Identification of Regions of α-Thrombin Involved in Its Interaction with Hirudin

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Received November 14, 1986; Revised Manuscript Received March 11, 1987

ABSTRACT: The contributions of various regions of human α-thrombin to the formation of the tight complex with hirudin have been assessed by using derivatives of thrombin. α-Thrombin in which the active-site serine was modified with diisopropyl fluorophosphate was able to bind hirudin, but its affinity for hirudin was decreased by 10^3-fold compared to unmodified α-thrombin. Modification of the active-site histidine with D-Phe-Pro-Arg-CH_2Cl resulted in a form of thrombin with a 10^6-fold reduced affinity for hirudin. γ-Thrombin is produced by proteolytic cleavage of α-thrombin in two surface loops corresponding to residues 65–83 and 146–150 in α-chymotrypsin [Berliner, L. J. (1984) *Mol. Cell. Biochem. 61*, 159–172; Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187–240]. The γ-thrombin–hirudin complex had a dissociation constant that was 10^6-fold higher than that of α-thrombin. Treatment of α-thrombin with pancreatic elastase resulted in a form of thrombin only cleaved in the loop corresponding to residues 146–150 in α-chymotrypsin, and this form of thrombin had only a slightly reduced affinity for hirudin. By using limited proteolysis with trypsin, it was possible to isolate β-thrombin which contained a single cleavage in the loop corresponding to residues 65–83 in α-chymotrypsin. This form of thrombin had a 100-fold decreased affinity for hirudin. Kinetic analysis of the binding of hirudin to β-thrombin indicated that the 100-fold decrease in affinity was predominantly due to a decrease in the rate of association of the two molecules. The results are discussed with respect to the contributions of various regions of thrombin to its tight-binding interaction with hirudin.

Thrombin is a serine protease which plays a central role in blood coagulation. It cleaves fibrinogen to produce fibrin monomers which polymerize to form the basis of a blood clot (Fenton, 1981). The inhibition of thrombin would provide an effective means of controlling thrombosis. The main circulating inhibitor for thrombin is antithrombin III, but thrombin is only slowly inactivated by this inhibitor in the blood (Rosenberg, 1977). Heparin increases the rate of inactivation of thrombin by antithrombin III by about 3 orders of magnitude (Olson & Shore, 1982), and for this reason, heparin is currently used as a therapeutic agent (Lundblad et al., 1980). The use of heparin, however, has disadvantages. Heparin has a short half-life in the blood (Simon, 1980) and is ineffective for patients with antithrombin III deficiencies. Because of these disadvantages, alternative ways to bring about the rapid inactivation of thrombin have been sought. A number of groups have attempted to design thrombin inhibitors which could be used in the control of thrombosis (Kettner & Shaw, 1981; Kikumoto et al., 1980a,b; Okamoto et al., 1980; Kaiser et al., 1985). Hirudin, which is found in the salivary glands of the medicinal leech *Hirudo medicinalis*, is a very specific and potent inhibitor of thrombin and could provide a useful alternative in the control of thrombosis (Walsmann & Markwardt, 1981). In addition, with the use of biotechnology, it now seems likely that sufficient quantities of this inhibitor will be available for possible therapeutic use (Harvey et al., 1986; Bergmann et al., 1986).

The interaction between thrombin and hirudin is also of interest from the point of view of the mechanisms involved in protein–protein interactions. Thrombin and hirudin interact rapidly to form an extremely tight noncovalent complex; the dissociation constant of this complex is about 20 fM (Stone & Hofsteenge, 1986), and it is of interest to determine the nature of the interactions involved in the formation of this complex.

This paper attempts to delineate the areas of thrombin which are involved in this interaction and the relative contributions of these various areas to the overall tightness of the complex. A number of derivatives of α-thrombin have been reported that possess some of the properties of α-thrombin but lack others. For example, autodegradation of α-thrombin or limited degradation by trypsin leads to the formation of γ-thrombin. This form of thrombin has amidolytic and esterolytic activities similar to α-thrombin but shows very little activity with fibrinogen as a substrate (Berliner, 1984). It has also been reported that γ-thrombin has a much reduced affinity for hirudin (Landis et al., 1978). However, the usefulness of γ-thrombin in identifying regions of thrombin that are important in its interaction with hirudin is limited because at least two regions of the molecule are altered through proteolytic cleavage (Fenton et al., 1977b; Boissel et al., 1984). Recently, methods have been developed for the isolation of thrombin derivatives that are only altered in one of these two regions (Kawabata et al., 1985; Braun et al., submitted for publication), and this paper presents the results of studies on the interaction of hirudin with these derivatives. By these means, it has been possible to assess the importance of two regions removed from the active site in the binding of hirudin.

Thrombin can also be modified at the active site to yield molecules that are catalytically inactive but retain the ability of α-thrombin to bind to cell-surface receptors (Tollefsen et al., 1974; Esmon et al., 1982; Carney et al., 1984). Thrombin with its active-site serine modified by diisopropyl fluorophosphate is still able to bind to hirudin (Fenton et al., 1979). Thrombin can also be inactivated by peptidyl chloromethanes through alkylation of the active-site histidine (Glover & Shaw, 1971). The inhibitor D-Phe-Pro-Arg-CH_2Cl has been shown to be a potent inhibitor of α-thrombin (Kettner & Shaw, 1981) and is thought to occupy an apolar binding site in addition to the primary specificity pocket (Sonder & Fenton, 1984; Walker et al., 1985). In the present paper, the binding of hirudin to α-thrombin inactivated with D-Phe-Pro-Arg-CH_2Cl

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0006-2960/87/0426-4617$01.50/0

or diisopropyl fluorophosphate has been examined. By comparing the affinity for hirudin of these two inactivated forms of thrombin with that of unmodified α-thrombin, it was possible to assess the contributions of the active-site and the apolar binding region to the binding of hirudin.

Experimental Procedures

Materials. The substrates D-Phe-Pip-Arg-pNA[1] (S-2238) and Tos-Gly-Pro-Arg-pNA (Chromozym TH) were from Kabi Vitrum, Molndal, Sweden, and Boehringer Mannheim, Mannheim, West Germany, respectively. Trypsin (Tos-Phe-CH_2Cl treated, 240 units/mg) and porcine pancreatic elastase were purchased from Worthington Biochemicals, Freehold, NJ. Tritiated diisopropyl fluorophosphate was from Amersham, Amersham, England. D-Phe-Pro-Arg-CH_2Cl and Eglin C were gifts from Dr. E. Shaw, Friedrich Miescher Institut, Basel, Switzerland, and Dr. H.-P. Schnebli, Ciba-Geigy, Basel, Switzerland, respectively. All other chemicals were of the highest purity available commercially. Hirudin (>2000 antithrombin units/mg) was obtained from Pentapharm AG, Basel, Switzerland, and further purified by HPLC as previously described (Stone & Hofsteenge, 1986). This material yielded one peak on HPLC and was homogeneous as judged by Edman degradation. The amino-terminal sequence corresponded to that of the major form previously described (Stone & Hofsteenge, 1986); that is, the amino acid sequence started with valine-valine. Thrombin was prepared as previously described (Stone & Hofsteenge, 1986) and was pure as judged by gel electrophoresis and amino acid sequence analysis.

Amidolytic assays were performed as previously described at 37 °C in 0.05 M Tris-HCl buffer, pH 7.8, which contained 0.1 M NaCl and 0.1% poly(ethylene glycol), M_r 6000 (Stone & Hofsteenge, 1986).

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

Preparation of Inactivated Forms of α-Thrombin. To produce α-thrombin in which the active-site serine was modified by diisopropyl fluorophosphate, α-thrombin (5–15 μM) was incubated at room temperature with 1.0 mM diisopropyl fluorophosphate in 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl for 30 min. After this period, the residual amidolytic activity was assayed, and another aliquot of diisopropyl fluorophosphate was added such that the nominal concentration in the inactivation mixture was 2.0 mM. This procedure was repeated every 30 min until the residual activity was less than 0.01% of the original activity. The enzyme was then dialyzed exhaustively against the incubation buffer and its concentration determined by measuring its absorbance at 280 nm (Fenton et al., 1977a). The residual activity of the preparation was again assayed after dialysis and remained less than 0.01%. The preparation also did not contain a significant concentration of diisopropyl fluorophosphate as incubation of the preparation with active thrombin did not cause any inactivation of thrombin. Essentially the same method as described above was used to prepare α-thrombin inactivated by D-Phe-Pro-Arg-CH_2Cl except that the incubation mixture contained 10.0 μM D-Phe-Pro-Arg-CH_2Cl instead of 1.0 mM diisopropyl fluorophosphate. The residual activity of this preparation was less than 0.001%.

Preparation of Proteolyzed Forms of Thrombin. For the preparation of β-thrombin, a 2-mL aliquot of α-thrombin (1.2 mg/mL) in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.3 M NaCl was treated with 0.3% (w/w) trypsin at 37 °C until approximately 40% of the clotting activity was lost (20–40 min). Soybean trypsin inhibitor was then added to a concentration of 50 μg/mL, and the mixture was incubated at 37 °C for an additional 5 min. This material was diluted to 10 mL with ice-cold 10 mM sodium phosphate buffer, pH 6.5, which contained 0.1% poly(ethylene glycol) 6000 and immediately applied to a Mono S 10/10 fast protein liquid chromatography (FPLC) column (Pharmacia, Uppsala, Sweden). The column was eluted with a linear gradient of 20–240 mM sodium phosphate buffer, pH 6.5, which contained 0.1% poly(ethylene glycol) 6000. β-Thrombin eluted between 50 and 70 mM phosphate, before α-thrombin. Fractions containing the major part of the β-thrombin activity free from α-thrombin were pooled and diluted with water to a concentration of about 20 mM sodium phosphate and then rechromatographed by using the gradient described above. Fractions containing β-thrombin were pooled, and NaCl was added to a final concentration of 0.5 M. This solution was frozen in an acetone–dry ice bath and stored at −70 °C.

For the preparation of γ-thrombin, a 6-mL aliquot of α-thrombin (1 mg/mL) in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.15 M NaCl and 0.1% poly(ethylene glycol) 6000 was treated with 3% (w/w) trypsin at 37 °C for 4 h. Soybean trypsin inhibitor was added to a concentration of 50 μg/mL, and the γ-thrombin was purified by FPLC by using the gradient system described above for the preparation of β-thrombin. In this system, γ-thrombin eluted slightly before β-thrombin. Details of the preparation and characterization of β- and γ-thrombin will be published elsewhere (Braun et al., submitted for publication).

A third proteolyzed derivative of thrombin can be prepared by treatment of thrombin with pancreatic elastase (Kawabata et al., 1985), and we have designated this form ε-thrombin. This form was produced by digestion of α-thrombin (0.72 mg/mL) with elastase (16 μg/mL) in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 0.1% poly(ethylene glycol) 6000 for 3 h at room temperature. The total reaction volume was 3.2 mL. At this time, the conversion of α-thrombin to ε-thrombin was complete as judged by gel electrophoresis, and no other protein bands were detected. This material retained about 60% of the original clotting activity. The digestion was stopped by adding 150 μg of Eglin C and then diluted to 10 mL with 20 mM sodium phosphate buffer, pH 6.5, containing 0.1% PEG 6000. This solution was applied to a Mono S FPLC column and the ε-thrombin purified by using the gradient system described above for β-thrombin. The fractions eluting between 75 and 90 mM sodium phosphate were pooled, dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, containing 0.3 M NaCl and 0.1% PEG 6000 at 4 °C, and stored at −70 °C.

All preparations of proteolyzed forms of thrombin were greater than 95% active as determined by active-site titration with methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973). The purity of these preparations was determined by labeling with tritiated diisopropyl fluorophosphate followed by sodium dodecyl sulfate gel electrophoresis and densitometry of autoradiograms or by liquid scintillation counting of sliced gel lanes (Lottenberg et al., 1982). The β-thrombin preparation was found to contain about 3% γ-thrombin and less than 2% α-thrombin whereas the γ-thrombin and ε-thrombin

[1] Abbreviations: DIP, diisopropylphosphoryl; Pip, pipecolyl; pNA, *p*-nitroanilide; Tos, tosyl; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; FPLC, fast protein liquid chromatography; PEG, poly(ethylene glycol).

preparations contained less than 1% of other thrombin forms. Amino acid sequence analysis (five cycles) detected only one additional amino terminus in the preparations of β-thrombin and ε-thrombin and allowed the cleavage sites to be identified as the B-chain residues Arg^{73}-Asn^{74} and Ala^{150}-Asp^{151} for β-thrombin and ε-thrombin, respectively. Similar analysis with γ-thrombin detected several N-termini in addition to the reported cleavage sites at Arg^{73}-Asn^{74} and Lys^{154}-Gly^{155} (Fenton et al., 1977; Boissel et al., 1984).

Analysis of Kinetic Data. The tight-binding inhibition of thrombin by hirudin was analyzed as previously described (Stone & Hofsteenge, 1986). Previous studies indicated that the substrate was able to compete with hirudin by binding at the active site and at a second lower affinity site (Stone & Hofsteenge, 1986). In most of the experiments presented, the concentration of substrate was maintained sufficiently low so that the binding of the substrate at the second site would not be significant. Under these conditions, the apparent inhibition constant ($K_{I'}$) obtained from the analysis can be related to the dissociation constant of the thrombin–hirudin complex (K_I) by eq 1 where S is the concentration of the substrate in the assay and K_m represents its Michaelis constant.

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

Under conditions where tight-binding inhibition is not observed, the inhibition caused by hirudin of a preparation which consists of only one species of thrombin will be described by eq 2 where v_0 is the velocity of substrate cleavage in the absence of inhibitor and I is the concentration of the inhibitor. If, however, there are two forms of the enzyme present and if the two forms have a different affinity for hirudin, then the variation of the velocity with the inhibitor concentration will be described by eq 3 (Segel, 1975) where v_{0_1} and v_{0_2} are the

$$v = \frac{v_0}{1 + I/K_{I'}} \quad (2)$$

$$v = \frac{v_{0_1}}{1 + I/K_{I_1'}} + \frac{v_{0_2}}{1 + I/K_{I_2'}} \quad (3)$$

velocities of the two enzyme species in the absence of inhibitor and $K_{I_1'}$ and $K_{I_2'}$ are the apparent inhibition constants for the two species. Data for the inhibition of γ-thrombin and β-thrombin preparations by hirudin were fitted to eq 2 and 3, respectively. Weighted linear regression (Cornish-Bowden & Endrenyi, 1981) was used for analysis according to eq 2 and weighted nonlinear regression (Duggleby, 1981) for eq 3. Weights were assigned on the assumption that the errors in the velocities were proportional to the velocities.

Slow-binding inhibition data were analyzed according to eq 4 (Morrison, 1982) where P represents the concentration of product at time t, v_i and v_s represent the initial and final steady-state velocity of the reaction, respectively, and k' is an apparent first-order rate constant. Nonlinear regression was used to fit data to this equation.

$$P = v_s t + (v_i - v_s)[1 - \exp(-k't)]/k' \quad (4)$$

RESULTS

Inhibition of Proteolyzed Forms of Thrombin by Hirudin. The proteolyzed form of thrombin which has been called γ-thrombin is readily obtained by treatment of α-thrombin with trypsin or by promoting the autolysis of thrombin with high salt concentrations. This form of thrombin contains structural alterations in at least two regions of the molecule (Fenton et al., 1977b; Boissel, 1984). Hirudin inhibited γ-thrombin only at much higher concentrations than those required to inhibit α-thrombin, and the inhibition was linear with respect to hirudin. Analysis of the data (7 points, 0–229 nM) according to eq 2 yielded an apparent inhibition constant of 22.2 ± 0.7 nM in the presence of 9.6 μM Tos-Gly-Pro-Arg-pNA. The value of the Michaelis constant of this substrate with γ-thrombin was determined to be 49 ± 3 μM, and by use of this value together with the relationship given in eq 1, a value of 18.6 ± 0.6 nM, was obtained for the dissociation constant of the γ-thrombin–hirudin complex (Table I). This value is 10^6-fold higher than the value of 22 fM previously obtained for α-thrombin (Stone & Hofsteenge, 1986).

Table I: Dissociation Constants for Interactions of Various Forms of Thrombin with Hirudin[a]

form of thrombin	dissociation constant
α-thrombin	22 fM[b]
β-thrombin	2.9 ± 0.2 pM[c]
γ-thrombin	19 ± 1 nM
ε-thrombin	56 ± 7 fM
DIP-thrombin	26 ± 6 pM
D-Phe-Pro-Arg-CH_2–thrombin	19 ± 2 nM

[a] Assays were performed as described under Experimental Procedures and the data were analyzed as described under Analysis of Kinetic Data to yield the values of the dissociation constants which are given together with their standard errors. [b] This value was previously determined (Stone & Hofsteenge, 1986). [c] This value is the weighted mean of five independent determinations.

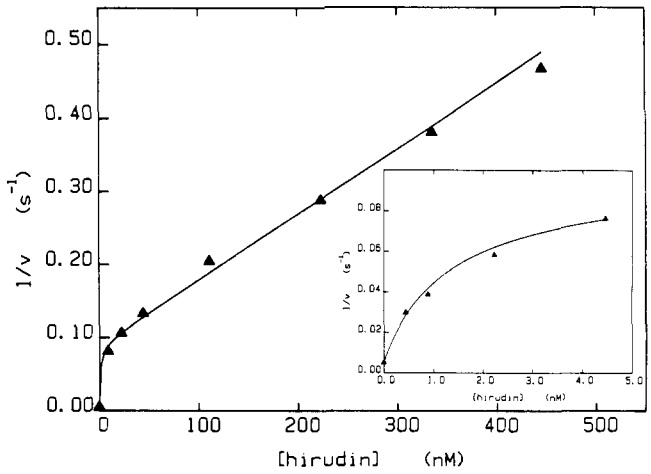

FIGURE 1: Effect of hirudin on the steady-state velocity of the β-thrombin preparation. Assays were performed as described under Experimental Procedures in the presence of 103 μM D-Phe-Pip-Arg-pNA. The concentrations of enzyme were 15, 30, 60, and 300 pM at hirudin concentrations of 0.45, 0.89–4.46, 8.9–44.6, and 111.5–446 nM, respectively. The data were fitted to eq 3, and the line in the figure shows the fit of the data to the reciprocal form of this equation. The inset shows data obtained at hirudin concentrations less than 5.0 nM.

The large decrease in the affinity of γ-thrombin for hirudin could be due to the proteolytic cleavages present in one or both of the two regions cleaved in α-thrombin, or it could be due to a more general alteration in the tertiary structure of thrombin. In order to address this problem, thrombin forms which contain cleavages in only one of the two regions cleaved in γ-thrombin have been isolated. The form designated β-thrombin is thought to be a precursor in the formation of γ-thrombin (Fenton et al., 1977b), but sufficient quantities of human β-thrombin to permit structural and kinetic studies have not previously been isolated. The effect of hirudin on the steady-state velocity of the β-thrombin preparation is shown in Figure 1. The nonlinearity of the plot of the reciprocal of the steady-state velocity against the concentration of hirudin is consistent with the preparation containing two enzyme

Scheme I[a]

$$\begin{array}{ccccc} E + A & \overset{K_m}{\rightleftharpoons} & EA & \xrightarrow{k_c} & E + P \\ + & & & & \\ I & & & & \\ k_1 \downarrow \uparrow k_2 & & & & \\ EI & & & & \end{array}$$

[a] $K_I = k_2/k_1$.

species with different affinities for hirudin (Segel, 1975), and the data were analyzed according to eq 3. Similar results have been obtained with three different preparations of β-thrombin. The concentration of hirudin was varied over a 1000-fold range in order to determine accurately the four parameters of eq 3, and the data have been presented in two parts to illustrate the effect at low and high hirudin concentrations. The enzyme concentration was varied over a 10-fold range to allow accurate measurement of the lower velocities. In all cases, however, the enzyme concentration was maintained at least 10-fold less than the concentration of hirudin so that the concentration of hirudin would not be significantly depleted by the formation of the thrombin–hirudin complex. The lower affinity component in the β-thrombin preparation accounted for 5% of the total activity. Labeling of the β-thrombin preparation with tritiated diisopropyl fluorophosphate indicated that the preparation contained about 3% γ-thrombin (see Experimental Procedures) and, thus, it was assumed that the lower affinity component corresponded to γ-thrombin. A value of 11.1 ± 0.5 μM was obtained for the Michaelis constant of D-Phe-Pip-Arg-pNA with γ-thrombin, and by using this value together with the relationship given in eq 1, it was possible to calculate a value of 10.5 ± 0.5 nM for the dissociation constant of hirudin with the lower affinity form. This value represents the weighted mean from five separate determinations of $K_{I_2'}$ and agrees with the values of 18.6 nM given above for γ-thrombin. A value of 9.7 ± 0.3 μM was obtained for the Michaelis constant of D-Phe-Pip-Arg-pNA with β-thrombin, and by use of this value, a weighted mean value from five determinations of 2.9 ± 0.2 pM was obtained for the dissociation constant of the β-thrombin–hirudin complex (Table I). This value is about 100-fold higher than the value previously obtained for α-thrombin.

At concentrations of hirudin less than 1 nM, the steady-state velocity of the reaction with β-thrombin was only slowly achieved as is shown for several inhibitor concentrations in Figure 2A. Such data were well described by the equation for slow-binding inhibition (eq 4); the lines in the figure represent the fit of the data to this equation. As the inhibitor concentration was increased, the initial velocity of the reaction did not vary significantly, but the steady-state velocity (v_s) decreased (data not shown), and the apparent first-order rate constant of the reaction (k') increased (Figure 2B). The variation of the steady-state velocity with the concentration of hirudin was similar to that shown in Figure 1. All the data for the slow-binding inhibition experiment were obtained at hirudin concentrations at least 100 times lower than the value of $K_{I'}$ for γ-thrombin, and the variation of v_s could be analyzed according to eq 3 assuming that $I/K_{I_2'}$ was equal to zero. This analysis yielded a value of 2.1 ± 0.2 pM for the dissociation constant of the β-thrombin–hirudin complex; this value is in good agreement with the value of 2.9 pM determined for this constant from the steady-state velocity experiment described above. As shown in Figure 2B, the value of k' increased in a linear fashion as the concentration of hirudin was increased. Such a dependence of the value of k' on the inhibitor concentration is consistent with the general mechanism outlined

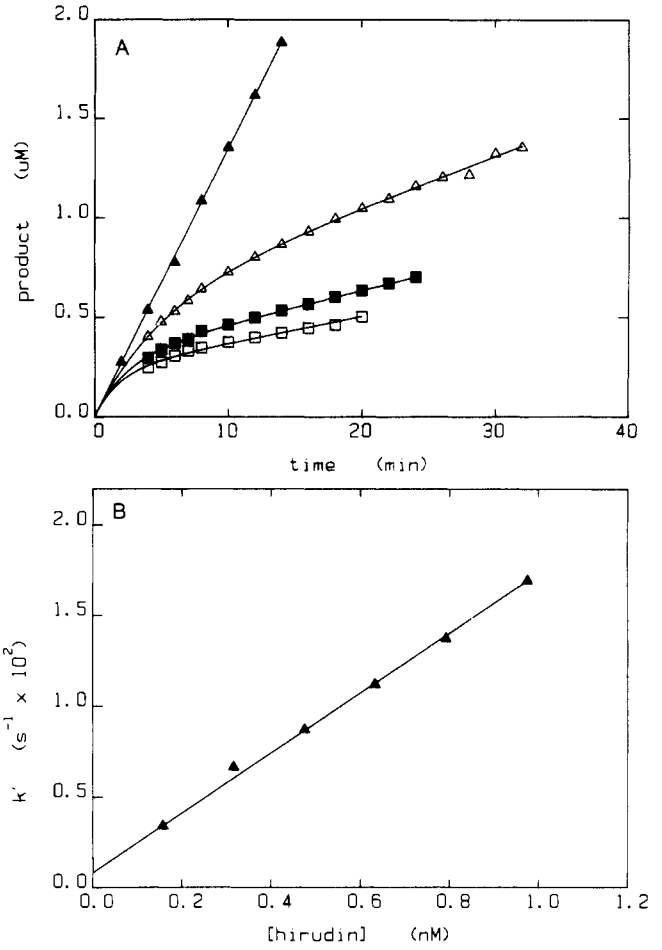

FIGURE 2: (A) Slow-binding inhibition of the β-thrombin preparation by hirudin. Assays were performed as described under Experimental Procedures in the presence of 104 μM D-Phe-Pip-Arg-pNA and 12 pM enzyme. The concentrations of hirudin were 0 (▲), 0.158 (△), 0.317 (■), and 0.475 nM (□). The data were fitted to eq 4, and the lines in the figure represent the fit to this equation. (B) Dependence of the apparent first-order rate constant for the slow-binding inhibition of β-thrombin on the concentration of hirudin. The value of k' was determined by analyzing data such as those shown in (A) according to eq 4. The line in the figure represents the fit of the data obtained by weighted linear regression to the equation $k' = k_2' + k_1'[\text{hirudin}]$. For this analysis, the values of k' were weighted according to the inverse square of their standard errors. Each point represents the mean of two determinations of k'.

in Scheme I (Morrison, 1982). For this mechanism, k' is equal to $k_2' + k_1'I$ where k_1' is the apparent association rate constant of the enzyme and inhibitor and k_2' is the apparent dissociation rate constant for the complex. Analysis of the data in Figure 2B by weighted linear regression according to this equation yielded a value of (1.65 ± 0.04) × 10^7 M^{-1} s^{-1} for k_1' and (8.1 ± 1.1) × 10^{-4} s^{-1} for k_2'. The values obtained for k_1' and k_2' do not represent the true values of the association and dissociation rate constants because the experiment was performed in the presence of substrate and the effect of this substrate on the rate constants has not been taken into account. Previous studies with α-thrombin indicated that the dependence of k_1' and k_2' on the substrate concentration could be described by eq 5 and 6, respectively (Stone & Hofsteenge, 1986), where

$$k_1' = \frac{k_1}{1 + S/K_{IS}} \quad (5)$$

$$k_2' = k_2(1 + S/K_m) \quad (6)$$

k_1 and k_2 are, respectively, the values of the association and dissociation rate constants in the absence of substrate, K_{IS} is

the dissociation constant of the substrate at a lower affinity site at which it competes for the formation of the enzyme–hirudin complex, and K_m is the Michaelis constant of the substrate. These relationships were also found to be valid for β-thrombin.[2] By using the relationships given in eq 5 and 6 together with the values of 9.7 μM for K_m and 0.64 mM for K_{IS} of D-Phe-Pip-Arg-pNA,[2] it was possible to calculate values of $(1.92 \pm 0.05) \times 10^7$ M^{-1} s^{-1} and $(6.7 \pm 1.1) \times 10^{-5}$ s^{-1} for k_1 and k_2 from the values of k_1' and k_2' obtained from the analysis of the data of Figure 2B. From these values of k_1 and k_2, a value of 3.5 ± 0.6 pM could be calculated for K_I by using the relationship given in Scheme I; this value is in agreement with the value of 2.9 pM determined from the steady-state velocity studies.

Treatment of α-thrombin with pancreatic elastase results in a form of thrombin that is cleaved between Ala^{150} and Asp^{151} (Kawabata et al., 1985) which is only four residues removed from Arg^{154}-Gly^{155} which is a major cleavage site in γ-thrombin (Fenton et al., 1977b; Boissel et al., 1984). We have termed this thrombin that has been treated with elastase ε-thrombin. Hirudin was found to be a tight-binding inhibitor of this form of thrombin with an apparent inhibition constant of 1.86 ± 0.22 pM in the presence of 90 μM D-Phe-Pip-Arg-pNA (data not shown). The Michaelis constant of D-Phe-Pip-Arg-pNA with ε-thrombin was 2.8 ± 0.1 μM, and by using this value, it was possible to calculate a value of 56 ± 7 fM for the dissociation constant of the ε-thrombin–hirudin complex (Table I). Thus, the cleavage between Ala^{150} and Asp^{151} has increased the dissociation constant of the thrombin–hirudin complex by less than 3-fold.

Interaction of Hirudin with Inactivated Forms of α-Thrombin. If hirudin were able to bind at sites distinct from the active site of thrombin, thrombin species with modified active sites would be expected to compete with α-thrombin in the formation of the complex with hirudin. The inclusion of D-Phe-Pro-Arg-CH_2–thrombin in the inhibition assays resulted in an increase in the observed dissociation constant for the α-thrombin–hirudin complex. With increasing concentrations of D-Phe-Pro-Arg-CH_2–thrombin, the dissociation constant of the α-thrombin–hirudin complex increased in a linear fashion (data not shown). The variation of the value of the dissociation constant with the concentration of D-Phe-Pro-Arg-CH_2–thrombin could be described by eq 1 as is expected for a competitive ligand, and analysis of the data (six points from 0 to 42 nM D-Phe-Pro-Arg-CH_2–thrombin) according to this equation yielded a value of 19.1 ± 2.4 nM for the D-Phe-Pro-Arg-CH_2–thrombin–hirudin complex. This value is 10^6-fold higher than the value obtained for α-thrombin (Table I).

Table II: Effect of DIP-thrombin on the Observed Steady-State Velocity of Thrombin in the Presence of Hirudin: Calculation of the Dissociation Constant for the Interaction between DIP-thrombin and Hirudin[a]

reaction conditions			
hirudin (pM)	DIP-thrombin (pM)	obsd velocity (nmol s^{-1} $nmol^{-1}$)	calcd dissociation constant for DIP-thrombin (pM)
0	0	208.7[b]	
57.4	0	8.7[b]	
57.4	13.6	9.0	13.5
		9.5	30.0
57.4	27.2	14.0	30.1
		15.7	36.8
57.4	40.8	17.9	27.9
		20.2	20.2
57.4	54.4	22.0	23.4
		21.1	26.0
		20.2	29.1
57.4	68.0	23.7	25.9
		24.8	22.9
		26.9	19.2
			av 26 ± 6

[a] Assays were performed as described under Experimental Procedures with a concentration of 45 pM α-thrombin in the presence of 106 μM D-Phe-Pip-Arg-pNA. For each assay, the dissociation constant for the DIP-thrombin complex was calculated as described in the text. [b] These values are the means of duplicate assays.

The interaction of DIP-thrombin with hirudin was much tighter than that observed for D-Phe-Pro-Arg-CH_2–thrombin, and the data could not be analyzed according to eq 1 because tight-binding interactions both between α-thrombin and hirudin and between DIP-thrombin and hirudin were observed. Moreover, with the concentrations of α-thrombin and DIP-thrombin of the same order, steady-state velocities were only slowly obtained such that substrate depletion became a problem. Thus, the following approach was taken to determine the dissociation constant of the DIP-thrombin–hirudin complex. The dissociation constant of the α-thrombin–hirudin complex was determined in the absence of DIP-thrombin. The effect of DIP-thrombin on the steady-state velocity at one particular hirudin concentration was then examined. The dissociation constants for the α-thrombin–hirudin complex (K_{I_1}) and the DIP-thrombin–hirudin complex (K_{I_2}) are given by eq 8a,b where I_t, E_t, and F_t are the total concentrations of

$$K_{I_1} = \frac{(E_t - EI)(I_t - EI - FI)}{EI} \quad (8a)$$

$$K_{I_2} = \frac{(F_t - FI)(I_t - EI - FI)}{FI} \quad (8b)$$

hirudin, α-thrombin, and DIP-thrombin, respectively, and EI

[2] The effect of substrate concentration on the association and dissociation rate constants for β-thrombin and hirudin was examined at one hirudin concentration. For the mechanism of Scheme I, the values of k_1' and k_2' can be calculated from the values of the parameters of eq 4 by using the relationships given in eq 7 (Morrison & Stone, 1985):

$$k_1' = \frac{k'}{I}\left(1 - \frac{v_s}{v_i}\right) \quad (7a)$$

$$k_2' = \frac{k'v_s}{v_i} \quad (7b)$$

For the β-thrombin preparation, however, the value of v_s must be corrected for the amount of γ-thrombin in the preparation which is not inhibited at the concentration of hirudin used. By using this procedure, it was possible to calculate values of k_1' and k_2' at various concentrations of D-Phe-Pip-Arg-pNA. The value of k_1' was found to decrease in a hyperbolic manner as the concentration of substrate was increased, and the data could be described by eq 5 (data not shown). Analysis of the data (six points in duplicate from 69 to 416 μM D-Phe-Pip-Arg-pNA) according to this equation yielded a value of 0.64 ± 0.1 mM for the dissociation constant of the substrate. This value does not agree with the value of 9.7 μM which was determined from initial velocity studies for the Michaelis constant of D-Phe-Pip-Arg-pNA. It agrees, however, with the value of 0.93 mM previously obtained for the dissociation constant of the lower affinity site at which the substrate competed in the interaction between α-thrombin and hirudin (Stone & Hofsteenge, 1986). The value of k_2' was found to be directly proportional to the concentration of substrate, and the data could be described by eq 6 (data not shown). Analysis of the data (six points in duplicate from 69 to 416 μM D-Phe-Pip-Arg-pNA) according to this equation yielded a value of 14.6 ± 5.9 μM for the Michaelis constant of the substrate, and this value agrees reasonably well with the value of 9.7 μM determined for its Michaelis constant. Analysis of the data yielded values of $(2.19 \pm 0.09) \times 10^7$ M^{-1} s^{-1} and $(4.4 \pm 1.8) \times 10^{-5}$ s^{-1} for k_1 and k_2, respectively, in the absence of substrate, and using the relationship given in Scheme I, it is possible to calculate a value of 2.03 ± 0.08 pM for the dissociation constant of the β-thrombin–hirudin complex. This value agrees well with the value of 2.9 pM determined from steady-state velocity studies.

Scheme II

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

and FI are the concentrations of the α-thrombin–hirudin and DIP-thrombin–hirudin complexes, respectively. By use of the relationships $E/E_t = v_I/v_0$ and $EI = E_t(1 - v_I/v_0)$ where v_I and v_0 are the steady-state velocities observed in the presence and absence of hirudin, respectively, and E is the concentration of free α-thrombin, eq 8a can be rearranged to yield eq 8c.

$$FI = I_t - E_t(1 - v_I/v_0) + K_{I_1}(1 - v_0/v_I) \qquad (8c)$$

Using the above relationships together with the previously determined value for K_{I_1}, it was possible to calculate the concentrations of EI and FI from the observed steady-state velocities in the presence and absence of hirudin. These values could then be substituted in eq 8b to obtain an estimate for K_{I_2}. The results obtained at five different concentrations of DIP-thrombin are given in Table II. Thirteen estimates of the dissociation constant were obtained, and the average value was 26 ± 6 pM which was 10^3-fold larger than that obtained with active α-thrombin (Table I). This value has been calculated on the basis that the substrate cannot compete with hirudin for binding sites on DIP-thrombin. This assumption seems justified on the basis of fluorescent titration experiments using dansylargininylmethylpiperidine. The binding of this compound to α-thrombin causes an increase in its fluorescence (Mann et al., 1981). No increase in fluorescence was observed, however, with DIP-thrombin even at concentrations of the compound 10-fold higher than those required to titrate fully the same concentration of α-thrombin (data not shown).

Discussion

With the three proteolytically modified forms of thrombin, three different types of inhibition by hirudin were observed. Tight-binding inhibition was observed with ϵ-thrombin, slow-binding inhibition with β-thrombin (Figure 2), and classical inhibition with γ-thrombin. In addition, because the preparation of β-thrombin contained a small fraction of γ-thrombin, hirudin appeared to be a hyperbolic inhibitor of this preparation (Figure 1). The mechanism of slow-binding inhibition of β-thrombin was unusual in that the association rate was independent of the binding of the substrate at the active site whereas the dissociation rate increased with increasing substrate concentration.[2] These observations are not consistent with any of the mechanisms commonly proposed to explain slow-binding inhibition (Morrison, 1982; Morrison & Stone, 1985; Morrison & Walsh, 1986). They are, however, consistent with a two-step binding mechanism previously proposed for the interaction of hirudin with α-thrombin (Stone & Hofsteenge, 1986) and outlined in Scheme II. In order to explain the data, it was proposed that the value of k_1 was rate limiting and that substrate binding at the active site was only competitive for the formation of the second complex (EI*); that is, the initial interaction of hirudin with the enzyme was not influenced by the presence of the substrate at the active site but, in order to form the tighter second complex, the active site could not be occupied by the substrate. The substrate could, however, compete in the formation of the initial complex by binding at a second lower affinity site. Further consideration of the model presented in Scheme II indicates that the initial interaction need not necessarily be rate limiting. If the steady-state assumption is made, that is, if the concentration of EI does not change during the course of the reaction and if its concentration is small relative to the total enzyme concentration, then the reaction at any concentration of hirudin will be described by eq 4 and the apparent first-order rate constant (k') will be given by eq 9. The values of the association and dissociation rate constants will be $k_1k_3/(k_2 + k_3)$ and $k_2k_4/(k_2 + k_3)$, respectively. If the value of k_3 is much greater than that of k_2, then the expressions for the association and dissociation rate constants are reduced to k_1 and k_2k_4/k_3. Under conditions where the substrate is only able to compete in the formation of the second complex, the observed value of k_3 would be $k_3/(1 + S/K_m)$, and the apparent association rate constant would be independent of the concentration of substrate, but the value of the dissociation rate constant would be directly proportional to the substrate concentration as was observed. Thus, the two-step mechanism of Scheme II is consistent with the data also if the steady-state assumption is made, the substrate can compete only in the second step, and $k_3 \gg k_2$. In such a case, hirudin would be analogous to a "sticky" substrate, which is defined as a substrate that undergoes forward reaction more rapidly than it dissociates from the enzyme; that is, $k_3 \gg k_2$ (Cleland, 1977).

$$k' = \frac{k_1k_3I + k_2k_4}{k_2 + k_3} \qquad (9)$$

It was not possible to measure the rate constants for the inhibition of α-thrombin by hirudin at the ionic strength used in this study. Values for these constants were, however, obtained by extrapolation from higher ionic strengths and were 1×10^9 M^{-1} s^{-1} and 2×10^{-5} s^{-1} for the association and dissociation rate constants, respectively (Stone & Hofsteenge, 1986). These values can be compared with the values of 2×10^7 M^{-1} s^{-1} and 4×10^{-5} s^{-1} obtained for β-thrombin. Thus, the 100-fold increase in the dissociation constant of the β-thrombin–hirudin complex is due predominantly to a 50-fold decrease in the rate of association. It was previously found that the rate of association of hirudin and α-thrombin was markedly dependent on ionic strength (Stone & Hofsteenge, 1986); such a dependence is consistent with the involvement of a charge–charge interaction in the association of hirudin with thrombin. The carboxyl terminal of hirudin contains a number of negatively charged residues which appear to be important to the activity of hirudin (Chang, 1983). When the sequences of α-thrombin and α-chymotrypsin are aligned, the cleavage site for β-thrombin is found within a sequence in which amino acid substitutions have introduced five additional basic residues into human α-thrombin (Jackson & Nemmerson, 1980). The sequence corresponds to the surface loop 63–85 in α-chymotrypsin (Birktoft & Blow, 1972). Comparison with other coagulation proteases indicates that α-thrombin also contains at least three more basic residues in this region than factor IX_a (Kurachi et al., 1982), factor X_a (Fung et al., 1985), and protein C_a (Foster & Davie, 1984). It seems possible that this basic region in α-thrombin makes a major contribution to its interaction with hirudin and to the specificity of this interaction. Disruption of the structure of this region in β-thrombin would lead to the observed decrease in the affinity for hirudin.

Although the data obtained with β-thrombin suggest that the region around the β-cleavage site is important for the binding of hirudin, it is not possible to conclude that the decrease in affinity is exclusively due to alterations in this region. In the absence of X-ray crystal structures of α- and β-thrombin, it is not certain that the cleavage that produces β-thrombin has not caused structural alterations in other regions of the enzyme. The fact that the Michaelis constant for D-Phe-Pip-Arg-pNA has increased about 2.5-fold suggests that there

may also be minor structural changes around the residues responsible for the binding of this substrate. It has been possible, however, to isolate an antibody against a synthetic peptide which contains the β-cleavage site, and this antibody was able to block the binding of hirudin to α-thrombin (G. Noë, J. Hofsteenge, and S. R. Stone, unpublished results). With γ-thrombin, the possibility of extensive structural alterations makes the interpretation of the data difficult. The data obtained with ϵ-thrombin indicate that the region around the second major cleavage site in γ-thrombin, which corresponds to residues 146–150 in α-chymotrypsin (Birktoft & Blow, 1972), does not play a major role in the binding of hirudin (Table I). In the γ-thrombin, a large portion of the loop containing the β-cleavage site has been removed by proteolysis, and a number of minor cleavages are present besides the cleavage in the γ-loop (Fenton et al., 1977b; Boissel et al., 1984; P. J. Braun, J. Hofsteenge, and S. R. Stone, unpublished results). Thus, the large difference in the affinity for hirudin of β- and γ-thrombin could be due to the removal of the β-loop or to more general structural alterations.

By using thrombin molecules with their active site modified, it has been possible to assess the importance of regions around the active site to the binding of hirudin. The affinity of DIP-thrombin for hirudin was 1000-fold less than that of α-thrombin. In DIP-thrombin, it has been presumed that the active-site serine was modified (Fenton, 1981) and the bulky diisopropyl group appears to block the primary specificity pocket since DIP-thrombin has a much reduced affinity for dansylargininyl-methylpiperidine. It can be calculated from the 1000-fold decrease in affinity that a maximum binding energy of 18 kJ/mol is contributed by the active-site serine and the primary specificity pocket. This value should be regarded as a maximum value since the blocking of the active-site binding regions may force hirudin to make less favorable contacts in regions distant from the active site. D-Phe-Pro-Arg-CH_2Cl inactivates thrombin by alkylating the active-site histidine (Glover & Shaw, 1971). In addition, the arginine of the tripeptide will be bound in the primary specificity pocket, and it has been proposed that the tripeptide also occupies an apolar binding region (Sonder & Fenton, 1984; Walker et al., 1985). Sonder and Fenton (1984) have demonstrated the existence of an apolar binding region that is able to bind proflavin with an affinity of 30 μM. Thrombin inactivated with D-Phe-Pro-Arg-CH_2Cl has about a 40-fold reduced affinity for proflavin which suggests an overlap in the binding regions of the two compounds. The affinity of D-Phe-Pro-Arg-CH_2–thrombin for hirudin was reduced by 10^6-fold compared with that of α-thrombin, and this corresponds to a decrease in the binding energy of 35 kJ/mol. Once again, this value should be regarded as a maximum contribution of the binding regions occupied by D-Phe-Pro-Arg. The value obtained for D-Phe-Pro-Arg-CH_2–thrombin when compared with DIP-thrombin indicates a major role for the apolar binding region in the binding of hirudin.

In summary, the data presented are consistent with hirudin making an ionic interaction with thrombin in the region of the β-cleavage site. Within the active-site region, the primary specificity pocket is required for an optimal interaction, and a major contribution is made by a binding region which binds the peptide portion of the inactivator D-Phe-Pro-Arg-CH_2Cl. It will be of interest to determine the regions of hirudin involved in the interaction with these regions of thrombin.

ACKNOWLEDGMENTS

We thank Drs. E. Shaw, B. A. Hemmings, H. Angliker, and E. J. Mackie for reading the manuscript.

Registry No. Thrombin, 9002-04-4; L-serine, 56-45-1; L-histidine, 71-00-1; hirudin, 8001-27-2.

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Inhibition of Factor XI_a by Antithrombin III

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Received November 10, 1986; Revised Manuscript Received February 19, 1987

ABSTRACT: The inactivation of human factor XI_a by human antithrombin III was studied under pseudo-first-order reaction conditions (excess antithrombin III) both in the absence and in the presence of heparin. The time course of inhibition was followed by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After electrophoresis, proteins were blotted onto nitrocellulose and stained either for glycoprotein or for antithrombin III using antibodies against antithrombin III. Concomitant with factor XI_a inactivation, two new slower migrating bands, one of which represented the intermediate complex consisting of one antithrombin III complexed with factor XI_a, appeared as a transient band. Complete inactivation resulted in a single band representing the complex of factor XI_a with two antithrombin III molecules. Quantitative analysis of the time course of inactivation was accomplished by measurement of the disappearance of factor XI_a amidolytic activity toward the chromogenic substrate S2366. Pseudo-first-order reaction kinetics were observed throughout. The rate constant of inactivation was found to be 10^3 M^{-1} s^{-1} in the absence of heparin and 26.7×10^3 M^{-1} s^{-1} in the presence of saturating amounts of heparin. From the kinetic data, a binding constant (K_d) of 0.14 μM was inferred for the binding of antithrombin III to heparin. The time course of inactivation and the distribution of the reaction products observed upon gel electrophoresis are best explained assuming a mechanism of inactivation in which the two active sites present in factor XI_a are inhibited in random order (i.e., independent of each other) with the same rate constant of inhibition.

Factor XI is a coagulation protein present in plasma in a precursor form (Bouma et al., 1977). It is the (pro)enzyme that links the contact phase to the intrinsic factor IX activation. Four proteins appear to be involved in contact activation reactions: factor XII, factor XI, prekallikrein, and high molecular kininogen (Cochrane & Griffin, 1982). Active factor XII with high molecular weight kininogen as a cofactor can convert factor XI to an active form (Griffin & Cochrane, 1979), which subsequently is able to activate factor IX by hydrolyzing two internal peptide bonds (Di Scipio et al., 1978).

Factor XI is a dimeric molecule which contains two identical protein chains (80 000 molecular weight) held together by (a) disulfide bond(s). Factor XI is converted to factor XI_a by the cleavage of an internal peptide bond in both precursor chains. Factor XI_a is composed of two heavy chains (M_r 50 000) and two light chains (M_r 33 000) and contains one active site at each light chain (Bouma et al., 1977; Fujikawa et al., 1986). It is the only known enzyme participating in blood coagulation that contains two active sites.

Four plasma protease inhibitors, α_1-antitrypsin, antithrombin III, C_1 inhibitor, and α_2-antiplasmin, have been reported to inactivate human factor XI_a. In plasma, α_1-antitrypsin is thought to be the main factor XI_a inhibitor followed by antithrombin III (Scott et al., 1982a). However, the inactivation by antithrombin III can be accelerated in the presence of heparin (Damus et al., 1973). Scott et al. reported a 4-fold enhancement while Beeler et al. reported a 40-fold acceleration of the inactivation at saturating heparin concentrations (Scott et al., 1982b; Beeler et al., 1986).

The stoichiometry of the complex formed between factor XI_a and antithrombin III has been shown to be 1 mol of factor XI_a to 2 mol of inhibitor (Kurachi & Davie, 1977), indicating that both active sites interact with antithrombin III. Thus, the presence of an intermediate, factor XI_a complexed with one antithrombin III, formed during the inactivation of factor XI_a is likely to be expected but has not been demonstrated yet.

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0006-2960/87/0426-4624$01.50/0