Kinetics of the Inhibition of Thrombin by Hirudin

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ABSTRACT: The dissociation constant for hirudin was determined by varying the concentration of hirudin in the presence of a fixed concentration of thrombin and tripeptidyl *p*-nitroanilide substrate. The estimate of the dissociation constant determined in this manner displayed a dependence on the concentration of substrate which suggested the existence of two binding sites at which the substrate was able to compete with hirudin. A high-affinity site could be correlated with the binding of the substrate at the active site, and the other site had an affinity for the substrate that was 2 orders of magnitude lower. Extrapolation to zero substrate concentration yielded a value of 20 fM for the dissociation constant of hirudin at an ionic strength of 0.125. The dissociation constant for hirudin was markedly dependent on the ionic strength of the assay; it increased 20-fold when the ionic strength was increased from 0.1 to 0.4. This increase in dissociation constant was accompanied by a decrease in the rate with which hirudin associated with thrombin. This rate could be measured with a conventional recording spectrophotometer at higher ionic strength and was found to be independent of the binding of substrate at the active site.

Thrombin plays a central role in the blood coagulation pathway. It cleaves fibrinogen to form fibrin that subsequently polymerizes to form the basis of a blood clot. Thrombin is also able to activate other blood coagulation factors such as factor V, factor VIII, and protein C. In addition, thrombin interacts with a number of different types of cells to cause a variety of responses including mitosis with fibroblasts and secretion with platelets (Fenton, 1981). Hirudin is a potent and highly specific inhibitor of thrombin and can be isolated from the salivary gland of the leech *Hirudo medicinalis* (Markwardt, 1970). Hirudin has been used as a specific inhibitor of thrombin in a number of different experimental systems. For example, it has been used to inhibit the mitogenic effect of thrombin on fibroblasts (van Obberghen-Schilling et al., 1982) and the thrombin activation of platelets (Hoffmann & Markwardt, 1984). Despite the use of hirudin in different experimental systems, the kinetics of the inhibition of thrombin by hirudin had not been thoroughly examined. This paper presents the results of such a study.

Experimental Procedures

Materials. The hirudin used in the majority of kinetic experiments was a gift from Plantorgan Werk, Bad Zwischenahn, West Germany, and had a specificity activity of 12000 antithrombin units/mg. Other preparations of hirudin were purchased from Sigma, St. Louis, MO, and Pentapharm, Basel, Switzerland. The substrates S-2238 (D-Phe-pipecolyl-Arg-pNA)[1] and S-2288 (D-Ile-Pro-Arg-pNA) were from Kabi Vitrum, Molndal, Sweden, and venom from *Oxyuranus scutellatus* was from Sigma, St. Louis, MO. Carboxypeptidase Y was purchased from Worthington, Freehold, NJ. All other chemicals were of the highest purity available commercially. Prothrombin was purified from human plasma (Miletich et al., 1980). Thrombin was formed from prothrombin by using the venom from *O. scutellatus* (Owen & Jackson, 1973) and was purified on SP-Sephadex (Lundblad, 1971). If necessary thrombin was further purified by using fast protein liquid chromatography with a Mono-S column (Pharmacia, Uppsala, Sweden) and the same gradient as described for SP-Sephadex. Thrombin prepared in this manner was pure and free from proteolytically degraded forms as judged by electrophoresis and by its amino-terminal sequence. The concentration of thrombin was determined by active site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973), and the enzyme was found to be 97% active.

Protein Chemistry. High-performance liquid chromatography (HPLC) of hirudin was performed with a chromatograph from Waters Associates, Milford, MA, equipped with a 5-μm Bakerbond C_8 column (Baker Chemical Co., Phillipsburg, NJ). The column was equilibrated with 0.1% TFA, and hirudin was eluted with a gradient of acetonitrile in the same solvent. Protein was detected by measuring absorbance at 214 nm. Solvent was removed from the purified samples under vacuum, and they were stored at −70 °C in water.

Amino acid sequence analysis was performed as described by Hewick et al. (1981). PTH-amino acids were analyzed as described by Hunkapiller and Hood (1983) with 5% tetrahydrofuran added to buffer A.

Partial desulfation of the sulfotyrosine residue of hirudin was achieved by incubation in 50% aqueous TFA for 30 min at 56 °C (Chang, 1983). The desulfohirudin was purified by HPLC as described earlier. Sequence analysis of the purified material yielded only one N-terminal sequence which indicated that the polypeptide chain of desulfohirudin prepared in this way was intact. The sulfotyrosine content of different hirudin preparations was determined by amino acid analysis after digestion with carboxypeptidase Y under the conditions described by Chang (1983).

Enzyme Assays. The release of *p*-nitroaniline that resulted from the hydrolysis of the peptidyl *p*-nitroanilide substrates was followed by measuring the increase in absorbance at 405 nm with a Shimadzu UV 240 spectrophotometer. The assays were performed in polystyrene cuvettes at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, which contained 0.1% poly(ethylene glycol) 6000 and, unless otherwise indicated, 0.1 M NaCl.

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[1] Abbreviations: pNA, *p*-nitroanilide; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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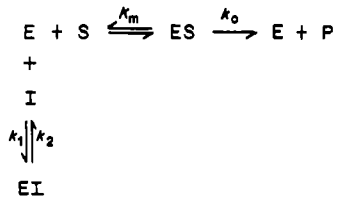
Scheme I



Assays were started by the addition of thrombin. Under the assay conditions, thrombin was stable for at least 40 min as determined by the progress-curve method of Selwyn (1965). The amount of product formed was calculated by using an absorption coefficient of 9920 M^{-1} cm^{-1} for *p*-nitroaniline at 405 nm, and the concentration of the substrate was determined spectrophotometrically at 342 nm by using an absorption coefficient of 8270 M^{-1} cm^{-1} (Lottenberg & Jackson, 1983).

Theory and Data Analysis

Tight-Binding Inhibition. The inhibition of an enzyme in the presence of a substrate can be represented by the mechanism in Scheme I. The dissociation of the inhibitor (K_I) is equal to k_2/k_1. If the binding of the inhibitor to the enzyme causes a significant depletion in the concentration of the free inhibitor, the variation of the steady-state velocity (v_s) with the total inhibitor concentration (I_t) is (Morrison, 1969; Henderson, 1972)

$$v_s = (v_0/2E_t)[[(K_{I'} + I_t - E_t)^2 + 4K_{I'}E_t]^{1/2} - (K_{I'} + I_t - E_t)] \quad (1)$$

where 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. If the inhibitor and the substrate compete for the active site, $K_{I'}$ is related to the dissociation constant of the inhibitor (K_I) by eq 2. If the concentration of the

$$K_{I'} = K_I(1 + S/K_m) \quad (2)$$

enzyme or inhibitor is not accurately known, an additional factor can be incorporated into eq 1 to allow for this fact (Williams & Morrison, 1979). For the inhibition of thrombin by hirudin, the concentration of thrombin was known from active-site titration, but the concentration of hirudin was only known by weight and the data could be analyzed as

$$v_s = (v_0/2E_t)[[(K_{I'} + xI_x - E_t)^2 + 4K_{I'}E_t]^{1/2} - (K_{I'} + xI_x - E_t)] \quad (3)$$

where I_x is the concentration of the inhibitor in terms of weight per volume and x is a factor that when multiplied together with I_x, will yield the molar concentration of the inhibitor.

In steady-state velocity experiments, the dissociation constant for hirudin was determined by holding the concentration of thrombin constant and varying the concentration of hirudin over a range that included several concentrations that were lower than the concentration of thrombin and several concentrations that were higher. The total number of data points was usually between 7 and 10. The steady-state velocities obtained at each hirudin concentration were then fitted by weighted nonlinear regression to eq 1 or 3. Nonlinear regression requires initial estimates of the parameters. These estimates are refined by an iterative process until the best estimates of the parameters for that set of data are obtained (Duggleby, 1980). The iterative process may fail to converge if the data are not adequately described by the equation or if the initial estimates of the parameters are not sufficiently precise. In order to circumvent the latter problem, the nonlinear regression program used incorporated a graphics subroutine to enable interactive adjustment of the initial estimates of the parameters. For the regression, the observed values for the steady-state velocity were weighted according to the reciprocal of their value. This type of weighting was empirically found to give the best results.

Slow, Tight-Binding Inhibition. If the rate of interaction of the inhibitor with the enzyme is slow so that the inhibited steady-state velocity is only slowly achieved, the progress curve of product formation for the mechanism of Scheme I will be described by (Cha, 1976; Williams et al., 1979)

$$P = v_s t + \frac{(v_0 - v_s)(1 - d)}{dk'} \log\left(\frac{1 - d\exp(-k't)}{1 - d}\right) \quad (4)$$

where P is the amount of product formed at time, t, d is a function of E_t, I_t, and $K_{I'}$, and k' is a function of these parameters and the observed second-order rate constant ($k_{I'}$) for the interaction between the inhibitor and enzyme (Cha, 1976). If the inhibitor and substrate compete for a binding site, the value of $k_{I'}$ will depend on the substrate concentration (Cha, 1976):

$$k_{I'} = \frac{k_1 K_S}{S + K_S} \quad (5)$$

where K_S is the binding constant for the substrate.

The type of inhibition that is observed when the enzyme and inhibitor interact slowly has been termed slow-binding inhibition (Morrison, 1982). When such inhibition of thrombin by hirudin was observed, the data were fitted to eq 4 by nonlinear regression. This analysis yielded estimates for v_0, $K_{I'}$, and $k_{I'}$.

Results

Previous studies have indicated that hirudin binds extremely tightly to thrombin (Walsmann & Markwardt, 1981; Fenton et al., 1979), and the following protocol was adopted in order to determine the dissociation constant of hirudin accurately: (a) the concentration of the enzyme was kept as low as practically possible; (b) a concentration of substrate that was at least an order of magnitude greater than its K_m was used as this should theoretically increase the observed value of the dissociation constant (see eq 2); (c) the concentrations of hirudin were chosen so that several were below the fixed concentration of thrombin (these points will define v_0 and x in eq 3) and several points were at concentrations about equal to the enzyme concentration or slightly above (these points will define $K_{I'}$); (d) a factor (x) was introduced to allow for the fact that the molar concentration of hirudin was not accurately known (see eq 3). Using this protocol and with a concentration of 45 pM thrombin, it was found that it was possible to determine reproducibly values for $K_{I'}$ as low as 0.2 pM by fitting the data to eq 3. Figure 1 shows a typical set of data. The standard errors of the estimated values for $K_{I'}$ were usually less than 20% of the value. Analysis of the data also yielded a value for factor x, and this value could be used to calculate the concentration of hirudin in the stock solution. The standard errors for the estimated values of factor x were always less than 5%. At higher substrate concentrations and higher values of ionic strength, where the value of $K_{I'}$ was more than 5% of the concentration of thrombin, the data were fitted to eq 1. In such experiments, the value of factor x was first determined at lower substrate concentration and ionic strength, and this factor was used to calculate the molar concentration of hirudin used in the experiments. With higher values of $K_{I'}$, this procedure was found to give more reproducible estimates of $K_{I'}$.

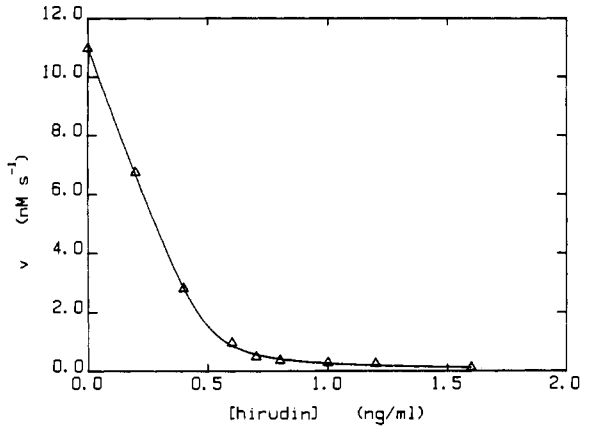


FIGURE 1: Effect of hirudin on the steady-state velocity of thrombin. Assays were performed as described under Experimental Procedure with a concentration of 45 pM thrombin and 163 μM S-2238. The observed steady-state velocities were fitted to eq 3 by using nonlinear regression. Values of 155 s^{-1}, 1.16 pM, and 91 μmol/g were obtained for v_0, $K_{I'}$, and x, respectively. The line in the figure has been drawn by using these values.

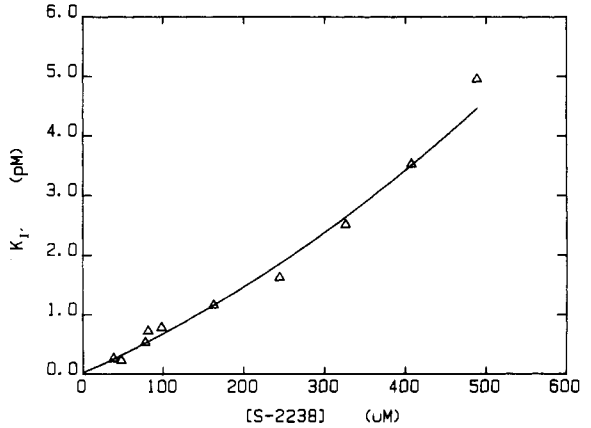


FIGURE 2: Effect of substrate concentration on the value of the apparent dissociation constant ($K_{I'}$) for hirudin. Assays were performed with a concentration of 45 pM thrombin as described under Experimental Procedures. The values of $K_{I'}$ were determined as described under Theory and Data Analysis. These values were weighted according to the squared inverse of their standard errors and fitted to eq 6 by using weighted linear regression. The line drawn in the figure represents the best fit to this equation; the values of 22 fM, 3.63 μM, and 0.93 mM were obtained for K_I, K_m, and K_{IS}, respectively.

Equation 2 predicts that the value of $K_{I'}$ should show a linear dependence on the concentration of substrate. The value of $K_{I'}$, however, showed a parabolic dependence on the concentration of the substrate S-2238 (D-Phe-pipecolyl-Arg-pNA) as shown in Figure 2. Such a parabolic dependence is not expected for any of the usually observed forms of inhibition. A possible explanation for the parabolic plot observed in Figure 2 is that a second molecule of substrate binds at an allosteric site on thrombin and causes a conformational change such that hirudin is not able to bind at the active site. Such a conformational change would also be expected to inhibit the binding of the substrate at the active site and substrate inhibition would be observed. Under the assay conditions used to obtain the data for Figure 2, no deviations from Michaelis–Menten kinetics were observed over a range of substrate concentrations from 1 to 1200 μM S-2238. Thus, the above allosteric mechanism seems to be excluded. The data could be explained by two other mechanisms. In both mechanisms, the substrate and hirudin compete for the active site. In the first mechanism, hirudin binds to an extended region on thrombin which includes other sites as well as the active site, and the second molecule of substrate competes with hirudin for part of this extended binding region. In the second mechanism, the substrate is able to bind to hirudin to form a substrate–hirudin complex which is unable to bind to the enzyme. The two mechanisms cannot be distinguished kinetically, and eq 6

$$K_{I'} = K_I(1 + S/K_m)(1 + S/K_{IS}) \quad (6)$$

describes the dependence of $K_{I'}$ on the substrate concentration for both mechanisms, where K_{IS} is the dissociation constant for the binding of the substrate at the second site on the enzyme or for its binding to hirudin, and other terms are as previously defined. The data of Figure 2 were fitted to eq 6 by weighted linear regression in which the values of $K_{I'}$ were weighted according to the squared inverse of their standard errors. The value of K_m was determined from initial velocity studies to be 3.63 ± 0.09 μM under the assay conditions, and this parameter was held fixed at this value for the analysis of the data of Figure 2. This analysis yielded values of 22 ± 1 fM for K_I and 0.93 ± 0.21 mM for K_{IS}. Data similar to those shown in Figure 2 were also obtained when S-2288 (D-Ile-Pro-Arg-pNA) was used as the substrate instead of S-2238. In this case, analysis of the data yielded values of 17 ± 4 fM and 0.46 ± 0.26 mM for K_I and K_{IS}, respectively. The value of K_m for S-2288 was determined from initial-velocity experiments to be 5.12 ± 0.1 μM, and the value of this parameter was again held constant in the analysis according to eq 6.

The above results were obtained by using a preparation of hirudin with a specific activity of 12 000 antithrombin units/mg of protein which has been designated hirudin 1 (see Table I). The value of about 20 fM for the dissociation constant of hirudin is considerably lower than some other values reported in the literature (Fenton, 1981; Walsmann & Markwardt, 1981). Therefore, it was of interest to determine whether the observed dissociation constant was peculiar to this particular preparation of hirudin. Less pure preparations of hirudin (hirudins 2 and 3) yielded essentially the same apparent dissociation constants in the presence of 85 μM S-2238

Table I: Apparent Dissociation Constants of Different Preparations of Hirudin[a]

hirudin	source	$K_{I'}$ (pM)	degree of sulfation
1[b]	Plantorgan	0.75 ± 0.11	0.99
1A[c,d]	Plantorgan	0.83 ± 0.08[f]	[g]
1B[c,d]	Plantorgan	0.77 ± 0.07[f]	0.98
2[e]	Sigma	0.89 ± 0.08	[g]
3[e]	Pentapharm	0.76 ± 0.14	[g]
4[c]	Pentapharm	0.87 ± 0.14	0.96
5[c]	Pentapharm	0.76 ± 0.10	0.99

[a] Assays were performed as described under Experimental Procedures with concentrations of 45 pM thrombin and 85 μM S-2238. The data were analyzed according to eq 3 to yield estimates of the apparent dissociation constant $K_{I'}$. The purity of the samples was assessed by HPLC and N-terminal amino acid sequence analysis as described under Experimental Procedures. The degree of sulfation is expressed as the fraction of tyrosine-63 that was sulfated as determined by using the method of Chang (1983). [b] This preparation displayed two peaks on HPLC and two N-terminal amino acid sequences (see text). [c] These preparations chromatographed as single peaks on HPLC and N-terminal amino acid sequence indicated that they were pure. [d] Hirudins 1A and 1B correspond to peaks A and B of Figure 3. [e] These preparations displayed multiple peaks on HPLC. [f] These values represent the weighted means of three separate determinations. [g] The degree of sulfation of hirudins 2 and 3 could not be determined because the samples were impure. For hirudin 1A, the amount available was not sufficient for a sulfotyrosine determination. Sulfotyrosine can be desulfated with 50% aqueous TFA (see Experimental Procedures); the elution position of both hirudin 1A and hirudin 1B on a C_8 reversed-phase column shifted in a similar fashion to a higher percentage of acetonitrile upon incubation in 50% aqueous TFA. This result suggests that hirudin 1A also contained sulfotyrosine.

Table II: Partial Amino Acid Sequence of Hirudin[a]

position no.	1				5					10					15
hirudin 1A	I	T	Y	T	D	X	T	E	S	G	(Q	N	L	X	L)
yield (pmol)	41	13	32	13	14	ND	3	4	ND	ND					
hirudin[b]	V	V	Y	T	D	C	T	E	S	G	Q	N	L	C	L

[a] Hirudin 1A (see Figure 3), with intact disulfide bridges, was sequenced as described under Experimental Procedures. The yield of each amino acid is given below the single letter code for the amino acid. For comparison, part of the sequence of hirudin as determined by Dodt et al. (1984) is also shown. "X" denotes that no amino acid was found and "ND" that the yield was not determined. The residues within parentheses were identified only tentatively as the yield was less than 2 pmol. [b] Dodt et al. (1984).

as that observed for hirudin 1 (Table I). Two different lots of hirudin were purified by HPLC (hirudins 4 and 5). The purified material rechromatographed as a single peak and was pure as determined by N-terminal amino acid sequence analysis. Both of these preparations yielded the same apparent dissociation constant as hirudin 1 (Table I). Thus, a number of different hirudin preparations of varying degrees of purity yielded essentially the same results as hirudin 1 (Table I).

Tyrosine-63 of hirudin is sulfated (Bagdy et al., 1976), and the degrees of sulfation of the different purified preparations of hirudin were determined. All preparations displayed almost complete sulfation (Table I). If the degree of sulfation were important to the inhibitory effect of hirudin, it might be possible to explain the discrepancies observed in dissociation constants between different laboratories in terms of variations in the degrees of sulfation of the hirudin preparations used. Purified desulfohirudin was prepared from hirudin 5. The apparent dissociation constant of this compound displayed a parabolic dependence on the concentration of substrate similar to that shown in Figure 2. Analysis of the data yielded values of 207 ± 15 pM and 1.13 ± 0.22 mM for K_I and K_{IS}, respectively. Thus, complete desulfation has increased the dissociation constant for hirudin by only 10-fold, and the large differences in dissociation constants observed between the present study and previous studies cannot be explained in terms of differences in the degrees of sulfation of the hirudin preparations.

Amino acid sequence analysis of hirudin 1 (270 pmol) indicated that the protein was of very high purity. A microheterogeneity was found, however, at the first two positions of the polypeptide chain. The major component (80% of the total, as determined from the yield of PTH-amino acids in the first cycle in two experiments) started with the sequence Val-Val-, whereas the minor one (20%) began with the sequence Ile-Thr-. The Edman degradation was carried on for 24 cycles with a repetitive yield of 93%, and no other heterogeneities were found. The two components could be separated by HPLC (Figure 3). The relative amounts of the two forms, as calculated from the ratio of peak heights, were the same as those calculated from the sequence analysis as mentioned earlier: 82% and 18%. The major component eluted at the same position as the purified hirudins 4 and 5. The individual peaks of the hirudin 1 were N-terminally sequenced. The results obtained for the minor component (peak A) are shown in Table II. It was observed that the yield of PTH-amino acid dropped significantly after cycle 6. This could be caused by the presence of a cystinyl residue at this position, since this phenomenon was not observed in a run on performic acid oxidized hirudin 1 (data not shown). The sequence obtained for the 24 N-terminal amino acids of the major component (hirudin 1B) was in exact agreement with that obtained by Dodt et al. (1984). The two forms hirudin 1A and 1B were kinetically indistinguishable. In the presence of 85 μM S-2238, hirudin 1A, hirudin 1B, and hirudin 1 yielded the same apparent dissociation constant (Table I). In addition, the apparent dissociation constants of hirudin 1A and 1B were not

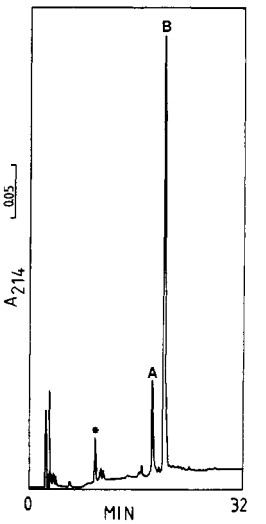


FIGURE 3: Fractionation of hirudin by HPLC. Hirudin 1 (see Table I) was separated into two components by chromatography on a C_8 reversed-phase column. The solvent was 0.1% TFA, and the protein was eluted with a gradient of acetonitrile in the same solvent (10–63% in 45 min). The relative amounts of hirudin A and hirudin B, as determined from the peak heights, were 18% and 82%, respectively. The peaks were collected separately and analyzed structurally and kinetically. The peak indicated with an asterisk was also found in a blank run and is caused by the buffer component of the sample.

significantly different at higher substrate concentration; values of 5.1 ± 0.2 and 5.5 ± 0.2 pM were observed for hirudin 1A and 1B, respectively, at 417 μM S-2238. Since hirudin 1A and hirudin 1B were kinetically indistinguishable from each other and hirudin 1, further studies were performed with hirudin 1. Other workers have also observed forms of hirudin that differed in their amino acid sequence but could not be distinguished on the basis of their affinity for thrombin (Dodt et al., 1984).

Under the standard assay conditions, it was not possible to measure the rate of interaction between hirudin and thrombin by using a conventional recording spectrophotometer. At concentrations of hirudin above 100 pM, steady-state velocities appeared to be obtained in less than 1 min, and even at concentrations of hirudin of 20 pM, a steady-state velocity was apparently achieved in about 5 min. Although these rates are within the range that can be measured with a conventional recording spectrophotometer, they were found impossible to measure because the change in absorbance during these times was too small to be used for accurate data analysis. The rate at which the steady-state velocity was obtained, however, was dependent on the ionic strength. At an ionic strengths above 0.2, the rate of interaction between hirudin and thrombin became markedly slower, and Figure 4 shows the data obtained at an ionic strength of 0.4. At this ionic strength, the kinetics of the inhibition caused by hirudin 1A and 1B were the same as those shown in Figure 4. At higher ionic strengths, hirudin could be classified as a slow-binding inhibitor of thrombin (Morrison, 1982). A concentration of about 100 μM S-2238

Table III: Effect of Ionic Strength on the Inhibition of Thrombin by Hirudin[a]

ionic strength	added salt	eq	$K_{I'}$ (pM)	$k_{1'}$ ($nM^{-1}s^{-1}$)	$k_{2'}$ ($s^{-1} \times 10^3$)
0.1	75 mM NaCl	3	0.29 ± 0.06	1.94[c]	0.56[b]
0.125	100 mM NaCl	3	0.53 ± 0.10	1.06[c]	0.56[b]
0.2	175 mM NaCl	4	1.61 ± 0.14	0.387 ± 0.011	0.62 ± 0.06
0.3	275 mM NaCl	4	3.11 ± 0.17	0.177 ± 0.004	0.55 ± 0.03
0.35	325 mM NaCl	4	4.25 ± 0.32	0.125 ± 0.003	0.54 ± 0.04
0.4	375 mM NaCl	4	6.48 ± 0.44	0.088 ± 0.002	0.57 ± 0.04
0.4	330 mM Na_2SO_4	4	5.72 ± 1.02	0.090 ± 0.007	0.51 ± 0.10

[a] Assays were performed as described in the text with a concentration of 93 μM S-2238 and in a buffer consisting of 50 mM Tris, pH 7.8, to which various concentrations of salt were added in order to vary the ionic strength. The values for ionic strength given in the table were calculated assuming a pK_a of 7.8 for Tris at 37 °C (Ellis & Morrison, 1982); the ionic strengths of the solutions determined from their conductance were within 5% of the calculated values. The values of the kinetic parameters were determined by fitting the data to eq 3 or 4 as indicated and are given together with their standard errors. [b] At these values of ionic strength, slow-binding inhibition was not observed, and this value of $k_{2'}$ represents the weighted mean of the values obtained with higher concentrations of NaCl. [c] These values for $k_{1'}$ were calculated by dividing the weighted mean value of $k_{2'}$ by the determined value of $K_{I'}$.

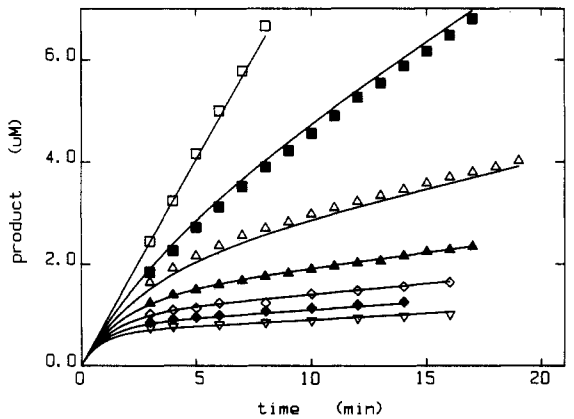


FIGURE 4: Slow-binding inhibition of thrombin by hirudin. Assays were performed as described under Experimental Procedures in 50 mM Tris buffer, pH 7.8, containing 375 mM NaCl and with concentrations of 45 pM thrombin and 93 μM S-2238. Hirudin was present at concentrations of 0 (□), 40 (■), 80 (△), 120 (▲), 160 (◇), 200 (◆), and 241 (▽) pM. The data were fitted to eq 4, and the lines shown in the figure were drawn by using the values of the parameters given in Table III. Data points at times less than 3 min have been omitted.

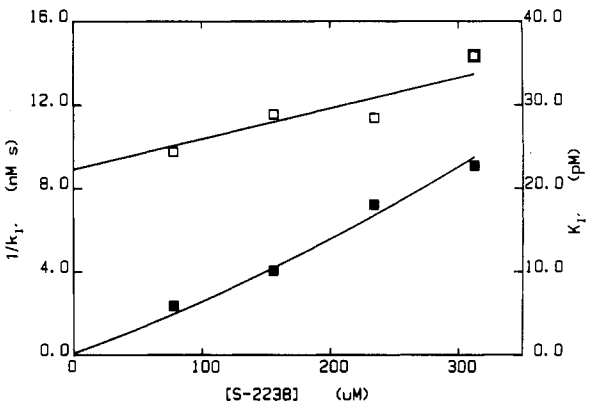


FIGURE 5: Effect of substrate concentration on the values of the parameters describing the slow-binding inhibition of thrombin by hirudin. Assays were performed and data analyzed as described in the legend of Figure 4 with four different concentrations of S-2238. The figure shows the variation of $k_{1'}$ (□) and $K_{I'}$ (■) with substrate concentration. The line was drawn through the reciprocal values of $k_{1'}$ using the reciprocal form of eq 5 and the values of 0.61 mM for K_S and 1×10^8 M^{-1} s^{-1} for k_1. The line was drawn through the values of $K_{I'}$ by using eq 6 and values of 0.27 pM, 3.63 μM, and 0.93 mM for K_I, K_m, and K_{IS}, respectively.

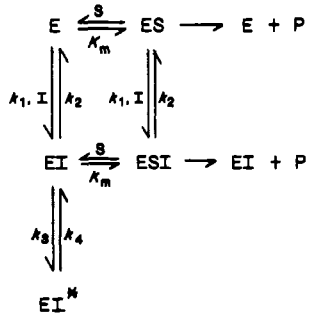
was used in most slow-binding inhibition experiments. At this concentration of substrate, there would not be significant binding of substrate at the proposed second substrate binding site. At least 10 μM substrate could, however, be consumed before there was a significant decrease in the rate of the enzyme due to substrate depletion.

Data obtained at several ionic strengths were analyzed according to eq 4, and the values for $k_{1'}$ and $K_{I'}$ obtained are given in Table III. The values obtained for both of these parameters were found to be highly dependent on ionic strength. The value of $K_{I'}$ increased 20-fold when the ionic strength was increased from 0.1 to 0.4. The effect seems to be an ionic strength effect rather than a specific effect of sodium or chloride ions since when Na_2SO_4 was used to adjust the ionic strength instead of NaCl, essentially the same values for $K_{I'}$ and $k_{1'}$ were observed (Table III). In addition, the observed effect was not due to a change in the affinity of the enzyme for the substrate since the K_m for the substrate did not change significantly in the ionic strength range used. The observed changes in the values of $k_{1'}$ and $K_{I'}$ with ionic strength are consistent with an ionic interaction being important in the binding of hirudin to thrombin (Eigen et al., 1964; Nolte et al., 1980).

The apparent rate of dissociation of the complex ($k_{2'}$) is equal to the product of $K_{I'}$ and $k_{1'}$ (see Scheme I). The value of $k_{2'}$ calculated in this manner did not change significantly when the ionic strength was increased from 0.2 to 0.4 (Table III). Assuming that the value of $k_{2'}$ is also constant at values of ionic strength below 0.2, it is possible to calculate a value for $k_{1'}$ at lower values of ionic strength. The weighted mean value of $k_{2'}$ determined at values of ionic strength above 0.2 was $(0.56 \pm 0.02) \times 10^{-3}$ s^{-1}. The value of $k_{1'}$ is given by $k_{2'}/K_{I'}$ and was found to be in excess of 1×10^9 M^{-1} s^{-1} at values of ionic strength of 0.125 or less (Table III).

If the substrate and hirudin compete for the same binding site, the value of $k_{1'}$ should vary with the concentration of substrate as described by eq 5; that is, a plot of $1/k_{1'}$ vs. the concentration of substrate should yield a straight line with an intercept of $1/k_1$ and a slope of K_S/k_1. To examine whether this relationship holds, data were obtained at an ionic strength of 0.4 with several different concentrations of S-2238. The data were analyzed according to eq 4 and Figure 5 shows the plot of $1/k_{1'}$ vs. the concentration of substrate. The values of $k_{1'}$ were weighted according to the squared inverse of their standard errors and fitted to eq 5. This analysis yielded a value of 0.61 ± 0.21 mM for K_S and $(1.1 \pm 0.2) \times 10^8$ M^{-1} s^{-1} for k_1. The value of K_S determined from this analysis is 2 orders of magnitude greater than the value of 3.63 μM obtained for the Michaelis constant from initial velocity experiments. It does, however, agree reasonably well with the value of 0.93 mM obtained for K_{IS} from the analysis of the data of Figure 2. Thus, the rate of interaction between thrombin and hirudin does not appear to depend on whether a tripeptidyl substrate is bound at the active site. Figure 5 also displays the effect

Scheme II[a]



[a] $K_r = k_4/k_3$.

of substrate concentration on the value of $K_{I'}$. The data could be adequately described by eq 6. Assuming that K_m and K_{IS} equal 3.63 μM and 0.93 mM, respectively, weighted linear regression according to eq 6 yielded a value of 0.21 ± 0.01 pM for K_I at an ionic strength of 0.4.

Discussion

Hirudin was found to form a very tight complex with thrombin, and the dissociation constant for the complex was determined to be about 20 fM. This value is about 3 orders of magnitude lower than some other values cited in the literature (Fenton, 1981; Walsman & Markwardt, 1981). Previous studies have, however, been less detailed, and the data have not been subjected to the same statistical analysis as has been performed in the current study. The value determined by Landis et al. (1978) appears to agree more closely with the value determined in this study. These workers determined the value of the dissociation constant to be less than 0.1 nM in the presence of 1.0 mM Tos-arginine methyl ester at 22 °C. The value of K_m for this substrate is 10.4 μM (Fenton et al., 1979), and thus, the dissociation constant for hirudin in the absence of the substrate would be less than 1 pM (see eq 2). Moreover, this value would be even lower at 37 °C since the dissociation constant for hirudin has been shown to decrease markedly as the temperature is increased (Landis & Waugh, 1975).

The data presented are consistent with the binding of hirudin being competitive with the binding of peptidyl *p*-nitroanilide substrates at the active site. The rate of interaction of hirudin and thrombin, however, appeared to be independent of the binding of the substrate at the active site (Figure 5). In addition, both the binding of hirudin and its rate of interaction with thrombin were inhibited by the binding of the substrate at a second, lower affinity site (Figures 2 and 5). As discussed earlier, this second site could be either on the enzyme or on hirudin. On the basis of data presented, the mechanism shown in Scheme II can be proposed: We propose that the binding of hirudin to thrombin involves at least two steps. At higher ionic strength, the rate-limiting step involves an interaction at a site distinct from the active site, and this complex rearranges to form a tighter complex in which hirudin is also bound at the active site. In order to form this tighter complex, the substrate must not be bound at the active site. This mechanism predicts that, at higher ionic strength, the rate-limiting step in the interaction between hirudin and thrombin will not be influenced by the binding of the substrate at the active site as was observed (Figure 5). The overall equilibrium constant for the formation of the complex should, however, depend on whether the substrate is bound at the active site, and this prediction is consistent with the observed dependence of the value of $K_{I'}$ on substrate concentration (Figures 2 and 5). This proposed mechanism is also consistent with data obtained with D-Phe-Pro-Arg chloromethyl ketone inactivated thrombin as a competitor for the interaction between active thrombin and hirudin. These data indicated that hirudin was able to bind to D-Phe-Pro-Arg-thrombin but the affinity of the inactivated thrombin for hirudin was much reduced; the dissociation constant was 19 nM (S. R. Stone and J. Hofsteenge, unpublished results). Thus, hirudin is still able to bind to thrombin when the active site is blocked but can bind more tightly when it is able to bind to the active site. In order to completely explain the data of Figures 2 and 5, it is necessary to propose a second binding site for the substrate. This second site could be on either thrombin or hirudin, and the binding of the substrate to this site inhibits the reaction between thrombin and hirudin. Although the rate-limiting step in the formation of the thrombin–hirudin complex at higher ionic strength appears to involve the interaction of hirudin with a site on thrombin distinct from the active site, the nature of the rate-limiting step at lower ionic strength cannot be determined from the data presented in this paper.

At concentrations of S-2238 where the binding of the substrate at the lower affinity site is not significant, the relationship given in eq 7 describes the apparent dissociation

$$K_{I'} = \frac{k_2 K_r (1 + S/K_m)}{k_1} \qquad (7)$$

constant for hirudin ($K_{I'}$). It follows from this equation that the value of the apparent dissociation rate constant ($k_{2'}$) given in Table III is equal to the numerator of eq 7. The rate of dissociation of hirudin from the complex will be equal to $k_2 K_r$ or $k_{2'}/(1 + S/K_m)$. Thus, from the mean value of $k_{2'}$ given in Table III, a value of 2×10^{-5} s^{-1} can be calculated for the dissociation rate constant of hirudin from the complex, and for all practical purposes, the formation of the complex can be considered irreversible.

The mechanism proposed in Scheme II is also consistent with results obtained with thrombomodulin. Thrombomodulin is a protein that is found on the surface of endothelial cells. When this protein binds to thrombin, thrombin becomes a more efficient activator of protein C but is no longer able to cleave fibrinogen. The ability of thrombin to cleave low molecular weight substrates is not, however, greatly affected. Thus, thrombomodulin has been proposed to bind at a site distinct from the catalytic site (Esmon, 1983). Thrombomodulin has been found to block the interaction between thrombin and hirudin (Hofsteenge et al., 1986). Moreover, the rate of interaction between hirudin and thrombin was dependent on the concentration of thrombomodulin as would be predicted for the mechanism of Scheme II if thrombomodulin and hirudin were competing for the same site. Other workers have suggested that binding sites distinct from the catalytic site are important in the binding of hirudin (Fenton, 1981; Fenton et al., 1979; Walsmann & Markwardt, 1981).

The rapid rate of formation of the thrombin–hirudin complex (>1×10^9 M^{-1} s^{-1} at low ionic strength) together with the strong dependence of this rate on ionic strength suggests that the initial interaction between hirudin and thrombin is diffusion-controlled and involves an ionic interaction (Nolte et al., 1980). The results of other studies also suggest that charged regions of thrombin and hirudin are important for the interaction between the two molecules. A region rich in acidic amino acids at the C-terminus of hirudin has been shown to be essential to the inhibitory activity of hirudin (Chang, 1983). The evidence with thrombin is less definitive. Acetylation of the free amino groups of thrombin results in an enzyme that retains its ability to cleave low molecular weight substrates

but is unable to bind hirudin (Markwardt, 1970). Thrombin is converted through limited proteolysis from the α-form to β- and γ-thrombin which are no longer able to cleave fibrinogen but retain amidolytic and esterase activity. These proteolytically altered forms of thrombin have a reduced affinity for hirudin (Landis et al., 1978). One of the regions of α-thrombin, which is altered in the proteolyzed forms, is a region rich in basic amino acids (Berlinger, 1984), and it is possible that this region is important in the binding of hirudin. In an attempt to define more precisely the regions of thrombin involved in the interaction, we are conducting protein chemical and immunochemical studies.

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