because hirulog-1 is hydrolysed, our previously reported value of 2.3 nM (Maraganore *et al.*, 1990) must be considered as a K_i' value, and the true K_i must be defined at time zero before any degradation. The turnover rate *k* thus describes the cleavage rate. Because of the nanomolar value for K_i', these values were determined by the tight-binding method (Morrison, 1969, 1982; Henderson, 1972), as employed for thrombin–hirudin binding (Stone & Hofsteenge, 1986; Stone *et al.*, 1987; Braun *et al.*, 1988; Witting *et al.*, 1991). Implicit in the derivation of the expressions in this method is the assumption of competitive inhibitions. That hirulog-1 behaved as a competitive inhibitor of Spectrozyme-TH hydrolysis by α-thrombin is shown by the common intercept on the ordinate of the plot of $1/v_s$ versus $1/S$ (Mahler & Cordes, 1966) (Fig. 2). Values for *k* were calculated on the basis of a consumptive inhibitor model (Goldstein, 1944).

Although the incubations of hirulog-1 and the Spectrozyme-TH amidolytic assays were carried out at approx. 23 °C, the fibrinogen-clotting assays were at 37 °C. In further contrast, the amidolytic assay is kinetic, whereas the clotting assay measures the time for an end point. Nevertheless, clotting I_{50} values (i.e. concentrations giving 50% inhibition) approximate K_i', which can be converted into a K_i estimate on the assumption that the fibrinogen Aα cleavage is rate-limiting in the clotting process (Fenton *et al.*, 1989). Such approximations are applicable for moderate-affinity inhibitors but not for those with very high affinities (Webb, 1963), such as hirudin (J. I. Witting & J. W. Fenton II, unpublished work). Thus we were pleased by the closeness of our K_i and *k* values obtained by either amidolytic or clotting assays (Table 3). Of note, the 'specificity constants' from

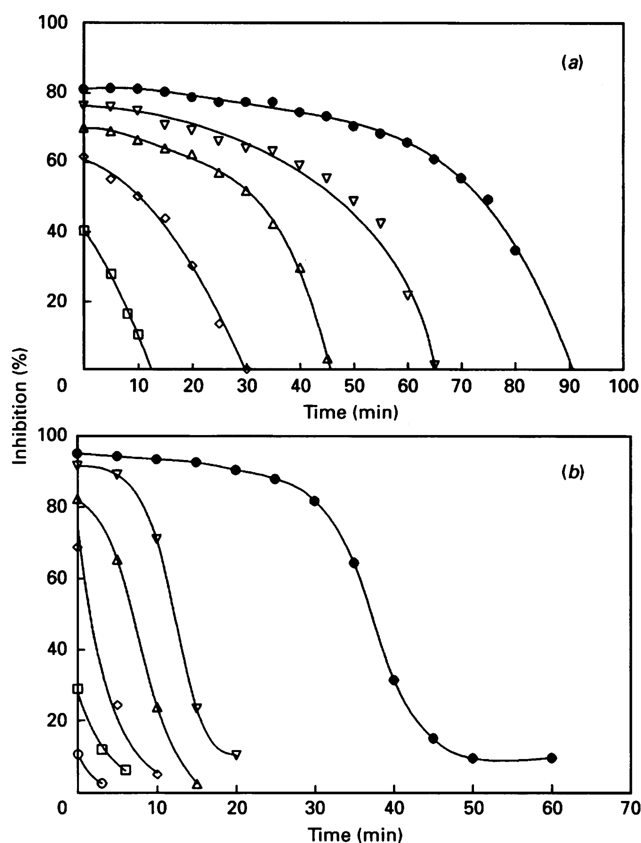

Fig. 1. Inhibition progress plots with hirulog-1

(*a*) Human α-thrombin at 27.6 nM was incubated with 95.0 nM- (□), 190 nM- (◇), 285 nM- (△), 380 nM- (▽) and 475 nM- (●) hirulog-1 in 150 mM-NaCl/10 mM-Hepes/10 mM-Tris buffer, pH 7.4, containing 2.5 g of poly(ethylene glycol) 6000/l at approx. 23 °C. Assays were performed by adding 200 μl of the incubation mixtures to 800 μl of 22.5 μM-Spectrozyme-TH in the preceding buffer at approx. 23 °C. A determination was made every 5 min until hirulog-1 was inactivated. Control samples without hirulog-1 were assayed before the zero-time incubation, between each determination and after the final determination. (*b*) Human α-thrombin at 32.8 nM was incubated with 5.96 nM- (○), 11.9 nM- (□), 47.6 nM- (◇), 95.0 nM- (△), 190 nM- (▽) and 380 nM- (●) hirulog-1 in 150 mM-NaCl/30 mM-$CaCl_2$/10 mM-imidazole buffer, pH 7.4, containing 8.8 g of poly(ethylene glycol) 6000/l at approx. 23 °C. Clotting assays were performed with a fibrometer at 37 °C by adding 100 μl of the incubation mixture to 200 μl of the preceding buffer warmed to 37 °C. Clotting was initiated with the addition of 100 μl of fibrinogen (8 g/l) in 150 mM-NaCl/10 mM-imidazole buffer, pH 7.4, warmed to 37 °C. Starting at the zero-time incubation, duplicate determinations were made every 5 min until hirulog-1 became inactive. Duplicate control samples without hirulog-1 were assayed before the zero-time incubation, between each determination and after the final determination.

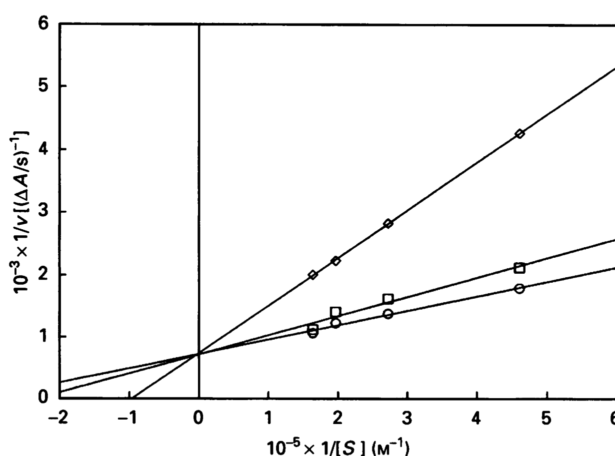

Fig. 2. Lineweaver–Burk plot of $1/v_0$ versus 1/[S] at different concentrations of hirulog-1

Human α-thrombin at 27.6 nM was mixed with 0 nM- (○), 155 nM- (□) and 255 nM- (◇) hirulog-1 in 150 mM-NaCl/10 mM-Hepes/10 mM-Tris buffer, pH 7.4, containing 2.5 g of poly(ethylene glycol) 6000/l at approx. 23 °C. Assays were performed by adding 200 μl of the α-thrombin and hirulog-1 mixtures to 800 μl of the preceding buffer containing 1.25 μM- to 6.25 μM-Spectrozyme, and data were analysed by linear regression. Respective correlation coefficients were 0.993, 0.981 and 0.999, and a common $1/v_s$ intercept was obtained for $1/v_s = (1/V_{max.}) + [K_m/V_{max.}\, S(1 + I/K_i)]$.

the k/K_i ratios for these data range from 2.1 to 3.6 $\mu M^{-1} \cdot s^{-1}$ and are an order of magnitude lower than those from the $k_{cat.}/K_m$ ratios for Spectrozyme-TH (Table 2) and other tripeptide *p*-nitroanilide substrates (Witting *et al.*, 1987), as well as fibrinogen Aα cleavage by α-thrombin (Higgins *et al.*, 1983). This implies that hirulog-1 has comparatively poor substrate properties. In this regard, *k* values of hirulog-1 are three to four orders of magnitude lower than the fibrinogen Aα cleavage $k_{cat.}$ value. However, the affinity of hirulog-1 for α-thrombin is about three orders greater than that of fibrinogen.

Clearly the binding of fibrinogen is unable to compete with the high affinity of hirulog-1 for α-thrombin, with the consequence that hirulog-1 is a highly effective inhibitor of fibrinogen clotting. On the other hand, the nanomolar K_i value of α-thrombin for hirulog-1 is similar to that for thrombomodulin (Owen & Esmon, 1981; Hofsteenge *et al.*, 1986; Jakubowski *et al.*, 1986) and to the K_d values for high-affinity binding sites on platelets (Martin *et al.*, 1976), fibroblasts (Glenn *et al.*, 1980; Perdue *et al.*, 1981) as well as endothelial cells (Awbrey *et al.*, 1979). This would suggest that high-affinity binding proteins or sites on various cell types should compete for α-thrombin *in vivo*. However, α-thrombin is actively incorporated into the forming fibrin clot (or thrombus), where it remains partitioned from the blood (Wilner *et al.*, 1981). Such thrombus-entrapped thrombin is protected from blood-borne inhibitors, such as antithrombin III, which cannot readily permeate the fibrin matrix (Weitz *et al.*, 1990) and is believed to serve as a source for thrombin in post-clotting and wound-healing events (Fenton, 1988; Fenton *et al.*, 1991).

Hirudin is approx. 3-fold larger than hirulog-1, and its complex with α-thrombin is essentially irreversible even though it is non-covalent because of the upper to mid femtomolar K_i values of the complex (Stone & Hofsteenge, 1986; Braun *et al.*, 1988; Witting *et al.*, 1991). The hirulog-1 complex in contrast has four to five orders of magnitude less affinity and presumably forms an enzyme–substrate (or inhibitor) complex that was found to turn over slowly (Table 3). Furthermore, the first cleavage product (residues 4–20) of hirulog-1 contains the desulphonated hirudin tail fragment 'hirugen', which has a micromolar K_i value for α-thrombin (Maraganore *et al.*, 1989). Thus hirulog-1 is hydrolysed to a mild thrombin inhibitor, which should not interfere with thrombin generation amplification (e.g. Factor V and Factor VIII activation) and should be less apt to cause any bleeding tendencies (Fenton *et al.*, 1991). Moreover, thrombin regenerated from the hirulog-1 complex could be cleared by inhibitors in blood (e.g. antithrombin III) or have beneficial functions in healing processes (Fenton, 1988).

Hirulog-1 was designed before there was any knowledge of the crystallographic structure of α-thrombin and complexes with

hirudins and was designed so that it binds to and bridges between the thrombin catalytic site and exosite for fibrinogen recognition (see the Introduction). Although the Pro-2–Arg-3–Pro-4 sequence in hirulog-1 resembles that of Pro-46–Lys-47–Pro-48 in hirudin HV1, Lys-47 is not a prerequisite for hirudin complex-formation, since it is replaced by asparagine in hirudin HV2 (Harvey *et al.*, 1986; Degryse *et al.*, 1989) and by other residues in various recombinant hirudins without substantial loss in inhibitory activities (Braun *et al.*, 1988; Dodt *et al.*, 1988; Degryse *et al.*, 1989). Furthermore, when Pro-48 is replaced by alanine, α-thrombin does not cleave this hirudin variant (Dodt *et al.*, 1990), as would be predicted by the present data if Lys-47 were accessible to the catalytic apparatus of the enzyme. In the crystallographic structure of the α-thrombin–hirudin complex, Lys-47 projects away from the specificity pocket to interact with Trp-60D (chlymotrypsin numbering) in the thrombin B-chain (Rydel *et al.*, 1990). Thus, in agreement with the long-recognized fact that the thrombin–hirudin complex is non-covalent (Markwardt, 1970; Fenton *et al.*, 1979), the hirudin Lys-47–Pro-48 bond is inaccessible to the thrombin catalytic apparatus and is consequently non-cleavable. Nevertheless, the present finding that thrombin can slowly cleave an imide Arg–Pro bond suggests that hirudin may have evolutionarily evolved from a protein with a Lys–Pro (or Arg–Pro) linkage, whose mechanism of inhibition was that of slow cleavage of an imide bond to one where such a linkage is inaccessible to proteolysis by thrombin. In these regards, examples of slowly cleaved imide bonds in proteins may be found where such proteins serve as slow turnover inhibitors or those with delayed activation functions.

In summary, hirulog-1 is a bivalent 20-residue peptide designed to bridge-bind at regions adjacent to the catalytic site and at the anion-binding exosite for fibrin(ogen) recognition. It is a specific inhibitor of thrombin forms with high clotting activities (α- and ζ-thrombins) and is slowly cleaved at its Arg-3–Pro-4 bond.

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