BIOCHEMICAL LETTERS

Hirulog-1 and -B2 thrombin specificity

In a preceding issue of this journal, we reported the tight-binding K_i values for the peptide inhibitor, hirulog-1 [(D-F)-PRP(G)₄NGDFEETPEEYL], with human α - and ζ -thrombins [1]. In contrast to the situation with both of these thrombin forms with high fibrinogen clotting activities, we were unable to detect inhibition of γ -thrombin (which has essentially no clotting activity) with up to ~ 1500 -fold molar excess of inhibitor to enzyme or of trypsin with > 1000 -fold excess of inhibitor. In subsequent experiments employing much greater inhibitor concentrations, we have found inhibition of γ -thrombin, but not cleavage, while trypsin still was not inhibited. We also report here K_i and k parameters for a more potent analogue, hirulog-B2, which has D-cyclohexylalanine substituted in the first position [2].

As with D-phenylalanine in hirulog-1, this substitution presumably interacts with the subsite formed by Y60a and W60d in the thrombin B chain, where F8 in the fibrinogen A α chain binds [3,4]. Furthermore, this subsite comprises a portion of the apolar binding site immediately preceding the thrombin catalytic site [5] and is independent of the subsequent anion-binding exosite for fibrin(ogen) recognition [6], hirudin tail fragments [6,7], thrombin receptors, and other substances [8,9]. Moreover, hirulogs are designed as bridged bivalent inhibitors interacting with both the apolar binding site and the preceding exosite [1,10].

Hirulogs were synthesized as described [2,3]. Spectrozyme-TH (D-hexahydrotyrosyl-AR *p*-nitroanilide) was a gift from Dr. Richard Hart, American Diagnostic, Greenwich, CT, U.S.A. The thrombin and trypsin preparations were the same as employed in our preceding paper [1]. Kinetic parameters were measured at $\sim 23^\circ\text{C}$ in 150 mM-NaCl/10 mM-Hepes/10 mM-Tris buffer at pH 7.4, containing 2.5 g/l of poly(ethylene glycol) 6000, and K_i and k values were determined for α - and ζ -thrombin by tight-binding and consumptive inhibitor methods [1].

Because γ -thrombin exhibited neither tight-binding nor inhibitor consumption, K_i values were determined by conventional methods [11]. Steady-state velocities were obtained with (22.8 or 20.8 μM -hirulog-1 or -B2, respectively) or without inhibitor for eight substrate concentrations (1–100 μM -Spectrozyme-TH and 5.9 nM- γ -thrombin). Velocities were then fitted by nonlinear regression to the relationship:

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

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. In the competitive situation, K_m is increased by the factor of $1 + I/K_i = K_p$, where I is the initial

Fig. 1. Lineweaver-Burke plots of $1/v_s$ versus $1/S$ for human α -, γ -, and ζ -thrombins with hirulog-B2 in buffer saline at pH 7.4 and $\sim 23^\circ\text{C}$ (see the text)

(a) Human α -thrombin. Assays were performed by adding 100 μl of buffer (○) or 10.9 nM-hirulog-B2 (□) to 800 μl of 6.22–150 μM -Spectrozyme-TH followed by 100 μl of 55.1 nM- α -thrombin. Respective correlation coefficients (r) were 0.895 and 0.909. (b) Human

γ -thrombin. The inhibitor concentration was the same; substrate concentration were 0.53–99.7 μM for 46.3 nM-enzyme. Correlation constants were 0.937 and 0.936. (c) Human ζ -thrombin. The inhibitor concentration was unchanged; substrate concentrations were 0.53–99.7 μM for 46.3 nM enzyme. The correlation constants were 0.972 and 0.999. In all three plots, a common $1/v_s$ intercept was obtained for:

$$1/v_s = (1/V_{\max}) + [K_m/V_{\max} S(1 + I/K_i)]$$

Table 1. Hirulog-1 and -B2 kinetic parameters determined by Spectrozyme-TH amidolytic activities with human thrombin and bovine trypsin preparations

Amidolytic activities were measured with Spectrozyme-TH in 150 mM-NaCl buffered with 10 mM-Hepes and 10 mM-Tris containing 2.5 g/l of PEG 6000 at pH 7.4 and 23 °C. Values for K_i were determined at time zero by tight binding method for α - and ζ -thrombin and by conventional kinetics for ζ -thrombin; k values were based on inhibitor consumption.

Enzyme* preparation	Enzyme† concn. (nM)	Hirulog-1		Hirulog-B2	
		K_i (nM)	k (min ⁻¹)	K_i (nM)	k (min ⁻¹)
α -Thrombin	5.51	2.56 ± 0.35 ($n = 3$)‡	0.326 ± 0.082 ($n = 12$)‡	0.077 ± 0.004 ($n = 4$)	0.197 ± 0.042 ($n = 11$)
γ -Thrombin	4.63	24 300 ± 831 ($n = 3$)	§	12 240 ± 460 ($n = 4$)	§
ζ -Thrombin	5.23	1.84 ± 150 ($n = 3$)‡	0.362 ± 0.056 ($n = 18$)‡	0.064 ± 0.003 ($n = 3$)	0.178 ± 0.018 ($n = 4$)
Trypsin	5.34	No inhibition with 22.8 μ M ($n = 3$)		No inhibition with 20.8 μ M ($n = 3$)	

* Enzyme preparations are those in Table 1 in [1].

† Enzyme concentrations were based on active-site titration.

‡ Values from Table 3 in [1].

§ There was no consumption of 22.8 or 20.8 μ M hirulog-1 or -B2, respectively, when incubated at 23 °C with γ -thrombin for up to 20 h.

inhibitor concentration. The least-squares analysis of the non-linear curve-fitting program of Tablecurve (Jandel Scientific, Corte Madera, CA, U.S.A.) was used to determine K_m (without inhibitor and K_p (with inhibitor). Then K_i is calculated from the relationship:

$$K_i = K_m I / (K_p - K_m).$$

As with hirulog-1, both α - and ζ -thrombins with hirulog-B2 gave linear plots, showing it to behave as a competitive inhibitor (Figs. 1a and 1c), and enabling the use of tight-binding analysis [1]. Competitive inhibition of γ -thrombin was also observed with hirulog-1 (data not shown) and -B2 (Fig. 1b), validating the kinetic methods employed [11]. On the other hand, such kinetic behaviour should be considered to be 'pseudo-competitive', since the hirulogs are divalent inhibitors binding at both the catalytic site and adjacent regions, as well as the anion-binding exosite for fibrinogen recognition [1,10]. Under other experimental conditions, hirulogs do not display competitive inhibition (E. Di Cera, personal communication).

Hirulog-B2 was bound 33.2- and 28.8-fold tighter than hirulog-1 to α - and ζ -thrombins, while the cleavage rates were decreased 1.65- and 2.03-fold, respectively (Table 1). As previously found for hirulog-1 [1], ζ -thrombin bound hirulog-B2 slightly tighter (but within ± 2 S.E.M.) than α -thrombin (Table 1). In marked contrast, hirulog-B2 was bound only ~ 2 -fold more tightly than hirulog-1 with γ -thrombin, while γ -thrombin bound hirulog-1 and -B2, respectively, ~ 4 and > 6 orders of magnitude less tightly than α -thrombin. Furthermore, no consumption of either inhibitor was observed within 20 h, implying that γ -thrombin cannot cleave the arginyl-prolyl bond in these hirulogs, as shown for α - and ζ -thrombins with hirulog-1 [1]. No inhibition with either hirulog was found for trypsin with up to ~ 4000 -fold inhibitor to enzyme (Table 1).

These data demonstrate that both hirulog-1 and -B2 are highly specific for thrombin forms with high fibrinogen clotting activities

(e.g. α - and ζ -thrombins). The potential pharmaceutical applications of hirulog-1 are presently under investigation [12].

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Joyce I. WITTING*, Paul BOURDON†, John M. MARAGANORE‡, and John W. FENTON II*§

*New York State Department of Health, Wadsworth Center for Laboratories and Research, P.O. Box 509, Albany, NY 12201, U.S.A.; †Biogen Inc., 14 Cambridge Center, Cambridge, MA 02142, U.S.A.; and §Department of Physiology and Cell Biology and Department of Biochemistry and Molecular Biology, Albany Medical College of Union University, Albany, NY 12208, U.S.A.

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