Kinetic Mechanism for the Interaction of Hirulog with Thrombin†

Marina A. A. Parry,‡ John M. Maraganore,§ and Stuart R. Stone*,‡

Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K., and Biogen Inc., 14 Cambridge Center, Cambridge, Massachusetts 02142

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ABSTRACT: Hirulog (D-FPRPGGGGDGDFEEIPEEYL) is a bivalent inhibitor of thrombin consisting of a moiety (D-FPRP) that binds to the active-site cleft and a hirudin-like C-terminal region (DGDFEEIPEEYL) that binds to the positively charged surface groove of thrombin known as the anion-binding exosite. The formation of the thrombin−Hirulog complex was studied using steady-state and rapid kinetics at 37 °C. The inhibition constant for Hirulog was found to be 1.9 nM. Hirulog was slowly degraded by thrombin with a k_{cat} value of 0.01 s^{-1}. The formation of the complex resulted in an enhancement of 44% in the intrinsic fluorescence of thrombin. The kinetics of the increase in thrombin fluorescence were described by a double-exponential decay. The dependence of the rate constant for the fast phase on the concentration of Hirulog could be described by the Michaelis−Menten equation with K_m and k_{max} values of 0.75 ± 0.12 μM and 325 ± 17 s^{-1}. The data were consistent with a mechanism in which the C-terminal region of Hirulog binds to the anion-binding exosite with a dissociation constant of 0.75 μM in the first step, followed by two intramolecular steps with rate constants of about 300 and 30 s^{-1}. A C-terminal fragment of hirudin was found to compete in the first step confirming that this process corresponded to the binding of the hirudin-like C-terminus of Hirulog to the anion-binding exosite. The results of experiments using the fluorescent probe *p*-aminobenzamidine suggested that an additional very rapid step, in which Hirulog is bound to the active site, occurs between the processes with rate constants of 300 s^{-1} and 30 s^{-1}. The steps following exosite binding resulted in 400-fold increase in the stability of the complex. The effective association rate constant at low Hirulog concentrations was 4×10^8 M^{-1} s^{-1}, while the effective dissociation rate constant was about 1 s^{-1}; thus, under the conditions used, the release of cleaved Hirulog was not a significant pathway for the dissociation of the complex.

Thrombin is a trypsin-like protease whose activity is central to both haemostasis and thrombosis. It is generated after activation of the coagulation cascade, and once produced, it is able to cleave fibrinogen to yield fibrin monomers which polymerize to form a hemostatic plug. Thrombin further stimulates its own production by activating factors V and VIII, essential cofactors in the cascade (Davie et al., 1991). The activity of thrombin is controlled by the inhibitor antithrombin III and by thrombin's activation of the protein C anticoagulant pathway. When complexed to the endothelial cell protein thrombomodulin, thrombin activates protein C, which turns off the coagulation cascade by degrading factors Va and VIIIa (Esmon, 1989). Thrombin's activity can be therapeutically controlled by the administration of heparin, which markedly increases the inhibitory potential of antithrombin III. However, there are certain conditions, such as arterial thrombosis, in which heparin is relatively ineffective. Thus, there has been considerable interest in developing thrombin inhibitors as therapeutic agents (Tapparelli et al., 1993). One such inhibitor is hirudin, a 65 residue polypeptide that was originally isolated from the medicinal leech. Recombinant hirudin (rhir)[1] rapidly forms a tight complex with thrombin (Dodt et al., 1988; Braun et al., 1988). The tightness of the complex can be attributed to the extended area of the interaction between thrombin and rhir. The C-terminal region of rhir binds to a positively charged surface groove of thrombin, termed the anion-binding exosite, while the N-terminus of the molecule binds to the active-site cleft in an orientation that had not previously been observed (Rydel et al., 1990, 1991; Grütter et al., 1990; Vitali et al., 1992). Conventional and stopped-flow kinetics have been used to investigate the mechanism for the inhibition of thrombin by hirudin (Stone & Hofsteenge, 1986; Stone et al., 1989; De Cristofaro et al., 1992; Jackman et al., 1992). The formation of the rhir−thrombin complex involves at least three steps; the initial interaction between the C-terminal region of rhir and the anion-binding exosite is followed by at least two intramolecular steps (Jackman et al., 1992).

Hirulog is another inhibitor of thrombin with therapeutic potential (Weitz & Hirsh, 1993; Lidon et al., 1993). It is composed of a moiety (D-FPRP) that binds to the active site of thrombin in a substrate-like manner and a C-terminal region (DGDFEEIPEEYL) based on rhir which binds to the anion-binding exosite (Maraganore et al., 1990). These two

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* Address correspondence to this author at the Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K. Tel: (+44-223) 336838; Fax: (+44-223) 336827.

‡ University of Cambridge.

§ Biogen Inc.

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[1] Abbreviations: rhir, recombinant hirudin variant 1; rhir(52−65), the C-terminal fragment of rhir comprising residues 52−65; PAB, *p*-aminobenzamidine; Hirulog, Hirulog®, which was originally described as Hirulog-1 (Maraganore et al., 1990) and has the sequence D-FPRPGGGGDGDFEEIPEEYL.

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regions of Hirulog are joined by a polyglycine linker. Hirulog inhibits thrombin with a K_i value of about 2 nM and is slowly cleaved by thrombin at the Arg-Pro bond with a k_{cat} value of 0.3 min^{-1} at 23 °C (Witting et al., 1992). The crystal structures of complexes of thrombin with cleaved Hirulog and a cleavage-resistant analog have been determined (Skrzypczak-Jankun et al., 1991; Qiu et al., 1992). The structures of D-FPR and the hirudin-like C-terminal region of Hirulog are very similar to those of the analogous regions of D-FPRCH$_2$- and rhir bound to thrombin (Bode et al., 1989; Rydel et al., 1991; Skrzypczak-Jankun et al., 1991; Qiu et al., 1992).

The current study investigates the steps involved in the formation of the Hirulog−thrombin complex. The results are consistent with a four-step mechanism for complex formation. The binding of Hirulog to thrombin was found to result in an increase in the intrinsic fluorescence of thrombin. The kinetics of the increase in fluorescence suggested that at least three steps were involved in the formation of the Hirulog−thrombin complex. By using a fragment of rhir that binds to the anion-binding exosite, it was possible to show that the initial step corresponded to the binding of the C-terminus of Hirulog to this exosite. The kinetics of displacement of the active-site probe *p*-aminobenzamidine (PAB) by Hirulog suggested the occurrence of an additional rapid step that corresponded to the binding of Hirulog to the active site.

EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin was prepared as described by Stone and Hofsteenge (1986) and was fully active as determined by active site titration using 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). Hirulog (D-FPRPGGGGDGDFEEIPEEYL) and the peptide corresponding to residues 52−65 of rhir (NDGDFEEIPEEYLQ), designated rhir(52−65), were prepared as described previously (Maraganore et al., 1990; Jackman et al., 1992). Amino acid analysis was used to confirm the composition of Hirulog and rhir(52−65) and to determine their concentrations. *p*-Aminobenzamidine (PAB) was purchased from Sigma (Poole, U.K.) and its concentration determined from its absorbance at 293 nm (Olson & Shore, 1982). The chromogenic substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide was from Chromogenix (Mölndal, Sweden).

Reaction Conditions. All kinetic and fluorescence experiments were performed at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% (w/v) poly(ethylene glycol), M_r 6000.

Amidolytic Assays. The inhibition constants (K_i) for PAB and Hirulog were determined in assays containing 20−50 pM thrombin and 50 μM D-Phe-pipecolyl-Arg-*p*-nitroaniline. The rate of cleavage of the substrate was followed at 400−410 nm in a Hewlett-Packard 8452A spectrophotometer (Stone et al., 1991).

Fluorescence Measurements. Experiments were performed using an Applied Photophysics SF17.MV stopped-flow fluorometer. For intrinsic protein fluorescence studies, an excitation wavelength of 280 nm and a bandwidth of 12 nm were used with an 320 cutoff filter between cell and fluorescence detector. The experimental conditions used with the fluorescent probe PAB were similar to those of Olson and Shore (1982); the excitation wavelength was 335 nm with a bandwidth of 5 nm, and a 345 nm cutoff filter was used for the emission. A dead time of 2.2 ± 0.2 ms between flow triggering and the start of data collection was determined for the stopped-flow fluorometer (Jackman et al., 1992). For data analysis, the average of 3−10 individual traces was used. All data were acquired under pseudo-first-order conditions with the ligand concentration being at least 8-fold greater than that of thrombin. Rate constants and amplitudes of the averaged traces were obtained by fitting the data to equations describing a single- or double-exponential decay using the SF17.MV nonlinear regression software. Only data acquired at times greater than 2.5 ms after the start of data collection were used in the analysis of intrinsic fluorescence decays. The amplitudes of the fluorescence change for the exponential decays (ΔF_{obs}) were determined by extrapolating the fit back to the beginning of the dead time using the following relationship: $\Delta F_{obs} = \Delta F_{fit}/\exp[-(2.2 \times 10^{-3})k_{obs}]$, where ΔF_{fit} is the fitted amplitude for the decay from the start of data collection. The intrinsic fluorescence of thrombin was linearly proportional to its concentration in the range studied.

Rate of Cleavage of Hirulog by Thrombin. The rate of cleavage at 37 °C was determined by incubating 2 μM Hirulog with 63 nM thrombin for 5−15 min in the reaction buffer at 37 °C. The reaction was stopped by adding 120 nM rhir, and the reaction products were separated by reverse-phase HPLC on a C_4 column. A gradient of 7−35% acetonitrile (v/v) in 0.1% trifluoroacetic acid over 30 min at a flow rate of 1 mL/min was used. The position of elution of the larger fragment of cleaved Hirulog (PGGGGDGD-FEEIPEEYL) was determined by incubating 2 μM Hirulog with 120 nM thrombin for 4 h; under these conditions, no intact Hirulog was observed. The amount of cleaved product was determined from the area of its peak at 214 nm using fully cleaved Hirulog as a standard.

DATA ANALYSIS

Steady-State Kinetics. The K_i values for Hirulog and PAB were determined from the inhibition of the steady-state velocity of thrombin's cleavage of D-Phe-pipecolyl-Arg-*p*-nitroaniline. At least 14 assays were performed for each K_i determination; the concentration range contained values above and below the apparent inhibition constant (K_i'). The data could be described by the Dixon equation (Segel, 1975), and nonlinear regression analysis according to this equation yielded values for K_i'. These values were corrected for the competing effect of substrate by using the relationship $K_i = K_i'/(1+[S]/K_m)$; previously determined K_m values for D-Phe-pipecolyl-Arg-*p*-nitroaniline in the absence (Stone & Hofsteenge, 1986) and presence of rhir(52−65) (Dennis et al., 1990) were used in these calculations.

Pre-Steady-State Kinetics. For reaction mechanisms involving more than one step, a hyperbolic dependence of one or more of the rate constants on the concentration of ligand can be observed (Hiromi, 1979; Hammes & Schimmel, 1970). This hyperbolic dependence of the observed rate constant (k_{obs}) will take the form of the following equation:

$$k_{obs} = \frac{k_{max}}{1 + K_m/[I]} + k_{int} \quad (1)$$

This equation has the same form as the Michaelis−Menten

equation except that a nonzero intercept (k_{int}) with the y-axis is observed; k_{max} is the maximum value of k_{obs} which occurs at infinite concentrations of ligand (I), and K_m is the concentration of ligand at which $k_{obs} = 0.5(k_{max} - k_{int})$. Equation 1 is equivalent to the Michaelis−Menten equation when k_{int} is equal to zero as in the equation:

$$k_{obs} = \frac{k_{max}}{1 + K_m/[I]} \quad (2)$$

When a hyperbolic dependence of the observed rate constant on the concentration of Hirulog was observed, the values of k_{obs} were fitted to eqs 1 and 2 using weighted nonlinear regression. In all cases, eq 2 yielded a better fit to the data.

RESULTS

Steady-State Kinetic Parameters for the Inhibition of Thrombin by Hirulog and PAB. The K_i values for Hirulog and PAB were determined using the amidolytic assay described in Experimental Procedures. The K_i value for Hirulog was found to be 1.9 ± 0.1 nM. This value compares well with the value of 2.6 nM determined by Witting et al. (1992) at 23 °C. Hirulog is slowly cleaved at its Arg^3-Pro^4 bond by thrombin (Witting et al., 1992). The catalytic constant for the cleavage of Hirulog at 37 °C was estimated by saturating the enzyme with the inhibitor and determining its rate of cleavage by HPLC analysis. When an enzyme is saturated with substrate, the k_{cat} value will be given by the rate of product release divided by the enzyme concentration. The k_{cat} value for Hirulog with thrombin was found to be $(1.2 \pm 0.1) \times 10^{-2}$ s^{-1} ($n = 9$) at 37 °C. This value is slightly higher than the value of 5×10^{-3} s^{-1} determined at 23 °C by Witting et al. (1992). Conditions were chosen in steady-state and rapid kinetic experiments such that no significant cleavage of the inhibitor would occur during the time course of the experiments. Under the conditions of the steady-state experiments, it could be calculated that less than 1% of Hirulog was cleaved, while less than 0.01% was cleaved during the short time courses (0.02−0.5 s) of the rapid kinetic experiments.

The K_i value determined for PAB was 117 ± 7 μM which is somewhat higher than the value of 67 μM determined at 25 °C by Olson and Shore (1982). The dissociation constant for rhir(52−65), which corresponds to the C-terminal region of rhir, is 1.5 μM (Jackman et al., 1992). In the presence of a saturating concentration (27 μM) of rhir(52−65), the K_i value for PAB decreased to 65 ± 5 μM. In a number of other studies, the binding of the C-terminal region of hirudin to the anion-binding exosite of thrombin has been shown to affect allosterically the interaction of substrates and inhibitors with the active site (Dennis et al., 1990; Hortin & Trimpe, 1991; Schmitz et al., 1991; De Cristofaro et al., 1993; Parry et al., 1993).

Pre-Steady-State Kinetics of the Interaction of Hirulog with Thrombin. The equilibrium fluorescence changes for the reaction of Hirulog with thrombin in the absence and presence of rhir(52−65) were determined as described previously for rhir (Jackman et al., 1992). Reaction of saturating concentrations of Hirulog with thrombin resulted in a 44% enhancement in the intrinsic fluorescence of thrombin, which is slightly higher than the 35% enhancement observed with rhir (Jackman et al., 1992). The C-terminal fragment of rhir [rhir(52−65)] caused a smaller increase (20%) in the intrinsic fluorescence of thrombin (Jackman et al., 1992). In the presence of a saturating concentration (27 μM) of rhir(52−65), the fluorescence increase caused by Hirulog was 21%. Thus, about half of the fluorescence enhancement observed on the formation of the Hirulog−thrombin complex can be attributed to the binding of the hirudin-like C-terminal region to the anion-binding exosite.

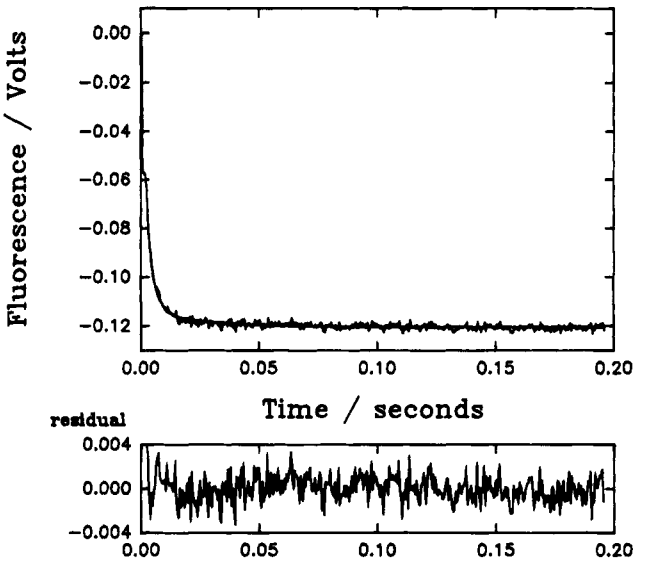


FIGURE 1: Double-exponential decay curve for the reaction of Hirulog with thrombin. Hirulog (12 μM) was reacted with thrombin (0.1 μM) and the increase in intrinsic fluorescence (decrease in volts) followed as described in the Experimental Procedures. The data were fitted to the equation for a double-exponential decay, and the fit of the data to this equation is also shown in the *upper panel*. The *lower panel* shows the residuals of the fit.

The reaction of Hirulog with thrombin was best described by a double-exponential decay over the concentration range 0.47−18.8 μM (Figure 1). Analysis of the data yielded two observed rate constants (k_{obs1} and k_{obs2}) and the fluorescence changes associated with the two processes (ΔF_{obs1} and ΔF_{obs2}). At lower concentrations of Hirulog (0.1−0.3 μM), the data fitted equally well to single or double-exponential decays. Such data are obtained when the two decay phases are not well separated (Cornish-Bowden, 1975), and these data were not used in the analysis of the dependence of the rate constants on the concentration of Hirulog. The value of k_{obs1} showed a hyperbolic dependence on the concentration of Hirulog (Figure 2). Equation 1 is the most general equation that describes the hyperbolic dependence of rate constants on ligand concentration (Hiromi, 1979; Hammes & Schimmel, 1970). However, it was not possible to fit these data to eq 1 using weighted nonlinear regression; large standard errors were obtained for all parameters. A reasonable fit to the data was obtained when the value of k_{int} was set to zero as in eq 2 (Figure 2). This analysis yielded values of 325 ± 17 s^{-1} and 0.75 ± 0.12 μM for k_{max} and K_m. The amplitude of the faster decay (ΔF_{obs1}) did not vary significantly with the concentration of Hirulog; the weighted mean value for the amplitude was 39.9 ± 0.3% of the thrombin fluorescence. The rate constant for the slow phase (k_{obs2}) and its amplitude (ΔF_{obs2}) also did not vary significantly with Hirulog concentration. The weighted mean values of the rate constant and amplitude were 30 ± 1 s^{-1} and 3.6 ± 0.2%. The sum of the amplitudes for the two decays was equal to the equilibrium fluorescence change for the reaction of

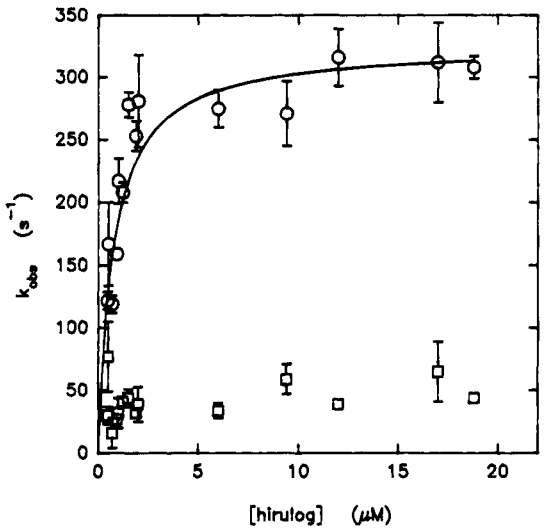


FIGURE 2: Variation of the estimates for the observed rate constants with the concentration of Hirulog. Data were obtained at the indicated concentrations of Hirulog and analyzed according to the equation for a double-exponential decay to yield values of k_{obs1} (○) and k_{obs2} (□), which are plotted together with the standard errors of the estimates. The variation of k_{obs1} with the concentration of Hirulog was analyzed according to eq 2 by weighted nonlinear regression. The curve illustrates the fit of the data to this equation.

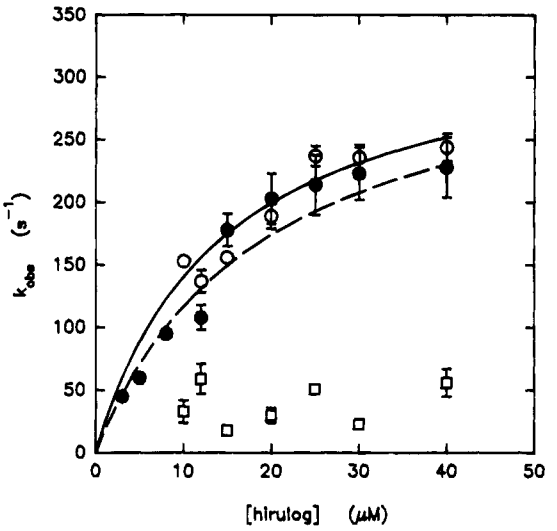


FIGURE 3: Effect of rhir(52−65) on the kinetic constants for the interaction of Hirulog with thrombin. Data were obtained at the indicated concentrations of Hirulog in the presence of 27 μM rhir-(52−65) ± 350 μM PAB. Analysis of intrinsic fluorescence data obtained in the absence PAB according to the equation for a double-exponential decay yielded estimates for k_{obs1} (○) and k_{obs2} (□); the standard errors of these estimates are indicated by the error bars. The dependence of k_{obs1} on the concentration of Hirulog was analyzed according to eq 2 by weighted nonlinear regression, and the solid curve shows the fit of the data to the equation. The data for PAB displacement were analyzed according the equation for a single-exponential decay to yield values of k_{obs} (●), which are plotted together with the standard errors of the estimates. These data were analyzed according to eq 2 by nonlinear regression, and the dashed curve shows the fit of the data to this equation.

Hirulog with thrombin. This result contrasts to the rhir−thrombin interaction, where a fraction of the fluorescence change was found to occur within the dead time of the spectrometer (Jackman et al., 1992).

In order to investigate the nature of the steps involved, the kinetics of complex formation in the presence of rhir-(52−65) were examined. In the presence of 27 μM rhir-(52−65), double-exponential decay traces were also observed. The value of k_{obs1} again displayed a hyperbolic dependence on the concentration of Hirulog (Figure 3), and analysis of the data according to eq 2 yielded estimates for k_{max} and K_m of 342 ± 52 s^{-1} and 14 ± 5 μM. The amplitude of the fast phase again did not vary with the concentration of Hirulog but was reduced in the presence of rhir(52−65); a weighted mean value of 19.6 ± 0.2% was observed. No significant variation of the rate constant for the slow phase and its amplitude with Hirulog concentration was seen (Figure 3). The weighted mean value of the rate constant for the slow phase (k_{obs2}) in the presence of rhir(52−65) was similar to that observed in the absence of this peptide (27 ± 2 compared with 30 ± 1 s^{-1}). The amplitude of the slow phase was somewhat smaller in the presence of rhir(52−65) (1.5 ± 0.1%).

The kinetic mechanism for the inhibition of thrombin by Hirulog was further investigated by examining the displacement of the active-site probe PAB in the presence of 27 μM rhir(52−65). PAB has previously been used to study the reaction of antithrombin III with thrombin. The displacement of PAB from thrombin's active site results in a decrease in its fluorescence (Olson & Shore, 1982). A relatively high concentration of PAB (350 μM) was used in the present study in order to saturate the active site with PAB. The kinetics for the displacement of PAB could be described by a single-exponential decay (Figure 4). The rate constants for displacement of PAB were not significantly different from those obtained for the fast phase of the intrinsic fluorescence enhancement in the absence of PAB with the same Hirulog concentrations (Figure 3). Intrinsic fluorescence changes could not be measured in the presence of PAB because of its high absorbance at 280 nm. The dependence of the rate constants for PAB displacement on the concentration of Hirulog was fitted to eq 2 by weighted nonlinear regression, and estimates for k_{max} and K_m of 340 ± 38 s^{-1} and 19 ± 3 μM were obtained. The amplitudes of the PAB displacement decays did not vary with the concentration of Hirulog.

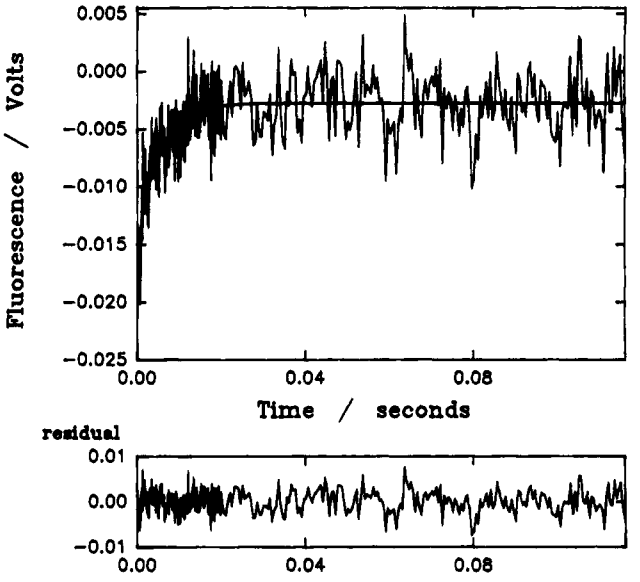


FIGURE 4: Decay curve for the displacement of PAB from the active site of thrombin by Hirulog. Hirulog (12 μM) was reacted with thrombin (0.2 μM) in the presence of 350 μM PAB. The change in fluorescence was followed as described in the Experimental Procedures. The data were fitted to the equation for a single-exponential decay, and the fit is shown together with the data in the *upper panel*. The *lower panel* shows the residuals of the fit.

DISCUSSION

The interaction of Hirulog with thrombin resulted in an increase in the intrinsic fluorescence of thrombin. The kinetics of this increase were best described by a double-exponential decay (Figure 1). The dependence of the observed rate constants on the concentration of Hirulog indicates that more than two steps are involved in the formation of the final complex. Although a double-exponential decay can be seen for a two-step mechanism if the first step is more rapid than the second, the observed rate constant for the faster initial step shows a linear dependence on the concentration of ligand (Hiromi, 1979). For the Hirulog−thrombin interaction, the faster step displayed a hyperbolic dependence on the concentration of Hirulog while the rate constant for the slower step did not vary with Hirulog concentration (Figure 2), and thus, the two-step mechanism can be excluded. It was possible to describe the kinetics of the reaction of rhir with thrombin by the three-step mechanism shown in Scheme 1 (Jackman et al., 1992). This mechanism is also consistent with the data of Figure 2 provided the formation of the initial complex (EI^1) occurs within the dead time of the spectrometer such that only the two following slower decays are observed. A similar situation was observed for rhir (Jackman et al., 1992).

Scheme 1

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI^3$$

Explicit equations defining the rate constants of the two observed decays under all conditions cannot be derived, but there are limiting conditions for which expressions for the observed rate constants can be obtained (Hammes & Schimmel, 1970). If the condition $k_1[I] + k_{-1} \gg k_2 + k_{-2} \gg k_3 + k_{-3}$ applies, as it did for the reaction of rhir with thrombin, the following equations can be derived for the rate constants of the two observed decays (Jackman et al., 1992):

$$k_{obs1} = \frac{k_2}{1 + K_{-1}/[I]} + k_{-2} \quad (3)$$

$$k_{obs2} = \frac{k_3}{1 + K_{-2} + K_{-1}K_{-2}/[I]} + k_{-3} \quad (4)$$

where K_{-1} and K_{-2} equal k_{-1}/k_1, and k_{-2}/k_2, respectively. Equations 3 and 4 predict that rate constants for the two observed decays (k_{obs1} and k_{obs2}) should display a hyperbolic dependence on the Hirulog concentration. The observed rate constant for the faster decay showed a hyperbolic dependence on the concentration of Hirulog (Figure 2), and analysis of the data yielded values of 0.75 ± 0.12 μM and 325 ± 17 s^{-1} for K_{-1} and k_2. The observed rate constant of the slower step did not vary significantly with Hirulog concentration; such a dependence is expected if $[I] \gg K_{-1}K_{-2}$. The observed lack of variation of the amplitudes of the two decays with the Hirulog concentration is also consistent with the mechanism presented in Scheme 1. For this mechanism, the observed amplitudes of the decays will be constant if the concentration of ligand is much greater than the overall K_i value (Hiromi, 1979; Jackman et al., 1992). Thus, since the lowest concentration of Hirulog used (0.5 μM) was 250-fold greater than the K_i value, no variation in the observed amplitudes was expected.

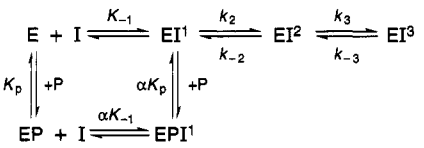
For the reaction of rhir with thrombin, it was proposed that the first step corresponds to the binding of the C-terminal region of rhir to the anion-binding exosite of thrombin and that this binding induces a conformational change in thrombin that is observed in the second step (Jackman et al., 1992). The C-terminal hirudin-like region of Hirulog binds to the anion-binding exosite in a manner similar to that of the corresponding region of rhir (Qiu et al., 1992; Rydel et al., 1991), and it seems probable that the first two steps with Hirulog also correspond to exosite binding and a conformational change induced by this binding. The interaction between thrombin and the C-terminal fragment of rhir [rhir(52−65)] involves the formation of an initial complex followed by a conformational change with an observed rate constant of about 300 s^{-1} (Jackman et al., 1992). The observed rate constant for the second step in the formation of Hirulog−thrombin complex was also about 300 s^{-1} (Figure 2), and this suggests that the same conformational change is being observed in both cases. The data of Figure 3 confirm that the first step involves the binding of the hirudin-like C-terminus of Hirulog to the anion-binding exosite. In the presence of 27 μM rhir(52−65), the value of the rate constant for the faster decay displayed a hyperbolic dependence on Hirulog concentration, and analysis of the data according to eq 2 yielded an increased value for K_m (14 ± 5 μM *versus* 0.75 ± 0.12 μM) while the value of k_{max} remained unchanged (342 ± 52 s^{-1} *versus* 325 ± 17 s^{-1}). These results are expected if rhir(52−65) competes with the C-terminal region of Hirulog in the first step. In such a case of competitive inhibition, the value of K_m determined from fitting the data to eq 2 will be related to K_{-1} by the following relationship:

$$K_m = K_{-1}(1 + [\text{rhir}(52-65)]/K_{CP}) \quad (5)$$

where K_{CP} is the dissociation constant for rhir(52−65). The value of K_{CP} determined previously was 1.5 μM (Jackman et al., 1992), and substitution of this value into the above expression yields a value for K_{-1} of 0.7 ± 0.3 μM, which corresponds to the value 0.75 ± 0.12 μM determined from the data of Figure 2. Thus, the data obtained in the presence of rhir(52−65) are both qualitatively and quantitatively consistent with the first step in the reaction of Hirulog with thrombin involving the binding of its hirudin-like C-terminal region to the anion-binding exosite.

The nature of the steps involved in the formation of the Hirulog−thrombin complex was further investigated using the fluorescent probe PAB. This molecule will bind to the primary-specificity pocket (Banner & Hadváry, 1991; Stürzebecher et al., 1992) and is displaced by ligands, such as antithrombin III (Olson & Shore, 1982), that bind to this pocket. The rate constants for displacement of PAB from the active site and the fast phase of the intrinsic fluorescence enhancement in the absence of PAB were not significantly different (Figure 3). This observation indicates that the second step is rate-limiting for the binding of Hirulog to the active site. However, the data indicate that Hirulog is not bound to the primary specificity pocket during this step. Two lines of evidence support this conclusion. First, a step with the same rate constant is also seen with rhir(52−65) which does not bind to the active site (Jackman et al., 1992). Second, if PAB were displaced in the second step as shown

Scheme 2



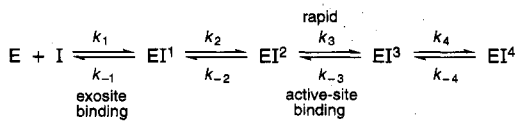
in Scheme 2, where P represents PAB, it would affect the rate of this step.

In the mechanism in Scheme 2, it is assumed that PAB and the C-terminal region of Hirulog can bind to thrombin simultaneously. The binding sites for the C-terminal region of Hirulog and PAB do not overlap; the C-terminal region of Hirulog binds to the anion-binding exosite while PAB binds to the active site. For this mechanism, PAB will act as a noncompetitive inhibitor in the second step, and the dependence of the observed rate constant for this step on the concentration of Hirulog will be given by the equation:

$$k_{obs} = \frac{k_2}{(1 + [P]/\alpha K_p) + K_{-1}(1 + [P]/K_p)/[I]} + k_{-2} \quad (6)$$

This equation predicts that the observed values of both k_{max} and K_m will be altered in the presence of PAB. Most importantly, k_{max} will be reduced by $(1 + [P]/\alpha K_P)$ in the presence of PAB. Since the first step corresponds to the binding of the C-terminal region of Hirulog to the anion-binding exosite, it can be assumed that αK_P will be about equal to the K_i value for PAB in the presence of rhir(52−65), i.e., 65 μM. Thus, if the mechanism shown in Scheme 2 applied to the Hirulog−thrombin interaction, the maximum value for observed rate constant for the second step should be 6-fold (1 + 350/65) lower in the presence of 350 μM PAB. However, the same value for k_{max} was observed in PAB displacement and fluorescence enhancement experiments, and the latter were conducted in the absence of PAB. Thus, the mechanism of Scheme 2 in which Hirulog binds to the primary specificity pocket and displaces PAB in the second step can be excluded. The data are consistent with a mechanism in which PAB is displaced in a rapid step which follows the second step, as shown in Scheme 3. Since the second step is much slower that the third step, the rate of PAB displacement will be equal to that of the rate-limiting second step. However, no rate reduction will be observed in the presence of PAB provided the second step remains rate-limiting. PAB will reduce the rate of the third step by a factor of $(1 + [P]/\alpha K_P)$ (see eq 6). Thus, the mechanism of Scheme 3 is consistent with the data if the magnitude of k_3 is at least 6-fold greater than that of k_2; otherwise, the third step would become rate-limiting in the presence of 350 μM PAB.

Scheme 3



In the presence of nearly saturating concentrations of PAB, the enzyme forms E, EI^1 and EI^2 will exist predominantly as complexes with PAB (PAB·E, PAB·EI^1, and PAB·EI^2). If it is assumed that the third step, in which PAB is displaced, is much more rapid than the second and fourth steps, the following equation can be derived for the observed rate constant for the displacement of PAB in the third step:

$$k_{obs} = \frac{\beta k_2}{1 + \alpha K_{-1}/[I]} + \frac{\beta k_{-2} k_{-3}}{k_3} \quad (7)$$

where αK_{-1} is the dissociation constant for Hirulog from the PAB·EI^1 complex and βk_2 and βk_{-2} are the rate constants for second step when PAB is bound to the active site. Equation 7 predicts that, in the presence of a saturating concentration of PAB, neither the k_{max} nor K_m values will necessarily be altered, which is consistent with the data of Figure 3. For the four-step mechanism in the absence of PAB, the following expressions can be derived for k_{obs1} and k_{obs2}, assuming that the second and fourth steps are rate-limiting:

$$k_{obs1} = \frac{k_2}{1 + K_{-1}/[I]} + \frac{k_{-2} k_{-3}}{k_3} \quad (8)$$

$$k_{obs2} = \frac{k_4}{1 + K_{-3} + K_{-2}K_{-3} + K_{-1}K_{-2}K_{-3}/[I]} + k_{-4} \quad (9)$$

where K_{-3} equals k_{-3}/k_3. These expressions for k_{obs1} and k_{obs2} are of the same form as those given in eqs 3 and 4 for the three-step mechanism. Thus, the data of Figure 2 are also consistent with the four-step mechanism of Scheme 3; the hyperbolic dependence of k_{obs1} on the concentration of Hirulog is in accord with eq 8, and the lack of variation of k_{obs2} with concentration is consistent with eq 9 provided [I] $\gg K_{-1}K_{-2}K_{-3}$.

Analysis of the data obtained in the presence of PAB and rhir(52−65) yielded a value of 19 ± 3 μM for K_m. The K_i value for PAB was reduced 1.8-fold by the binding of rhir-(52−65) to the anion-binding exosite. Thus, it follows that the binding of PAB must decrease the dissociation constant for rhir(52−65) by a similar amount. In the absence of PAB, the dissociation constant for rhir(52−65) is 1.5 μM (Jackman et al., 1992), and thus, in the presence of 350 μM PAB, its dissociation constant will be about equal to 0.8 μM. By substituting this value for K_{CP} together with the estimate of 19 ± 3 μM for K_m into eq 5, it can be calculated that the dissociation constant for the initial complex (αK_{-1}) is 0.5 ± 0.1 μM when PAB is bound to the active site.

In view of the demonstration of a four-step mechanism for Hirulog−thrombin complex formation, the three-step mechanism proposed for the rhir−thrombin interaction should be re-evaluated (Jackman et al., 1992). The fast third step in which Hirulog is bound to the active site was only detected because of the observed effects of PAB. Because this step is faster than both the second and fourth step, any change in intrinsic fluorescence for this step will not be detected. The signal for this more rapid step will be incorporated into the signal for the slower preceding step (Hiromi, 1979), and thus, the rapid third step will not be detected by following changes in intrinsic fluorescence alone. The kinetics of PAB displacement by rhir are currently being investigated. The incorporation of the signal for the rapid third step into the signal for the slower second step is probably the explanation for the differences in the observed amplitudes of the 300 s^{-1} step between rhir(52−65) and Hirulog. Thus, the observed fluorescence enhancement of 40% for this step with Hirulog will represent the sum of the

amplitudes for the second and third steps, whereas the amplitude of 20% observed with rhir(52−65) will be only due to the second step; the third step, in which Hirulog is bound to the active site, will not occur in the case of rhir-(52−65) which only binds to the exosite.

In addition to elucidating a kinetic mechanism for the interaction of Hirulog with thrombin, the data obtained in the present study also yield some information about the rates of the various steps involved and their contribution to the stability of the complex. A value of 4.3×10^8 M^{-1} s^{-1} was obtained for k_2/K_{-1} from analysis of the data of Figure 2. This represents the effective second-order rate constant for the association of Hirulog with thrombin at low inhibitor concentrations. The corresponding value for rhir was slightly lower (2.9×10^8 M^{-1} s^{-1}; Jackman et al., 1992). The value obtained also provides a lower limit for the true association rate constant (k_1) for Hirulog since k_2/K_{-1} cannot be greater than k_1. The estimate obtained for K_{-1}, which equals k_{-1}/k_1, was 0.75 μM, and therefore, it can be calculated that k_{-1} must be greater than 300 s^{-1} ($>0.75 \times 10^{-6} \times 4.3 \times 10^8$ s^{-1}). At the lowest Hirulog concentrations used (0.5 μM), $k_1[I] + k_{-1}$ would be greater than 500 s^{-1}, which is consistent with the initial binding step occurring within the dead time of the spectrometer. An estimation of the contribution of the second, third, and fourth steps to the tightness of the complex can be obtained by comparing the values of the overall inhibition constant (K_i) and the dissociation constant for the initial complex (K_{-1}). This comparison indicates that the tightness of the complex is increased 400-fold (0.75 μM/1.9 nM) by the steps following the initial complex formation. While the dissociation constant for the initial complex between thrombin and rhir is about equal to that for the initial thrombin−Hirulog complex (Jackman et al., 1992), the overall inhibition constant for rhir is 10^4-fold lower (0.23 pM; Braun et al., 1988). Thus, the steps following the initial binding at the anion-binding exosite make a much greater contribution to the stability of the thrombin−rhir complex. These steps involve the binding of the N-terminal core domain of rhir in and around the active site of thrombin, and the greater contribution of these steps to the stability of the final complex in the case of rhir can be attributed to the more extensive interactions made by the N-terminal core domain of rhir compared with the D-Phe-Pro-Arg-Pro portion of Hirulog (Rydel et al., 1991; Skrzypczak-Jankun et al., 1991; Qiu et al., 1992).

The rapid kinetic data can also be used to calculate the contribution of the dissociation of cleaved Hirulog to the reversibility of the complex observed in steady-state kinetics. The K_i value represents the ratio of the effective dissociation and association rate constants. At the nanomolar concentrations of Hirulog used to determine the K_i value, the second step will be rate-limiting in the formation of the stable complex, and the effective association rate constant (k_{assoc}) will be k_2/K_{-1}, i.e., 4.3×10^8 M^{-1} s^{-1}. The effective dissociation rate constant (k_{diss}) can be calculated from the expression $k_{diss} = K_i k_{assoc}$ to be 0.8 s^{-1}. Since the rate of cleavage of Hirulog was 0.01 s^{-1}, it can be concluded that, under the conditions of the assay, the major pathway of dissociation of Hirulog from the complex does not involve the release of cleaved inhibitor.

In conclusion, the data presented in this paper are consistent with a four-step mechanism for the inhibition of thrombin by Hirulog. In the first step, the C-terminal region of Hirulog binds to the anion-binding exosite of thrombin. This step is followed by a conformational change prior to interaction of the P_1 arginine of Hirulog with the primary-specificity pocket in a very rapid step. After occupation of this binding pocket, a further conformational change takes place. The rate constants for the first and second conformational changes were 300 and 30 s^{-1}, respectively, and the occurrence of steps with similar rate constants in the rhir−thrombin interaction (Jackman et al., 1992) suggests that both Hirulog and rhir induce similar conformational changes in thrombin. It seems possible that such conformational changes also play a role in the specific interaction of thrombin with other ligands, such as fibrinogen and the thrombin receptor, which bind to the enzyme's exosite and active site, and in the switch in thrombin's specificity that occurs upon binding of thrombomodulin (Esmon, 1989).

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