Effect of thrombomodulin on the kinetics of the interaction of thrombin with substrates and inhibitors

Jan HOFSTEENGE, Hayao TAGUCHI and Stuart R. STONE
Friedrich Miescher Institute, P.O. Box 2543, CH 4002 Basel, Switzerland

1. Thrombomodulin decreased by 20–30% the Michaelis constant of two tripeptidyl *p*-nitroanilide substrates of thrombin. Thrombomodulin increased the rate of inactivation of thrombin by two peptidyl chloromethane inhibitors by a similar amount. This effect appeared to be due to a decrease in the dissociation constants of the inhibitors. 2. An improved method for the separation of fibrinopeptides A and B by h.p.l.c. was developed, and this method was used to study the effect of thrombomodulin on the thrombin-catalysed cleavage of fibrinogen. In this reaction, thrombomodulin was a competitive inhibitor with respect to the A α -chain of fibrinogen. The release of fibrinopeptide B was also inhibited by thrombomodulin. Analysis of the inhibition caused by thrombomodulin with respect to fibrinopeptides A and B yielded the same dissociation constant for the thrombin–thrombomodulin complex. 3. In the presence of thrombomodulin, the rate of inactivation of thrombin by antithrombin III was stimulated 4-fold. This stimulation showed saturation kinetics with respect to thrombomodulin. 4. Thrombomodulin was found to compete with hirudin for a binding site on thrombin. As a result of this competition, hirudin became a slow-binding inhibitor of thrombin at high thrombomodulin concentrations. 5. Estimates of the dissociation constant for thrombomodulin were obtained in several of the above experiments, and the weighted mean value was 0.7 nM.

INTRODUCTION

Thrombin is formed from prothrombin in the penultimate step of the blood-coagulation pathway through the action of activated Factor X in the presence of activated Factor V, Ca²⁺ and phospholipid. Thrombin is able to cleave fibrinogen to form fibrin monomers, which subsequently polymerize to form the basis of a blood clot. Thrombin is also able to activate Factors V and VIII, which act earlier in the coagulation pathway, and to activate platelets (Jackson & Nemerson, 1980). In this manner, thrombin is able to stimulate its own production.

When thrombin is bound to a protein called thrombomodulin, which is found on the surface of vascular endothelial cells, some of its procoagulant activities are lost. Its ability to cleave fibrinogen and to activate factor V is inhibited (C. T. Esmon *et al.*, 1982; Maruyama *et al.*, 1985), and it is no longer able to activate platelets (Esmon *et al.*, 1983; Maruyama *et al.*, 1985). The degree of inhibition of these activities by thrombomodulin appears to depend on the source of the thrombomodulin (Maruyama *et al.*, 1985). In addition, the ability of thrombin to activate Protein C is greatly enhanced in the presence of thrombomodulin (N. L. Esmon *et al.*, 1982; Salem *et al.*, 1984). Activated Protein C is a serine proteinase which is capable of inactivating Factors V and VIII through proteolytic cleavage of these coagulation factors (Esmon, 1983). Thus, when bound to thrombomodulin, thrombin not only loses some of its procoagulant activities but also activates an enzyme that degrades factors essential for the formation of thrombin.

In order to investigate the mechanism by which thrombomodulin changes the specificity of thrombin, we

have examined the effect of thrombomodulin on the interaction of thrombin with various substrates and inhibitors. It is thought that different regions of thrombin are involved in some of these interactions. Synthetic tripeptidyl substrates and inhibitors interact with regions in the immediate vicinity of the catalytic centre, but macromolecular substrates and inhibitors are thought to have additional binding sites that are distant from the catalytic centre (Fenton, 1981). Thus the effect of thrombomodulin on the interaction of thrombin with various substrates and inhibitors could indicate the regions of thrombin to which thrombomodulin is bound.

EXPERIMENTAL

Materials

D-Phe-Pro-ArgCH₂Cl and D-Phe-Phe-ArgCH₂Cl were gifts from Dr. E. Shaw, Friedrich Miescher Institute, Basel, and hirudin (specific activity 12000 AT units/mg) was a gift from Plantorgan Werk, Bad Zwischenahn, West Germany. Snake venom from *Oxyuranus scutellatus* was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and frozen rabbit lungs were from Biogenzia Lemania, Lausanne, Switzerland. The peptidyl *p*-nitroanilide substrates S-2238 (D-Phe-Pip-Arg-Nan) and S-2266 (D-Val-Leu-Arg-Nan) and human fibrinogen were from Kabi Vitrum, Molndal, Sweden. Chromozym TH (Tos-Gly-Pro-Arg-Nan) and alkaline phosphatase were from Boehringer Mannheim, Mannheim, West Germany. All other chemicals were of the highest quality available commercially.

Purification of proteins

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 by fast protein liquid chromatography with a Mono-S column (Pharmacia, Uppsala, Sweden) and the same gradient as described by Lundblad (1971) for SP (sulphopropyl)-Sephadex. Thrombin prepared in this manner was pure and free from proteolytically degraded forms as judged by gel electrophoresis and by its *N*-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. Thrombomodulin was purified from frozen rabbit lungs by the method described by Salem *et al.* (1984) for purification of thrombomodulin from human placenta. Human antithrombin III was isolated as described by Miller-Andersson *et al.* (1974). Human Protein C was purified essentially by the method of Suzuki *et al.* (1983). Human fibrinogen from Kabi Vitrum was further purified by chromatography on lysine-Sepharose (Matsuda *et al.*, 1972). If this additional purification was not performed, the degradation products des-Ala-fibrinopeptide A and des-Arg-fibrinopeptide B were observed (nomenclature of Blombäck *et al.*, 1972). The α -chain of fibrinogen was subsequently dephosphorylated with alkaline phosphatase (Blombäck *et al.*, 1963). Fibrinogen prepared in this manner was 94% clottable. The concentrations of antithrombin III, Protein C and fibrinogen were determined spectrophotometrically at 280 nm (Nordenman *et al.*, 1977; Kisiel & Davie, 1981; Dellenback & Chien, 1970). All proteins were free from other contaminating proteins as judged by SDS/polyacrylamide-gel electrophoresis.

Amidolytic assay of thrombin

The release of *p*-nitroaniline that resulted from the hydrolysis of the peptidyl *p*-nitroanilide substrates was followed by measuring the increase in A_{405} 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) (M_r 6000), 0.005% Lubrol PX and 0.1 M-NaCl. 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 $9920 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for *p*-nitroaniline at 405 nm, and the concentration of the substrate was determined spectrophotometrically at 342 nm by using an absorption coefficient of $8270 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Lottenberg & Jackson, 1983).

Fibrinogen cleavage by thrombin

The protocol for fibrinogen-cleavage experiments was essentially as described by Higgins & Shafer (1981). Reactions were performed at 37 °C in 50 mM-Tris/HCl buffer, pH 7.4, to which 117 mM-NaCl had been added to give *I* 0.15. The buffer also contained 0.1% poly(ethylene glycol) (M_r 6000) and 0.003% Lubrol PX. Thrombin was added to start the reaction. The concentration of thrombin used was 0.22 nM for progress-curve experiments and 0.08 nM for initial-velocity experiments.

The cleavage reaction was stopped by the addition of D-Phe-Pro-ArgCH₂Cl (final concn. 170 μM). A zero-time point was obtained by adding the tripeptidyl chloromethane before the addition of thrombin. The end point of each reaction was determined by incubating the fibrinogen with 7.0 nM-thrombin for 60 min. For initial-velocity experiments, the time of incubation was adjusted so that less than 10% of the fibrinogen was consumed. Fibrinopeptide A (FPA) and fibrinopeptide B (FPB) were separated by h.p.l.c. as described below, and their concentrations were calculated from their peak heights. The peak heights of FPA and FPB obtained on total conversion of different concentrations of fibrinogen were used to construct standard curves. These curves were linear up to at least 1.0 nmol of fibrinopeptide and had correlation coefficients indistinguishable from 1 (> 0.99). The amounts of fibrinopeptide determined were always in this linear range.

Separation of fibrinopeptide A and fibrinopeptide B by h.p.l.c.

This was done by using a Whatman ODS 3 column at 40 °C. The gradient system utilized 20 mM-sodium phosphate buffer, pH 5.8; buffers A and B contained 2 and 25% (v/v) acetonitrile respectively. The following elution protocol was used: 0 min, 75% buffer A/25% buffer B; 10 min, 56% buffer A/44% buffer B; 20 min, 30% buffer A/70% buffer B. The flow rate was 1.0 ml/min throughout. Peptides were detected by measuring the A_{214} . For analytical purposes, the use of a system with high-pH phosphate buffer had two advantages when compared with other published methods for separating FPA and FPB (Kehl *et al.*, 1981; Higgins & Shafer, 1981): first, very sharp peaks were obtained, and, secondly, a very low noise level was observed, owing to the high u.v.-transparency of this buffer (Fig. 1). With this system, it was also possible to separate several other fibrinopeptide derivatives as shown in Fig. 1 (des-Ala-FPA, [3-phosphoserine]FPA and des-Arg-FPB).

Activation of Protein C

The activation of Protein C was performed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.05 M-NaCl, 1.0 mM-CaCl₂, 0.1% Lubrol PX and 0.2 mg of bovine serum albumin/ml. The reaction mixture contained 1.0 μM -Protein C and various concentrations of thrombin and thrombomodulin in a final volume of 90 μl . The activation reaction was started by the addition of thrombin and was stopped by adding 5 AT units of hirudin. This amount of hirudin was sufficient to inactivate thrombin completely and did not affect the amidolytic activity of activated Protein C. The time for which the activation reaction was allowed to proceed was adjusted so that less than 10% of the Protein C was activated. The amount of activated protein C produced was measured spectrophotometrically with the substrate S-2266 as previously described (Stone & Hofsteenge, 1985).

THEORY AND DATA ANALYSIS

Determination of the molar concentration of thrombomodulin

If the activation of Protein C by the thrombin-thrombomodulin complex was performed under conditions where the binding of thrombomodulin to thrombin

Fig. 1. Separation of fibrinopeptides by h.p.l.c.

Fibrinopeptides were separated by using a Whatman ODS 3 column at 40 °C. The buffer used was 20 mM-sodium phosphate, pH 5.8. Elution was accomplished with a gradient of acetonitrile in this buffer (see the Experimental section). Abbreviations: A, fibrinopeptide A; B, fibrinopeptide B; AP, fibrinopeptide A phosphorylated at serine-3; AY, des-alanine-fibrinopeptide A; Des-ArgB, des-arginine-fibrinopeptide B.

caused a significant decrease in the concentration of free thrombomodulin, the variation of the velocity with the concentration of thrombomodulin could be described by eqn. (1) (Henderson, 1973):

$$v = \frac{k_0 \cdot \{[E]_t + K_m + x \cdot [TM]_t\}}{2} - \sqrt{([E]_t + K_m + x \cdot [TM]_t)^2 - 4[E]_t \cdot x \cdot [TM]_t} \quad (1)$$

where $[E]_t$, $[TM]_t$ and k_0 are the total enzyme concentration, total concentration of thrombomodulin and catalytic constant respectively. The factor x converts the concentration of thrombomodulin from arbitrary units such as $\mu\text{g/ml}$ into molar units. The value of x

obtained by fitting data to eqn. (1) by using non-linear regression was then used to calculate the molar concentration of thrombomodulin in stock solutions.

Progress-curve kinetics at sub-saturating concentrations of fibrinogen

At concentrations of fibrinogen much lower than its Michaelis constant, the thrombin-catalysed release of FPA from fibrinogen can be described by eqn. (2) (Higgins *et al.*, 1983):

$$[\text{FPA}] = [\text{A}\alpha]_0 [1 - \exp(k_A' \cdot t)] \quad (2)$$

where $[\text{A}\alpha]_0$ is the initial concentration of the $\text{A}\alpha$ -chain (twice the fibrinogen concentration) and k_A' is the apparent first-order rate constant for the release of FPA. Progress-curve data for the release of FPA were fitted to eqn. (2) by non-linear regression in which both $[\text{A}\alpha]_0$ and k_A' were treated as unknown parameters. The values determined for $[\text{A}\alpha]_0$ by regression analysis were always within 10% of the values of $[\text{A}\alpha]_0$ determined experimentally by incubating the fibrinogen for an extended period with a high concentration of thrombin. At sub-saturating concentrations of fibrinogen, the kinetics of the release of FPB are consistent with a sequential model in which FPA must be released before FPB (Higgins *et al.*, 1983). Eqn. (3) describes this model:

$$[\text{FPB}] = [\text{B}\beta]_0 \left(1 + \frac{k_B'}{k_A' - k_B'} \cdot \exp(-k_A' \cdot t) - \frac{k_A'}{k_A' - k_B'} \cdot \exp(-k_B' \cdot t) \right) \quad (3)$$

where $[\text{B}\beta]_0$ is the initial concentration of the $\text{B}\beta$ -chain and k_B' is the apparent first-order rate constant for the release of FPB. Data were fitted to eqn. (3) by non-linear regression in which $[\text{B}\beta]_0$, k_A' and k_B' were the unknown parameters. At any particular thrombomodulin concentration, there was always good agreement between the value of k_A' estimated by analysis of FPA release according to eqn. (2) and that estimated from analysis of FPB release according to eqn. (3).

Tight-binding inhibition of thrombin by hirudin

In steady-state-velocity experiments, the dissociation constant for hirudin was determined by holding the concentrations of thrombin constant and varying the concentration of hirudin over a range including several concentrations that were lower than the concentration of thrombin and several concentrations that were higher (seven to ten points in total). Under these conditions, the variation of the steady-state velocity (v_s) with the total hirudin concentration ($[I]_t$) could be described by eqn. (4) (Williams *et al.*, 1979):

$$v_s = \frac{v_0}{2[E]_t} \cdot \left\{ \sqrt{(K_i' + x \cdot [I]_t - [E]_t)^2 + 4K_i' \cdot [E]_t} - (K_i' + x \cdot [I]_t - [E]_t) \right\} \quad (4)$$

where v_0 is the velocity observed in the absence of the inhibitor, $[E]_t$ is the total enzyme concentration, K_i' is the apparent inhibition constant and x is a factor that, when multiplied together with $[I]_t$, will yield the molar concentration of the inhibitor. Hirudin was found to be a competitive inhibitor with respect to the substrate (S. R. Stone & J. Hofsteenge, unpublished work), and

therefore K_i' can be related to the dissociation constant of the inhibitor (K_i) by eqn. (5):

$$K_i' = K_i(1 + [S]/K_m) \quad (5)$$

The steady-state velocities obtained at each hirudin concentration were fitted by weighted non-linear regression to eqn. (4). 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 of thrombin by hirudin

If the rate of interaction of an inhibitor with an enzyme is slow so that the inhibited steady-state velocity is only slowly achieved, the progress curve of product formation will be described by eqn. (6) (Cha, 1976; Williams *et al.*, 1979):

$$[P] = v_s \cdot t + \frac{(v_0 - v_s)(1 - d)}{d \cdot k'} \cdot \log\left(\frac{1 - d \cdot \exp(-k' \cdot t)}{1 - d}\right) \quad (6)$$

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). When slow-binding inhibition of thrombin by hirudin was observed, the data were fitted to eqn. (6) by non-linear regression. This analysis yielded estimates for K_i' and k_i' .

Inactivation of thrombin by peptidyl chloromethanes and antithrombin III

Progress curves for the inactivation of thrombin by peptidyl chloromethanes and antithrombin III in the presence of substrate were analysed as described previously (Stone & Hofsteenge, 1985). For the chloromethanes, the value of the apparent first-order rate constant for the inactivation (k') displayed a hyperbolic dependence on the concentration of inhibitor. Thus it was possible to determine values for the apparent dissociation constant for the initial enzyme-inhibitor complex (K_i') and for the rate constant for the formation of the inactive enzyme (k_i). With antithrombin III, the value of k' varied in a linear fashion with the concentration of the inhibitor, and it was possible to estimate only a value for the apparent second-order rate constant (k_i/K_i'). With both the chloromethanes and antithrombin III, the value of $1/k'$ showed a linear dependence on the substrate

concentration, as is expected for a competitive inhibitor (Morrison & Stone, 1985). Therefore the relationship given in eqn. (5) was used to calculate the values of K_i and k_i/K_i .

RESULTS

Effect of thrombomodulin on the interaction of thrombin with peptidyl substrates and inhibitors

Table 1 shows the effect of thrombomodulin on the kinetics of the thrombin-catalysed hydrolysis of two tripeptidyl *p*-nitroanilide substrates. Both substrates show a high specificity for thrombin (Lottenberg *et al.*, 1981) and thrombomodulin had caused a 20–30% decrease in the Michaelis constant without significantly affecting the catalytic constant. These findings were similar to those obtained with bovine thrombin (C. T. Esmon *et al.*, 1982). The irreversible inhibition of thrombin by two tripeptidyl chloromethanes was examined in the presence of the substrate S-2238. Two parameters are associated with the inactivation by peptidyl chloromethanes: the dissociation constant for the initial enzyme-inhibitor complex (K_i) and the rate at which this complex forms an inactive enzyme (k_i). Table 2 shows the effect of thrombomodulin on the values of these two parameters and on the value of the apparent second-order rate constant for the interaction between the inhibitor and the enzyme (k_i/K_i). Thrombomodulin had also caused a 20–30% increase in the value of k_i/K_i for both inhibitors. The values of k_i and K_i could not be accurately determined. Accurate determination of these parameters relies on data obtained at concentrations of inhibitor that are high relative to the value of the apparent inhibition constant (K_i'). At such concentrations, the rate of inactivation was too rapid to be measured with a conventional recording spectrophotometer. Consequently, although it appeared that the increase in k_i/K_i was due to a decrease in the value of K_i , a definitive judgement could not be made because of the observed errors in the value of K_i .

Effect of thrombomodulin on the cleavage of fibrinogen by thrombin

It has previously been reported that thrombomodulin is able to inhibit the ability of thrombin to clot fibrinogen (C. T. Esmon *et al.*, 1982; Maruyama *et al.*, 1985). The exact mechanism by which thrombomodulin inhibits this

Table 1. Effect of thrombomodulin on the kinetic parameters of two amidolytic reactions of thrombin

Assays were performed as described in the Experimental section. The concentration of D-Phe-Pip-Arg-Nan (S-2238) was varied from 1 to 19 μM (six concentrations in duplicate) and that of Tos-Gly-Pro-Arg-Nan (Chromozym TH) was varied from 3 to 26 μM (five concentrations in duplicate). Experiments were performed in the absence and in the presence of 3.9 nM-thrombomodulin (TM). Initial velocities were fitted to the Michaelis-Menten equation by using weighted linear regression (Cornish-Bowden & Endrenyi, 1981). These analyses yielded estimates of the catalytic constant (k_{cat}), Michaelis constant (K_m) and k_{cat}/K_m , which are given together with their standard errors.

Substrate	k_{cat} (s^{-1})		K_m (μM)		$10^{-7} \times k_{\text{cat}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	
	– TM	+ TM	– TM	+ TM	– TM	+ TM
D-Phe-Pip-Arg-Nan (S-2238)	196 $\pm$ 3	194 $\pm$ 7	3.6 $\pm$ 0.1	2.6 $\pm$ 0.2	5.4 $\pm$ 0.2	7.6 $\pm$ 0.3
Tos-Gly-Pro-Arg-Nan (Chromozym-TH)	229 $\pm$ 7	250 $\pm$ 7	9.0 $\pm$ 0.5	7.0 $\pm$ 0.4	2.5 $\pm$ 0.1	3.7 $\pm$ 0.1

Table 2. Effect of thrombomodulin on the inactivation of thrombin by two peptidyl chloromethanes

Inactivation assays were performed in the presence of the substrate D-Phe-Pip-Arg-Nan (S-2238) as described in the Experimental section. Each experiment was performed in the absence and in the presence of 3.9 nM-thrombomodulin (TM). D-Phe-Phe-ArgCH₂Cl concentration was varied from 59 to 250 μ M (one progress curve at each of five concentrations) in the presence of 245 μ M-S-2238. D-Phe-Pro-ArgCH₂Cl concentration was varied from 25 to 80 nM in the presence of 392 μ M-S-2238 (six different concentrations, with a total of 9 and 11 points with and without thrombomodulin respectively). The observed inactivation progress curves were analysed as described in the Theory and data analysis section. These analyses yielded the values of k_i , K_i and k_i/K_i that are given in the Table together with their standard errors.

Inhibitor	$10^{-3} \times k_i$ (s ⁻¹)		K_i (nM)		k_i/K_i (M ⁻¹ ·s ⁻¹)	
	- TM	+ TM	- TM	+ TM	- TM	+ TM
D-Phe-Pro-ArgCH ₂ Cl	52 ± 12	57 ± 20	2.5 ± 0.7	2.0 ± 0.8	(2.0 ± 0.1) × 10 ⁷	(2.9 ± 0.1) × 10 ⁷
D-Phe-Phe-ArgCH ₂ Cl	5.8 ± 1.9	7.0 ± 2.8	11700 ± 4100	9300 ± 4100	(4.9 ± 0.2) × 10 ²	(7.6 ± 0.3) × 10 ²

Fig. 2. Effect of thrombomodulin on the initial rate of release of fibrinopeptide A

The lines represent the best fit to the equation for competitive inhibition with values for k_{cat} , K_m and K_i of 78 s⁻¹, 11.2 μ M and 1.6 nM respectively. Thrombomodulin concentrations: Δ , 0 nM; ∇ , 1.7 nM; $\diamond$, 3.3 nM; $\square$, 6.6 nM.

activity of thrombin has not, however, been established. Thrombomodulin could be a competitive, uncompetitive or mixed-type inhibitor of the fibrinogen-cleavage reaction of thrombin. Thus initial-velocity experiments were performed to determine the nature of the inhibition caused by thrombomodulin. Fig. 2 shows that thrombomodulin was a competitive inhibitor with respect to the A α -chain of fibrinogen. Analysis of these data by weighted linear regression according to the equation for competitive inhibition (Segel, 1975) yielded a value for the dissociation constant of thrombomodulin of 1.6 ± 0.2 nM. Analysis of these data also yielded values of 11.2 ± 2.0 μ M and 78 ± 10 s⁻¹ for the Michaelis constant of the A α -chain and the catalytic constant. These values agreed with the values of 7.2 μ M and 83 s⁻¹ determined by Higgins *et al.* (1983).

In order to determine whether thrombomodulin inhibited the release of both FPA and FPB from fibrinogen, progress-curve experiments were performed at low concentrations of fibrinogen (0.3 μ M). Under these

conditions, the progress curve of the release of FPA will be described by a single exponential (eqn. 2), whereas the progress curve of FPB release is described by a double exponential (eqn. 3). In the absence of thrombomodulin, the apparent first-order rate constants for the release of FPA (k_A') and FPB (k_B') varied in a linear fashion with the concentration of thrombin over a 10-fold range. Regression analysis of this dependence of the first-order constant on the concentration of thrombin yielded values of $(1.1 \pm 0.1) \times 10^7$ M⁻¹·s⁻¹ and $(6.0 \pm 0.2) \times 10^6$ M⁻¹·s⁻¹ for the second-order rate constants for the interaction with thrombin of FPA and FPB respectively. These values were in good agreement with the values of 1.2×10^7 M⁻¹·s⁻¹ and 4.2×10^6 M⁻¹·s⁻¹ obtained by Higgins *et al.* (1983). Fig. 3 shows the effect of thrombomodulin on the release of FPA and FPB; the rate of release of both FPA (Fig. 3a) and FPB (Fig. 3b) was inhibited by increasing concentrations of thrombomodulin. The effect of thrombomodulin on the apparent first-order constants for the release of FPA and FPB was analysed by the Dixon equation for enzyme inhibition (Segel, 1975). Values of 0.63 ± 0.14 and 0.66 ± 0.14 nM for the inhibition constant of thrombomodulin were obtained from analyses of the effect of thrombomodulin on k_A' and k_B' respectively. Thus it appeared that the binding of thrombomodulin to a particular site was able to inhibit the release of both FPA and FPB from fibrinogen.

Effect of thrombomodulin on the inhibition of thrombin by antithrombin III

At the concentrations of antithrombin III used in the present study (> 0.4 μ M), the inhibitor forms a functionally irreversible complex with thrombin (Jesty, 1979), and it was possible to analyse the data according to the equation describing enzyme inactivation (Stone & Hofsteenge, 1985). Fig. 4 shows the variation of the apparent first-order inactivation rate constant (k') with the concentration of the inhibitor. The linear plot indicates that the concentrations of inhibitor used were far below the value of the apparent inhibition constant (K_i'). This result was in accord with the estimate of 1.4 mM for the dissociation constant of the initial thrombin-antithrombin III complex obtained by Olson & Shore (1982). In the presence of a concentration of 20 nM-thrombomodulin, the rate of inactivation of thrombin (k_i/K_i) increased by more than 4-fold, from $(1.48 \pm 0.02) \times 10^4$ to $(6.48 \pm 0.07) \times 10^4$ M⁻¹·s⁻¹. The

Fig. 3. Influence of thrombomodulin on the kinetics of the release of fibrinopeptides A and B

Fibrinogen ($0.3 \mu\text{M}$) was cleaved by thrombin in the presence of different concentrations of thrombomodulin (see Fig. 2 for the meaning of the symbols). (a) Rate of release of FPA. The lines are the best fit of the data to eqn. (2). (b) Rate of release of FPB. The lines are the best fit of the data to eqn. (3). Points at 1, 2 and 4 min are omitted for the sake of clarity.

value obtained for k_i/K_i in the absence of thrombomodulin agreed with the value reported by Olson & Shore (1982) for this parameter. The extent to which thrombomodulin stimulated the inactivation rate was dependent on the concentration of thrombomodulin, as shown in Fig. 5. These data were consistent with the mechanism shown in Scheme 1, in which free thrombin and thrombin complexed to thrombomodulin were inactivated at different rates. The dependence of k' on the concentration of thrombomodulin for this mechanism will be described by eqn. (7):

$$k' = \frac{(k_{i1}K_{tm} + k_{i2}[TM])[AT-III]}{[TM] + K_{tm}} \quad (7)$$

Values of k' obtained at different thrombomodulin concentrations were weighted according to the squared inverse of their standard errors and fitted by non-linear regression to eqn. (7). This analysis yielded values of $(1.45 \pm 0.04) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $(5.53 \pm 0.28) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$

Fig. 4. Effect of thrombomodulin on the rate of inactivation of thrombin by antithrombin III

Assays were performed as described in the Experimental section in the presence of $126 \mu\text{M}$ -Tos-Gly-Pro-Arg-Nan and with 0 (Δ) or 20 nM (∇) -thrombomodulin. The apparent first-order rate constant for the inactivation (k') was calculated as described in the Theory and data analysis section, and the Figure shows the variation of this parameter with the concentration of antithrombin III. The lines represent the best fit of the data to eqn. (5).

Fig. 5. Dependence of the rate of inactivation of thrombin by antithrombin III on the concentration of thrombomodulin

Assays were performed and data analysed as described in the legend to Fig. 2. The values of k' obtained were fitted to eqn. (7) by weighted non-linear regression, and the curve represents the best fit to this equation.

Scheme 1.

Abbreviations: TM, thrombomodulin; AT-III, antithrombin III; IIa, thrombin.

and 0.56 ± 0.10 nM for k_{11} , k_{12} and K_{tm} respectively. Similar results have been obtained with three different preparations of thrombomodulin.

The thrombomodulin used in this study was purified from rabbit lung and lung is known to contain relatively large amounts of heparin (Jacques, 1980). This highly sulphated glycosaminoglycan greatly stimulates the rate of inactivation of thrombin by antithrombin III (Rosenberg & Damus, 1973). Thus the following controls have been performed to exclude the possibility that the effect of thrombomodulin is due to contaminating heparin.

(1) The fraction of heparin molecules that enhances the inhibitory effect of antithrombin III binds strongly to antithrombin III ($K_d = 10^{-7} - 10^{-8}$ M) (Björk & Lindahl, 1982). Consequently, the thrombomodulin preparation was chromatographed on a column of immobilized antithrombin III (Höök *et al.*, 1976). Thrombomodulin did not bind to this column and enhanced the rate of inhibition of thrombin by antithrombin III to the same extent as the untreated material.

(2) Polybrene is known to neutralize the stimulatory effect of heparin (Danielsson & Björk, 1983). Polybrene at a concentration of 0.1 mg/ml did not alter the values of k' observed in the presence of thrombomodulin.

(3) C. T. Esmon *et al.* (1982) have shown that treatment of thrombomodulin with pepsin at pH 2.5 completely destroys its ability to stimulate the activation of Protein C by thrombin. Thrombomodulin that had been inactivated in this way (95% as measured by the Protein C activation assay) had also lost its ability to enhance the inactivation of thrombin by antithrombin III. The activity of heparin, a glycosaminoglycan, should not be affected by pepsin. These control experiments excluded the possibility that the enhanced rate of inactivation was due to a small amount of heparin in the thrombomodulin preparation.

Effect of thrombomodulin on the inhibition of thrombin by hirudin

In the absence of thrombomodulin, the rate of interaction between hirudin and thrombin could not be measured with 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 were within the range that can be measured with a conventional recording spectrophotometer, the change in absorbance in the pre-steady-state phase was too small to be used for accurate data analysis. In the presence of thrombomodulin concentrations greater than 10 nM, however, slow-binding inhibition by hirudin was observed. Fig. 6 shows the data obtained with a concentration of 24.9 nM-thrombomodulin. The effect of thrombomodulin on the value of the dissociation constant of hirudin (K_i) was also examined. Experiments conducted with 74 pM-thrombin in the presence of 186 μ M-S-2238 yielded tight-binding-inhibition data at 0 and 8.3 nM-thrombomodulin and slow tight-binding-inhibition data at 16.6 nM- and 24.9 nM-thrombomodulin. Analysis of these data according to eqns. (4) and (6) respectively provided estimates for K_i at each thrombomodulin concentration. Fig. 7 shows that the value of K_i increased in a linear manner with increasing concentrations of thrombomodulin, as

Fig. 6. Slow-binding inhibition of thrombin by hirudin in the presence of thrombomodulin

Assays were performed as described in the Experimental section in the presence of 74 pM-thrombin, 186 μ M-D-Phe-Pip-Arg-Nan and 24.9 nM-thrombomodulin. The Figure shows data obtained at hirudin concentrations of 0 ($\blacktriangle$), 63 ($\triangle$), 129 ($\blacktriangledown$), 192 ($\triangledown$), 257 ($\blacklozenge$) and 321 ($\lozenge$) pM. Additional data were obtained at concentrations of 32 pM- and 96 pM-hirudin. Data at times less than 2 min are not shown. The data were analysed according to eqn. (6), and values of 13.2 nM $\cdot$ s $^{-1}$, 3.96×10^7 M $^{-1}$ $\cdot$ s $^{-1}$ and 21.3 pM were determined for the initial velocity, the apparent second-order rate constant for the interaction between hirudin and thrombin and the dissociation constant of the hirudin-thrombin complex.

Fig. 7. Effect of thrombomodulin on the value of the dissociation constant for hirudin

Values for the dissociation constant for hirudin (K_i) were determined from tight-binding-inhibition data at 0 and 8.3 nM-thrombomodulin and from slow tight-binding-inhibition data at 16.6 nM- and 24.9 nM-thrombomodulin. The dependence of these values on the concentration of thrombomodulin could be described by eqn. (5), and the line in the Figure was drawn according to this equation, with the determined values of 20 fM and 1.8 nM for the dissociation constants of hirudin and thrombomodulin respectively.

would be expected if hirudin and thrombomodulin were competing for the same site. The data of Fig. 7 were analysed by weighted linear regression, assuming that both ligands were competing for the same site (eqn. 5), and values of 20 ± 5 fm and 1.8 ± 0.5 nm were obtained for the dissociation constants of hirudin and thrombomodulin respectively. The value obtained for the dissociation constant for hirudin agreed well with values previously determined (S. R. Stone & J. Hofsteenge, unpublished work). If thrombomodulin and hirudin compete for the same site, the rate of interaction between thrombin and hirudin should decrease with increasing concentrations of thrombomodulin (Morrison & Stone, 1985). Thus the fact that hirudin became a slow-binding inhibitor as the concentration of thrombomodulin was increased supports the conclusion that thrombomodulin and hirudin compete for the same site. Indeed, at concentrations of 74 nM-thrombin and 342 nM-hirudin, the reciprocal of the rate constant for the interaction between these two molecules showed a linear dependence on the concentration of thrombomodulin (results not shown). Such a dependence would be expected if hirudin and thrombomodulin were competing for the same site (Morrison & Stone, 1985).

DISCUSSION

From the data presented in this paper, it is possible to obtain some indication of how the binding site for thrombomodulin overlaps with other binding sites on thrombin. The binding of thrombomodulin to thrombin did not inhibit the binding of tripeptidyl substrates and inhibitors (Tables 1 and 2). Thus thrombomodulin does not appear to bind to regions important in binding the amino acid residues immediately *N*-terminal to the scissile bond. In fact, the binding of thrombomodulin to thrombin caused a decrease in the Michaelis constant for two tripeptidyl *p*-nitroanilide substrates (Table 1) and possibly also in the dissociation constant of two tripeptidyl chloromethane inhibitors (Table 2). Thrombomodulin did not cause a significant change in the catalytic constant of thrombin with the two substrates tested (Table 1). Therefore it seems that the binding of thrombomodulin does not cause large changes in the environment of the residues involved in the catalytic steps, i.e. histidine-57, aspartate-102 and serine-195 (chymotrypsin numbering; Birktoft & Blow, 1972). This proposal is supported by the observation that the rate constant for the inactivation of thrombin by the peptidyl chloromethanes tested did not change significantly in the presence of thrombomodulin (Table 2). The peptidyl chloromethanes inactivate serine proteinases by alkylating histidine-57 (Glover & Shaw, 1971), and thus the rate constant for inactivation should be sensitive to any large changes in the environment of this residue.

The binding of thrombomodulin to thrombin caused an increase in the rate of inactivation of the enzyme by antithrombin III (Figs. 4 and 5). Because of the low affinity of thrombin for antithrombin III, it was only possible to determine the value of the second-order rate constant for the inhibition (k_i/K_i). Consequently, these data did not indicate the manner by which the increased rate of inactivation of thrombin was achieved. It appears, however, that thrombomodulin and antithrombin III did not compete for the same binding site. In contrast, thrombomodulin and hirudin competed for a common

binding site on the thrombin molecule (Figs. 6 and 7). The results presented in Tables 1 and 2 indicate that this overlap did not occur in the region of the catalytic centre. The data of Figs. 2 and 3 indicated that thrombomodulin and fibrinogen also competed for a common binding site. It has been proposed that fibrinogen has a number of secondary binding sites adjacent to the catalytic centre (Fenton, 1981), and it seems that one or more of these sites is also involved in the binding of thrombomodulin. In this respect, it is noteworthy that the enzymic properties of the thrombin-thrombomodulin complex resemble those of proteolytically degraded forms of thrombin. The proteolytically degraded β - and γ -forms of thrombin are produced through autodegradation or by trypsinolysis. The amidolytic activity of the degraded forms is similar to that of native α -thrombin, and they exhibit similar inactivation kinetics with antithrombin III. The degraded forms of thrombin are, however, unable to cleave fibrinogen, and have a much decreased affinity for hirudin (Fenton, 1981). These similarities in the enzymic properties of the degraded forms of thrombin and the thrombin-thrombomodulin complex suggest that a region which is proteolytically altered in β - and/or γ -thrombin might be involved in the binding of thrombomodulin to thrombin. This suggestion is also supported by the finding that a peptide comprising residues 106–121 of the thrombin B-chain inhibited the activation of Protein C by the thrombin-thrombomodulin complex (J. Hofsteenge & S. R. Stone, unpublished work). This region is close to the cleavage site for the conversion of α -thrombin into β -thrombin (Boissel *et al.*, 1984). Protein-chemical and immunochemical studies can be used to identify more precisely the regions of thrombin involved in interactions with thrombomodulin and hirudin.

The results of the current study confirmed the importance of thrombomodulin in the control of coagulation. Analysis of data obtained in this study indicate that thrombin is bound tightly to thrombomodulin; the weighted mean of the five separate determinations of the dissociation constant was 0.7 ± 0.1 nM. Thrombomodulin enhanced the rate of inactivation of thrombin by antithrombin III about 4-fold. In addition, if thrombin were bound in the complex, fibrinogen would not be able to compete with antithrombin III. Thus the rate of inactivation of complexed thrombin would be further enhanced over that of uncomplexed thrombin in plasma. By using the known concentrations of fibrinogen and antithrombin III (Thompson & Harker, 1983), it can be calculated that the half-times for the inactivation of uncomplexed thrombin and complexed thrombin would be 2.3 and 0.25 s respectively. The value 0.25 s for the thrombin-thrombomodulin complex is close to the value of 0.1 s suggested by Travis & Salvesen (1983) for effective control of an enzyme.

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