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Enzymatic Properties of Proteolytic Derivatives of Human α-Thrombin

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ABSTRACT: The use of derivatives of α-thrombin obtained by limited proteolysis, that have only a single peptide bond cleaved, allowed the unequivocal correlation between the change in covalent structure and alteration of the enzymatic properties. β_T-Thrombin contains a single cleavage in the surface loop corresponding to residues 65-83 of α-chymotrypsin [Birktoft, J. J., & Blow, D. M. (1972) *J. Mol. Biol. 68*, 187-240]. Compared with α-thrombin, this modification had a minor effect on the following: (1) The Michaelis constant (K_m) for two tripeptidyl *p*-nitroanilide substrates increased 2-3-fold, whereas the catalytic constant (k_{cat}) remained unaltered. (2) A 2-3-fold increase in the binding constant (K_I) of a tripeptidyl chloromethane inhibitor was observed, but the inactivation rate constant (k_i) was the same, which indicated that the nucleophilicity of the active-site histidyl residue had not changed. (3) The second-order rate constant for the inhibition by antithrombin III decreased 2-fold. Heparin accelerated the inactivation, and the degree of acceleration was similar to that obtained with α-thrombin. Pronounced effects of the cleavage of this loop were found. (1) The cleavage of fibrinogen was approximately 80-fold slower than that with α-thrombin. This was mainly due to a 40-fold decrease in k_{cat}. In contrast, only a 1.9-fold increase in the Michaelis constant was observed. (2) The affinity for thrombomodulin had decreased 39-fold compared to α-thrombin. ϵ-Thrombin contains a single cleaved peptide bond in the loop corresponding to residues 146-150 in α-chymotrypsin. The kinetic parameters for all of the above-mentioned ligands, except fibrinogen, were not affected by this modification. With fibrinogen, a 40% reduction in k_{cat} was observed, but the K_m was found to be unaltered. The same set of parameters was also obtained with γ_T-thrombin which contained cleavages in both loops. These parameters have been compared with those for β_T- and ϵ-thrombin, and the results indicated that γ_T-thrombin was more similar to β_T-thrombin than to ϵ-thrombin.

Thrombin is a serine protease that interacts with a number of substrates, inhibitors, and receptors that are essential to the process of hemostasis (Fenton, 1981). The specificity of these interactions is determined not only by the active site and its specificity pocket but also by secondary binding sites distinct from the active site (Magnusson, 1972).

One approach to study the structure–function relationship of thrombin has been the use of proteolytically cleaved derivatives (Berliner, 1984). With human thrombin, these studies have used γ-thrombin, which is derived from α-thrombin by the cleavage of at least two peptide bonds. Comparison of the primary structures of human α-thrombin and α-chymotrypsin indicates that these cleavages probably occur in surface loops

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which correspond to residues 65–83 and 146–150 in chymotrypsin (Furie et al., 1982; Birktoft & Blow, 1972). γ-Thrombin is active against tripeptidyl *p*-nitroanilide substrates (Lottenberg et al., 1982; Sonder & Fenton, 1986) and can be inhibited by antithrombin III (Chang et al., 1979) but retains little activity with fibrinogen [Fenton et al., 1977; for a review, see Berliner (1984)].

The precise covalent structure of human γ-thrombin seems to depend on the method of preparation. Whereas Boissel et al. (1984) have shown that autolysis can produce a derivative that has been cleaved at Arg-62, Arg-73, Arg-123, and Lys-154, Fenton et al. (1977) have tentatively identified the major sites of trypsinolysis to be Arg-73 and Lys-154 [see Berliner (1984) for the numbering scheme used here]. More recently, it has been shown that both autolysis and trypsinolysis can occur in the region preceding Arg-73 (Chang, 1986; Braun et al., 1987). This variability in covalent structure, as well as the fact that at least two peptide bonds have been cleaved, has complicated the interpretation of the results of enzymological studies using γ-thrombin. A better correlation between the structural alteration and the enzymatic properties would be possible with derivatives that have only a single peptide bond cleaved. Kawabata et al. (1985) have described that the cleavage of human α-thrombin by pancreatic elastase occurs exclusively at Ala^{150}–Asn^{151}, yielding ε-thrombin. In addition, we have recently been able to produce, on a preparative scale, a derivative of human α-thrombin that is cleaved by trypsin at Arg^{73}–Asn^{74} only (Braun et al., 1987). This derivative is different from human β-thrombin obtained by autolysis. Whereas β-thrombin obtained by trypsinolysis still contains the peptide 63–73, this undecapeptide is missing in autolyzed β-thrombin (Boissel et al., 1984). Similarly, in bovine β-thrombin produced by autolysis, a small peptide has been excised (Lundblad et al., 1979). To avoid confusion, we have proposed (Braun et al., 1987) to call the human β-thrombins obtained by trypsinolysis and autolysis β_T- and β-thrombin, respectively.

In this study, we have used β_T- and ε-thrombin together with α-thrombin and γ-thrombin produced by limited trypsinolysis (γ_T-thrombin) to assess the importance of the regions around the cleavage sites for the interaction of thrombin with tripeptidyl *p*-nitroanilide substrates and a tripeptidyl chloromethyl ketone inhibitor, fibrinogen, antithrombin III in the presence and absence of heparin, and thrombomodulin.

Experimental Procedures

Materials. The inhibitors D-Phe-Phe-ArgCH_2Cl, hirudin (12000 units/mg), and Eglin C were gifts from Dr. E. Shaw (Friedrich Miescher Institut, Basel, Switzerland), Plantorgan AG (Bad Zwischenahn, West Germany), and Dr. H.-P. Schnebli (Ciba-Geigy AG, Basel, Switzerland), respectively. Human fibrinogen and the substrates D-Phe-Pip-Arg-pNA[1] (S-2238) and D-Ile-Pro-Arg-pNA (S-2288) were from Kabi-Vitrum, Molndal, Sweden, whereas tosyl-Gly-Pro-Arg-pNA (Chromozym TH) was from Boehringer, Mannheim, West Germany. Porcine pancreatic elastase and trypsin were purchased from Worthington, Freehold, NJ, and heparin (176 units/mg) was from Sigma, St. Louis, MO. All other chemicals were of the highest grade available commercially. Rabbit thrombomodulin, human antithrombin III, human α-thrombin, and human fibrinogen were purified and characterized as described previously (Hofsteenge et al., 1986).

Preparation of Proteolyzed Forms of Thrombin. β_T-Thrombin and γ_T-thrombin were prepared by limited digestion with trypsin as described elsewhere (Braun et al., 1987). A third proteolyzed derivative of thrombin was prepared by treatment of thrombin with pancreatic elastase (ε-thrombin; Kawabata et al., 1985) as described in detail by Stone et al. (1987).

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). This titration yielded the enzyme concentrations used to calculate the catalytic constant (k_{cat}). The purity of these preparations was determined by labeling with tritiated DFP followed by sodium dodecyl sulfate gel electrophoresis and densitometry of autoradiograms, or liquid scintillation counting of sliced gel lanes (Mann et al., 1973). The β_T-thrombin preparation was found to contain about 3% γ_T-thrombin and less than 2% α-thrombin whereas the γ_T-thrombin and ε-thrombin preparations contained less than 2% of other thrombin forms. Amino acid sequence analysis (five cycles) was performed as described previously (Stone & Hofsteenge, 1986) and detected only one additional amino terminus in the preparations of β_T-thrombin and ε-thrombin and allowed the cleavage sites to be identified as Arg^{73}–Asn^{74} and Ala^{150}–Asn^{151} for β_T-thrombin and ε-thrombin, respectively. Details of the determination of the covalent structure of β_T-thrombin have been reported elsewhere (Braun et al., 1987). Similar analysis with γ_T-thrombin detected the reported cleavage sites at Arg^{73}–Asn^{74} and Lys^{154}–Gly^{155} (Boissel et al., 1984; Fenton et al., 1977). Moreover, this derivative did not bind to an antibody specific for residues 62–73 of the α-thrombin B-chain, indicating that this peptide was missing.

Amidolytic Assays. Amidolytic assays were conducted at 37 °C as described previously (Hofsteenge et al., 1986) in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% poly(ethylene glycol) (M_r 6000). The concentration of the substrates was determined spectrophotometrically at 342 nm by using an absorption coefficient of 8270 $M^{-1}\cdot cm^{-1}$ (Lottenberg & Jackson, 1983).

Fibrinogen Cleavage Assays. Fibrinogen assays were performed at 37 °C as previously described (Hofsteenge et al., 1986) in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.117 M NaCl and 0.1% poly(ethylene glycol) (M_r 6000). The concentrations of fibrinopeptides A (FPA) and B (FPB) were determined after separation on a reverse-phase C_{18} column using standard curves of peak heights versus known concentrations of FPA and FPB (Hofsteenge et al., 1986).

Stability Studies. Denaturation studies of the proteolytic derivatives of human thrombin (approximately 500 pM) were performed by incubation in 1 mL of 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.1% poly(ethylene glycol) (M_r 6000), and 0–6 M deionized urea for 1 h at 25 °C. Thrombin activity was then measured by adding 2 mM S-2238 (50 μL) and monitoring the change in absorbance at 405 nm.

Binding of Thrombin to Thrombomodulin: Solid Phase Binding Assay. Microtiter wells were coated with 50 μL of thrombomodulin solution (0.2 μg/mL in PBS) overnight at 4 °C. Subsequent steps were performed at room temperature. Excess thrombomodulin was removed by washing 3 times with PBS containing 0.05% Tween 20 (PBS–Tween). The plates were then treated with 1% bovine serum albumin in PBS (50 μL) for 1 h and washed again. Solutions containing varying concentrations of α-thrombin or derivative forms (50 μL) were added to the coated wells and incubated for 2 h. Unbound

[1] Abbreviations: FPA, fibrinopeptide A; FPB, fibrinopeptide B; PBS, phosphate-buffered saline; pNA, *p*-nitroanilide; Pip, pipecolyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DFP, diisopropyl fluorophosphate.

Table I: Kinetic Parameters of Various Forms of Thrombin with Two Tripeptidyl *p*-Nitroanilide Substrates[a]

	α-thrombin	β_T-thrombin	γ_T-thrombin	ϵ-thrombin
D-Phe-Pip-ArgpNA (S2238)				
K_m (μM)	3.4 ± 0.1	9.7 ± 0.3	11.1 ± 0.5	2.8 ± 0.1
k_{cat} (s^{-1})	202 ± 2	233 ± 4	209 ± 6	196 ± 3
$(k_{cat}/K_m) \times 10^{-7}$ ($M^{-1}\cdot s^{-1}$)	6.1 ± 0.1	2.8 ± 0.1	2.0 ± 0.1	7.0 ± 0.2
Tos-Gly-Pro-ArgpNA (Chromozym TH)				
K_m (μM)	13.0 ± 0.6	36.0 ± 1.2	49.8 ± 2.6	12.6 ± 0.5
k_{cat} (s^{-1})	323 ± 10	322 ± 8	275 ± 11	272 ± 7
$(k_{cat}/K_m) \times 10^{-7}$ ($M^{-1}\cdot s^{-1}$)	2.5 ± 0.1	1.0 ± 0.1	0.55 ± 0.01	2.21 ± 0.04

[a] Assays were performed and the data analyzed as described previously (Stone & Hofsteenge, 1985). For each experiment, the initial velocity was determined in duplicate using at least five substrate concentrations. The range of substrate concentrations was chosen such that at least one concentration was more than twice the value of the Michaelis constant and at least one concentration was less than half this value.

thrombin was removed by washing as described above, and bound thrombin was assayed by the addition of 300 μM S-2288 (100 μL). The absorbance at 405 nm was measured after 1–3 h by using a Dynatech MR 6000 plate reader. Over this period, the rate of formation of pNA was linear with time. Blanks obtained by substituting buffer for thrombin or by omitting the coating with thrombomodulin gave readings smaller than 0.005 absorbance unit.

Data Analysis. Values of k_{cat} and K_m for tripeptidyl *p*-nitroanilide substrates and the Aα-chain of fibrinogen were determined by using weighted linear regression to fit the data to the Michaelis-Menten equation (Cornish-Bowden, 1977). The weighting assumed that the errors in the velocities were proportional to the observed values. At subsaturating concentrations of fibrinogen, the release of FPA and FPB from fibrinogen can be described by eq 1 and 2, respectively

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

$$[\text{FPB}] = [\text{B}\beta]_0\left[1 + \frac{k'_\text{B}}{k'_\text{A} - k'_\text{B}}\exp(-k'_\text{A}t) - \frac{k'_\text{A}}{k'_\text{A} - k'_\text{B}}\exp(-k'_\text{B}t)\right] \quad (2)$$

(Higgins et al., 1983), where $[\text{A}\alpha]_0$ and $[\text{B}\beta]_0$ are the initial concentrations of the Aα- and the Bβ-chain, respectively, and k'_A and k'_B are the apparent first-order rate constants for the release of FPA and FPB, respectively. Data were fitted to these equations by nonlinear regression as previously described (Hofsteenge et al., 1986). The inactivation of thrombin by D-Phe-Phe-ArgCH_2Cl and antithrombin III was studied in the presence of substrate as previously described (Stone & Hofsteenge, 1985). In the absence of heparin, micromolar concentrations of antithrombin III were used, and the inhibition appeared to be irreversible. The inhibition of thrombin by D-Phe-Phe-ArgCH_2Cl also appeared to be irreversible, and the data for this compound and antithrombin III in the absence of heparin were analyzed as described previously according to the equations for irreversible enzyme inactivation (Stone & Hofsteenge, 1985). These analyses yielded values of the dissociation constant (K_i) and inactivation rate constant (k_i) for D-Phe-Phe-ArgCH_2Cl and the second-order inactivation rate constant for antithrombin III.

In order to measure accurately the rate of inhibition of thrombin by antithrombin III in the presence of heparin, it was necessary to use low (nanomolar) concentrations of antithrombin III. At these concentrations, the inhibition of thrombin by antithrombin III appeared to be reversible, and the progress curve of product release was best described by eq 3 which is the equation for slow binding inhibition (Morrison, 1982) where P is the concentration of product *p*-nitroaniline, v_0 and v_s are the initial and steady-state velocities, respectively, and k is an apparent first-order rate constant. The data were fitted to this equation by nonlinear regression.

$$P = v_s t + \frac{(v_0 - v_s)(1 - e^{-kt})}{k} \quad (3)$$

The dissociation constant for the binding of thrombin to immobilized thrombomodulin will be given by eq 4 where A_t

$$K_d = \frac{(A_t - EA)(E_t - EA)}{EA} \quad (4)$$

is the amount of thrombomodulin bound to the microtiter plate capable of binding thrombin, E_t is the total thrombin concentration, and EA is the concentration of the thrombin–thrombomodulin complex. Under the conditions of the assay, the activity measured (v) is due to the thrombin–thrombomodulin complex, and it can be shown that

$$v = (V/2)[E_t + A_t + K_d - (E_t + K_d + A_t)^2 - 4E_tA_t]^{1/2} \quad (5)$$

where V is the rate of formation of pNA at an infinite concentration of thrombin. Optimized values for A_t, V, and K_d were determined for α-thrombin by weighted nonlinear regression, and the value of A_t was kept constant in subsequent calculations of V and K_d for β_T- and ϵ-thrombin data obtained in the same experiment.

Results

Stability Studies. γ_T-Thrombin, which contains at least two cleaved peptide bonds, has been shown to be more susceptible to denaturation by urea than α-thrombin (Bauer et al., 1980). It was, therefore, of interest to examine whether a single proteolytic cleavage in the thrombin B-chain would result in a similar destabilization. The effect of increasing concentrations of urea on the activity of α-, β_T-, γ_T-, and ϵ-thrombin is shown in Figure 1. With α-, β_T-, and ϵ-thrombin, it was observed that low concentrations of urea had a small (10%) stimulatory effect. The concentration of urea that caused 50% loss of activity was determined from these data to be 3.3, 2.2, 2.1, and 2.2 M for α-, β_T-, γ_T-, and ϵ-thrombin, respectively.

Interaction of Thrombin Derivatives with Tripeptidyl pNA Substrates and a Tripeptidyl Chloromethane Inhibitor. The kinetic parameters of α-, β_T-, γ_T-, and ϵ-thrombin with two thrombin-specific substrates were determined, and the results are shown in Table I. With both substrates, there was less than 20% variation in the value of k_{cat} compared with α-thrombin. The value for the Michaelis constant for both substrates had increased 3–4-fold with β_T- and γ_T-thrombin, whereas the value for ϵ-thrombin had remained essentially unaltered.

Table II shows the kinetic parameters associated with the inactivation of α-, β_T-, γ_T-, and ϵ-thrombin by D-Phe-Phe-Arg-CH_2Cl. The dissociation constant (K_I) for this compound followed a similar trend to that observed with the Michaelis constant for the substrates. In comparison with α-thrombin,

Table II: Inactivation of α-, β_T-, γ_T-, and ε-Thrombin by D-Phe-Phe-ArgCH$_2$Cl[a]

	α-thrombin	β_T-thrombin	γ_T-thrombin	ε-thrombin
K_I (μM)	14.6 ± 2.2	45.6 ± 5.3	41.1 ± 1.4	10.5 ± 1.3
$k_i \times 10^2$ (s^{-1})	9.0 ± 1.2	8.8 ± 0.9	7.9 ± 0.2	8.8 ± 0.1
k_i/K_I ($mM^{-1}\cdot s^{-1}$)	6.1 ± 0.1	1.93 ± 0.03	1.91 ± 0.01	8.4 ± 0.2

[a] Assays were performed as described under Experimental Procedures in the presence of 150 μM D-Phe-Pip-ArgpNA. D-Phe-Phe-ArgCH$_2$Cl was varied over a 4-fold range from 62.5 to 250 μM, and a minimum of four duplicate assays were performed for each thrombin derivative. Data were analyzed as described previously (Stone & Hofsteenge, 1985) to yield values of K_I and k_i which are given together with their standard errors.

Table III: Kinetic Parameters for the Cleavage of Fibrinogen by α-Thrombin and Proteolytically Modified Thrombins[a]

	α-thrombin	β_T-thrombin (+hirudin)	β_T-thrombin (−hirudin)	ε-thrombin
K_{mA} (μM)	11.4 ± 0.5	21.7 ± 3.9	19.0 ± 1.4	10.1 ± 0.6
k_{catA} (s^{-1})	103 ± 3	2.7 ± 0.4	6.6 ± 0.4	59 ± 6
$(k_{catA}/K_{mA}) \times 10^{-5}$ ($M^{-1}\cdot s^{-1}$)	90 ± 5	1.20 ± 0.03	3.50 ± 0.03	58.4 ± 3.5
$k_A' \times 10^{-5}$ ($M^{-1}\cdot s^{-1}$)[b]	110 ± 10[c]	1.65 ± 0.03	ND[d]	ND
$k_B' \times 10^{-5}$ ($M^{-1}\cdot s^{-1}$)[b]	60 ± 2[c]	0.90 ± 0.03	ND	ND
k_A'/k_B'	0.55 ± 0.19	0.55 ± 0.02	ND	ND

[a] Rates of release of fibrinopeptides were determined as described under Experimental Procedures. The range of fibrinogen concentrations used in initial velocity experiments was 0.7–12 μM (1.4–24 μM Aα-chain). The values shown are the weighted means of at least two experiments with their standard errors. [b] Determined from progress curves under first-order conditions (see Figure 2). [c] From Hofsteenge et al. (1986). [d] ND, not determined.

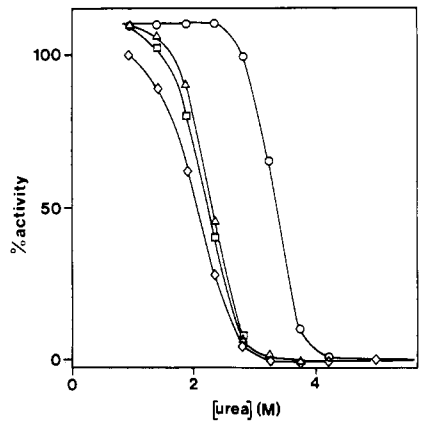

FIGURE 1: Effect of urea concentration on the amidolytic activity. α-Thrombin and its proteolytic derivatives were incubated at 25 °C with varying amounts of deionized urea in 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.1% poly(ethylene glycol) for 1 h. The residual thrombin activity was measured with the substrate D-Phe-Pip-Arg-pNA (100 μM). The activity in the absence of urea was taken as 100%. α-Thrombin (○); β_T-thrombin (□); γ_T-thrombin (◇); ε-thrombin (△).

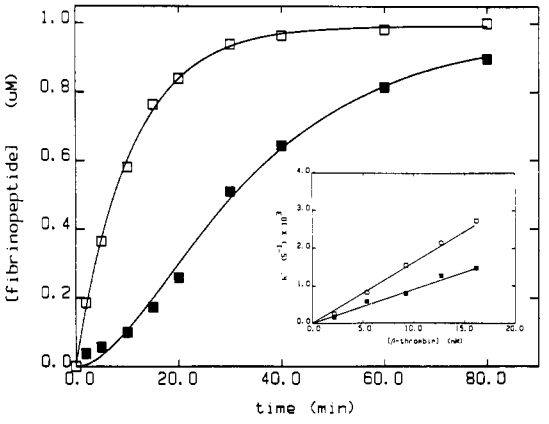

FIGURE 2: Time course of the release of FPA and FPB by β_T-thrombin under first-order conditions. The rate of release of the fibrinopeptides FPA (□) and FPB (■) from subsaturating concentrations of fibrinogen (0.5 μM) by β_T-thrombin (2.2–16.2 nM) was measured in the presence of a small amount (5% mol/mol) of hirudin as described under Experimental Procedures. The data for 9.2 nM β_T-thrombin are shown. The curves represent the best fit of the data to eq 1 and 2. Good agreement (less than 5% difference) was observed between the calculated infinite concentrations of product and the experimentally determined values from the incubation (60 min) of the same amount of fibrinogen with 40 nM α-thrombin. The inset shows the linear dependence of the apparent first-order rate constant on the thrombin concentration. From these data, average second-order rate constants for the release of FPA and FPB were obtained by weighted linear regression: $k_A = (1.65 \pm 0.03) \times 10^5$ $M^{-1}\cdot s^{-1}$; $k_B = (0.90 \pm 0.03) \times 10^5$ $M^{-1}\cdot s^{-1}$.

the value of K_I increased about 3-fold with β_T- and γ_T-thrombin whereas the value observed with ε-thrombin was not markedly different. The inactivation rate constant (k_i) did not vary significantly between the various thrombin derivatives.

Cleavage of Fibrinogen. The preparation of γ_T-thrombin retained less than 0.05% of the clotting activity of α-thrombin, whereas β_T- and ε-thrombin retained 4% and 60% of the activity, respectively. The kinetics for the cleavage of fibrinogen by β_T- and ε-thrombin were further investigated. The preparation of β_T-thrombin contained 1.5% α-thrombin, and in order to eliminate this activity, 0.05 pmol of hirudin/pmol of β_T-thrombin was included in all assays. The inhibition constants of hirudin with α- and β_T-thrombin are 20 fM and 2 pM, respectively (Stone & Hofsteenge, 1986; Stone et al., 1987). Thus, at the concentration of hirudin used, α-thrombin will be preferentially inhibited, and any hirudin in excess of the concentration of α-thrombin will inhibit β_T-thrombin. A contamination of α-thrombin as great as 5% would be totally inhibited by the added hirudin. On the other hand, if no contamination of α-thrombin were present, only 5% of the β_T-thrombin would be inhibited. Initial rate data obtained for the release of FPA by using this protocol did not show any deviation from Michaelis–Menten kinetics, and it was possible to obtain the estimates of k_{catA} and K_{mA} given in Table III. Comparison of these values with those obtained with α-thrombin indicated that the value for K_{mA} for the cleavage of the Aα-chain by β_T-thrombin had increased only about 2-fold but a decrease of 40-fold in the value for k_{cat} was observed. The omission of hirudin from the reaction mixture resulted in a 2-fold increase in the value of k_{cat} but did not significantly alter the value of K_{mA} (Table III). In order to determine whether the rate of release of both FPB and also FPA from fibrinogen had decreased, progress curve experiments were performed at subsaturating levels of fibrinogen. Under these conditions, the release of FPA was described by a single exponential (eq 1), whereas the progress of the release of FPB showed a lag phase and was described by a double exponential (eq 2). This lag phase has also been observed with α-thrombin (Blombäck et al., 1978; Higgins et al., 1983; Hofsteenge et al., 1986). The data obtained are shown in Figure 2. Analysis

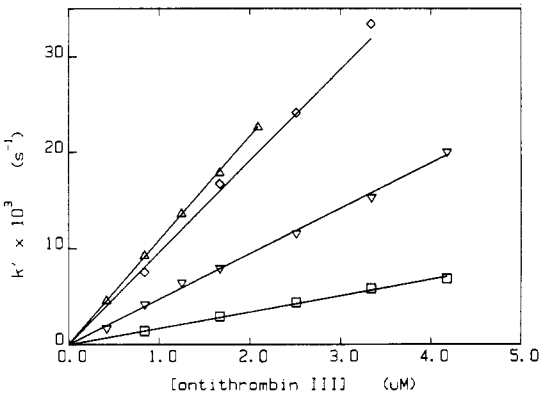

FIGURE 3: Inactivation of thrombin derivatives by antithrombin III. Assays were performed as described under Experimental Procedures in the presence of a known concentration of tosyl-Gly-Pro-Arg-pNA (about 200 μM). The data were analyzed as described previously (Hofsteenge et al., 1986) to yield values for the apparent first-order rate constant for the inactivation (k'). The dependence of k' on the concentration of antithrombin III is shown for α- (△), β_T- (▽), γ_T- (□), and ϵ-thrombin (◇).

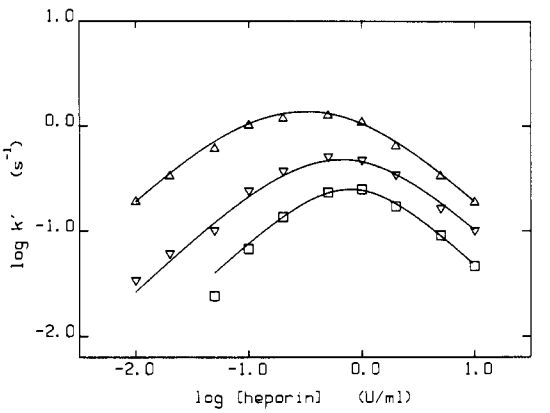

FIGURE 4: Inactivation of thrombin derivatives by antithrombin III in the presence of heparin. Assays were performed as described under Experimental Procedures in the presence of a known amount (100–300 μM) of D-Phe-Pip-Arg-pNA and 26.7 nM antithrombin III. The progress curve data obtained were analyzed according to eq 3. The values obtained for the rate constant (k'_{obsd}) were corrected for the presence of substrate as described elsewhere (Hofsteenge et al., 1986) to yield the value for the apparent first-order rate constant for the association of the thrombin derivatives with antithrombin III (k'). The dependence of this value is shown for α- (△), β_T- (▽), and γ_T-thrombin (□).

of these data according to the appropriate equations yielded estimates for the apparent first-order rate constants k_A' and k_B'. The values of these constants varied linearly with the thrombin concentration (Figure 2, inset), and an average value for the second-order rate constant was obtained from these data (k_A and k_B in Table III). The value of k_A was in agreement with the value obtained for k_{catA}/K_{mA} in the initial rate experiments. Compared with α-thrombin, k_A and k_B decreased a similar amount; that is, the ratio of the two constants remained constant (Table III).

Initial rate data were obtained with ϵ-thrombin, and analysis of the data yielded the values of K_{mA} and k_{catA} given in Table III. In comparison with α-thrombin, ϵ-thrombin had the same value for K_{mA} for the Aα-chain of fibrinogen and a value for k_{catA} which was about 40% lower.

Inhibition by Antithrombin III. The inhibition of thrombin by antithrombin III is of importance, since this inhibitor is one of the major inactivators of thrombin in plasma (Rosenberg, 1977). We have investigated the kinetics of inhibition of the proteolyzed forms of thrombin by this inhibitor. Figure 3 shows the dependence of the rate of inactivation of the various thrombin derivatives on the concentration of antithrombin III. Analysis of these data yielded the following second-order inactivation rate constants: 10.8 ± 0.1, 4.7 ± 0.1, 1.7 ± 0.1, and 9.5 ± 0.1 mM^{-1} s^{-1} for α-, β_T-, γ_T-, and ϵ-thrombin, respectively. Thus, α-thrombin and ϵ-thrombin were inactivated at approximately the same rate by antithrombin III, whereas the rates of inactivation of β_T- and γ_T-thrombin were reduced 2.3- and 6.4-fold, respectively, compared with α-thrombin.

Heparin, a glycosaminoglycan, has been shown to accelerate the inactivation of α-thrombin by antithrombin III (Björk & Lindahl, 1982). A model for its mechanism of action has been proposed in which a molecule of heparin can bind to both thrombin and antithrombin III, thereby increasing the rate of interaction of the two proteins. The model predicts a bell-shaped curve for the plot of the apparent first-order rate constant as a function of heparin concentration (Nesheim, 1983). In addition, it has been shown that heparin induces a conformational change in the antithrombin III molecule (Nordenman & Björk, 1978). We have examined the effect of heparin on the inhibition of α-, β_T-, γ_T-, and ϵ-thrombin by antithrombin III. The dependence of the rate of inhibition of thrombin by antithrombin III on the concentration of heparin is shown in Figure 4. For all forms of thrombin, a bell-shaped dependence of the first-order rate constant of inactivation on the concentration of heparin was observed. The curve for ϵ-thrombin was superimposable upon that for α-thrombin (data not shown), but the curves for β_T- and γ_T-thrombin appeared to be shifted slightly toward a higher concentration optimum. At the optimal concentration, the rate of inactivation of β_T-thrombin was 2.2-fold less than that of α-thrombin, whereas that of γ_T-thrombin was 5.4-fold lower.

Binding to Thrombomodulin. The binding of thrombin to the endothelial surface protein thrombomodulin causes a major change in the specificity of thrombin for macromolecular substrates. The thrombin–thrombomodulin complex catalyzes the activation of protein C but is inactive toward fibrinogen and platelets (Esmon & Owen, 1981; Esmon, C. T., et al., 1982; Esmon et al., 1983; Esmon, N. L., et al., 1982). Therefore, it was of interest to investigate the areas of thrombin that interact with this protein. The binding of α-, β_T-, γ_T-, and ϵ-thrombin to thrombomodulin was studied using a solid phase binding assay with thrombomodulin immobilized to the wells of microtiter plates. To test the validity of this method, we examined the binding of α-thrombin and its inhibition by chemically inactivated thrombin. Increasing amounts of α-thrombin resulted in saturable binding to the immobilized thrombomodulin as shown in Figure 5A, and a good fit of the data to eq 5 was observed. Moreover, thrombin that had been inactivated with D-Phe-Pro-ArgCH_2Cl acted as a competitive inhibitor for the binding of active α-thrombin, since the observed K_d increased linearly with increasing concentration of D-Phe-Pro-ArgCH_2–thrombin. Analysis of these data yielded an inhibition constant of 0.6 ± 0.2 nM (data not shown). The results of a representative experiment using α-, β_T-, γ_T-, and ϵ-thrombin are shown in Figure 5A,B. Analysis of the data according to eq 5 yielded values for K_d (averages of five independent experiments) of 0.40 ± 0.13, 15.5 ± 3.7, and 0.58 ± 0.18 nM for α-, β_T-, and ϵ-thrombin, respectively. The value for α-thrombin is in good agreement with the value obtained by Owen and Esmon (1981) (0.48 nM) and by ourselves, in a study examining the effect of thrombomodulin on the binding of four different ligands to thrombin (0.7 ± 0.1 nM; Hofsteenge et al., 1986). The affinity of γ_T-thrombin for thrombomodulin was too low to allow the calculation of a value for K_d. Even at concentrations as high as 5 μM, very little binding

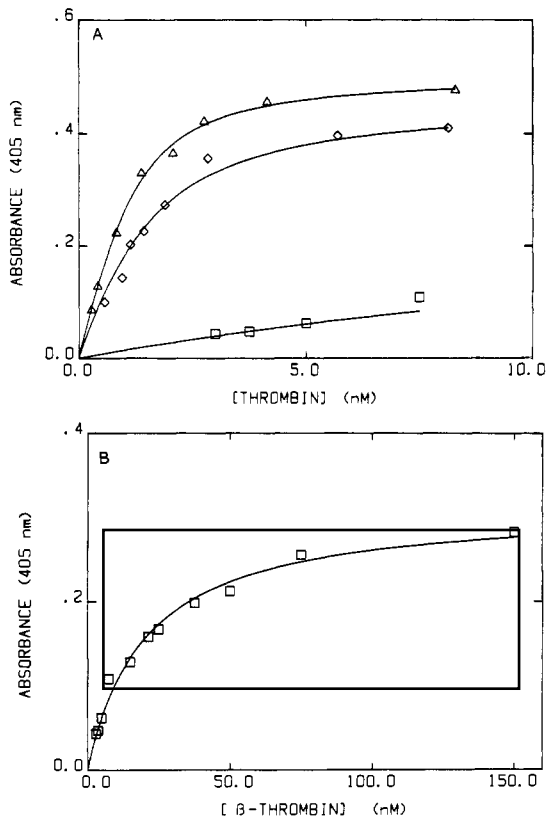

FIGURE 5: Binding of thrombin derivatives to thrombomodulin. (A) Binding of α- (△), ε- (◇), and β_T-thrombin (□) to immobilized thrombomodulin in microtiter wells. For β_T-thrombin, only the first part of the curve is shown (see Panel B for the entire binding curve). Bound thrombin was assayed with 200 μM S-2288 as described under Experimental Procedures. (B) Binding of β_T-thrombin to immobilized thrombomodulin.

was observed. Moreover, we found that γ_T-thrombin in the presence of thrombomodulin was a very poor activator of protein C (data not shown).

DISCUSSION

In order to delineate areas of the thrombin molecule that are important for the interaction of the enzyme with substrates, inhibitors, and receptors, we have studied the enzymatic properties of proteolytic derivatives of human α-thrombin.

Human γ_T-thrombin that has been used in structure–function relationship studies until now (Berliner, 1984) has the disadvantage that its precise covalent structure varies with the method of preparation and that it contains cleavages in two areas of the molecule (Boissel et al., 1984; Fenton et al., 1977). This complicates the correlation of alterations in covalent structure to changes in enzymatic properties. With the availability of two well-characterized thrombin derivatives that contain only one cleaved peptide bond, β_T-thrombin, cleaved at Arg-73 (Braun et al., 1988), and ε-thrombin, cleaved at Ala-150 (Kawabata et al., 1985), it was now possible to make a more unequivocal correlation. Previously, the usefulness of these derivatives was demonstrated in studies on the identification of areas of thrombin that interact with the thrombin-specific inhibitor hirudin (Stone et al., 1987). The dissociation constant for the hirudin–β_T-thrombin complex had increased 100-fold in comparison with α-thrombin, whereas that of the ε-thrombin–hirudin complex was unchanged.

It has been shown that γ_T-thrombin is more susceptible to denaturation by urea than α-thrombin (Bauer et al., 1980). The results obtained in our studies are in agreement with this observation (Figure 1). Moreover, the fact that the midpoint of denaturation (as measured by the loss of amidolytic activity) for both β_T- and ε-thrombin was very similar to that for γ_T-thrombin indicated that a single cleavage in the B-chain was sufficient to destabilize the molecule. The location of the cleavage did not seem to have any influence.

The specificity constant (k_{cat}/K_m) for the hydrolysis of tripeptidyl pNA substrates by γ_T-thrombin appeared to be approximately 3-fold lower than that of α-thrombin (Table I), in agreement with findings from other laboratories (Lottenberg et al., 1982; Sonder & Fenton, 1986). A similar decrease was found with β_T-thrombin and was in both cases due to an increase in the Michaelis constants, whereas the catalytic constants remained constant. Since the values of the kinetic parameters were measured at 37 °C, they were found to be consistently higher than those obtained at 25 °C by Lottenberg et al. (1982) and Sonder and Fenton (1986). No change in the kinetic parameters was found in the case of ε-thrombin (Table I). Therefore, it seems likely that the cleavage of the peptide bond 73–74 caused the decrease in amidolytic activity. Model-building studies have suggested that these residues are relatively far removed from the catalytic center (Furie et al., 1982) and therefore are not likely to participate directly in the binding of the tripeptidyl pNA substrates. In order to explain this small increase in K_m, one has to assume that proteolysis at Arg-73 causes a small conformational change in the molecule which affects the residues that are involved in the binding of these substrates.

Previously, it has been shown that the rate of loss of clotting activity on conversion of α-thrombin to β_T-thrombin parallels the rate of cleavage of the peptide bond 73–74 (Braun et al., 1988). It seems, therefore, likely that this single proteolytic cleavage diminishes the ability of thrombin to cleave fibrinogen (Fenton et al., 1977; Braun et al., 1988). We have found that the specificity constant of human β_T-thrombin for the cleavage of FPA from the Aα-chain of fibrinogen was reduced about 80-fold. This reduction was mainly due to a 40-fold decrease in the catalytic constant (Table III). In contrast, the Michaelis constant had increased only 2-fold. These findings are consistent with the observation of Lundblad et al. (1984) that fibrinogen binds equally well to bovine α- and β-thrombin, as judged from competitive inhibition studies using pNA substrates. These authors attribute the decrease in catalytic efficiency to a decreased nucleophilicity of the active-site histidine residue. In bovine β-thrombin, this residue reacts 3-fold slower with tosyl-LysCH_2Cl than in α-thrombin. Using D-Phe-Phe-ArgCH_2Cl, we did find a 3-fold reduction in the affinity of thrombin for this inhibitor (Table II), but we did not observe a reduced rate of reaction of the histidine residue in the β_T-thrombin–inhibitor complex; i.e., the value of k_i was the same for human α- and β_T-thrombin (Table II). It was concluded that the nucleophilicity of the active-site histidine residue has not changed in human β_T-thrombin. The use of D-Phe-Phe-ArgCH_2Cl has the advantage over tosyl-LysCH_2Cl in that one can estimate both the binding (K_I) and the rate of reaction after the initial complex between enzyme and inhibitor has been formed (k_i). A change in the value for k_i reflects more precisely a change in the nucleophilicity of the histidyl residue than the overall rate of inactivation in the case of tosyl-LysCH_2Cl. The reduction in k_{cat} for the Aα-chain of fibrinogen could thus be due to a less optimal alignment of the scissile bond relative to the catalytic residues. A similar mechanism could apply to the cleavage of the fibrinogen Bβ-chain, since the catalytic efficiency of cleavage of FPB from the Bβ-chain was affected to exactly the same extent as that of FPA from the Aα-chain (Table III). γ_T-Thrombin has nearly completely lost its ability to cleave fibrinogen (0.05%

of the activity of α-thrombin). ε-Thrombin, which is cleaved at Ala-150, only four residues away from the second cleavage point in γ_T-thrombin, showed only a minor decrease in k_{cat} (40%). Thus, it seems unlikely that γ_T-thrombin had lost its clotting activity as a result of the cleavage in the loop corresponding to residues 146–150 in α-chymotrypsin. Either the excision of peptide 63–73 (Braun et al., 1988) or a major conformational change as a result of the combination of the proteolytic cleavages eliminated the fibrinogen clotting activity of this derivative.

The effect of proteolysis of thrombin on its rate of inactivation by antithrombin III was small. A similar explanation as described above could apply to the results obtained on the inactivation by antithrombin III. A 6-fold decrease of the second-order rate constant for inhibition was found with γ_T-thrombin, but a 2-fold decrease was observed with β_T-thrombin. On the other hand, the rate constant for ε-thrombin remained unaltered. Thus, it seems that none of the regions cleaved in γ_T-thrombin by itself was of major importance for the inhibition of thrombin by antithrombin III. The inactivation of thrombin by antithrombin III has been found to be accelerated by the glycosaminoglycan heparin (Björk & Lindahl, 1982). For all three proteolytic derivatives, a bell-shaped curve was found for the dependence of the first-order rate constant for inhibition on the concentration of heparin, although the optimal concentrations of heparin for β_T- and γ_T-thrombin seemed to be slightly higher than for α-thrombin (Figure 4). These results indicated that the mechanism of action of heparin was the same in all cases. Moreover, at the optimal concentration of heparin, the relative rate of inactivation compared with α-thrombin was the same as found in the absence of heparin. Thus, proteolysis of the loops corresponding to residues 65–83 and 146–150 in α-chymotrypsin did not affect the binding and action of heparin.

The binding of thrombin to the endothelial cell surface protein thrombomodulin causes major changes in the specificity of thrombin for macromolecular substrates (Esmon & Owen, 1981; Esmon, C. T., et al., 1982; Esmon et al., 1983; Esmon, N. L., et al., 1982). We have shown previously that thrombomodulin competitively inhibits the binding of fibrinogen and hirudin to thrombin but that it has a minimal effect on the binding of tripeptidyl substrates and inhibitors (Hofsteenge et al., 1986). It was of interest to study the interaction of this protein with proteolyzed forms of thrombin, since some of the properties of β_T-thrombin and γ_T-thrombin resembled those of the thrombin–thrombomodulin complex, e.g., impaired cleavage of fibrinogen and binding of hirudin (Stone et al., 1987), and a small decrease in the affinity for tripeptidyl substrates and inhibitors. It was found that γ_T-thrombin had nearly completely lost its ability to bind to thrombomodulin. This observation is in agreement with the studies of Bezeaud et al. (1985), in which it is shown that γ-thrombin inactivated with diisopropyl fluorophosphate does not displace α-thrombin from the complex with thrombomodulin. Cleavage at Arg-73 (β_T-thrombin) resulted in a 39-fold increase in the dissociation constant of the thrombomodulin–thrombin complex. In contrast, the affinity of ε-thrombin for thrombomodulin was essentially the same as that of α-thrombin. These results could indicate that the region around Arg-73 is important for the interaction with thrombomodulin. Alternatively, it is conceivable that the cleavage at Arg-73 caused conformational alterations that resulted in a lower affinity of β_T-thrombin for thrombomodulin. It has been possible, however, to isolate a polyclonal antibody that binds specifically to residues 62–73 of α-thrombin (G. Noë, J. Hofsteenge, and S. R. Stone, unpublished results). This antibody inhibited not only the cleavage of fibrinogen and the binding of hirudin but also the activation of protein C by α-thrombin in the presence of thrombomodulin.

On the basis of the above results, it seems likely that the area around Arg-73 of the B-chain of thrombin (corresponding to loop 65–83 in α-chymotrypsin) contributes significantly to the specific interaction with fibrinogen and hirudin (Stone et al., 1987). The modulation of the specificity of thrombin by thrombomodulin could also take place by binding to this region. In contrast, a modification in the region corresponding to loop 146–150 in α-chymotrypsin, as it occurs in ε-thrombin, does not seem to have a major effect on these interactions.

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Registry No. S2238, 62354-65-8; chromozym TH, 61876-61-7; D-Phe-Phe-ArgCH_2Cl, 74392-49-7; antithrombin, 9000-94-6.

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Platelet Activation by Thrombin in the Absence of the High-Affinity Thrombin Receptor†

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ABSTRACT: The receptor status of the moderate-affinity platelet binding site for α-thrombin has been established by treating platelets with *Serratia marcescens* protease under conditions causing cleavage of 95–97% glycoprotein Ib (2.5 μg for 30 min). High-affinity binding was lost under these conditions, but the platelets continued to show moderate-affinity binding (K_{d_1} = 16 ± 5 nM; 930 ± 300 sites/platelet) and low-affinity binding (K_{d_2} = 4.6 ± 3 μM; 170 000 ± 90 000 sites/platelet), in good agreement with the values previously obtained for moderate- and low-affinity binding in intact platelets [Harmon, J. T., & Jamieson, G. A. (1986) *J. Biol. Chem. 261*, 15928–15933]. Platelets treated with *Serratia* protease under these conditions were about 4-fold less sensitive to activation by α-thrombin, as measured by serotonin secretion. Crossover studies with analogues showed that binding of α-thrombin was competable by both D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone treated thrombin, and both analogues were capable of inhibiting activation of *Serratia*-proteolyzed platelets by α-thrombin. These studies establish that the moderate-affinity platelet binding site for α-thrombin is a receptor, occupancy of which is required for platelet activation in the absence of the high-affinity receptor.

The definition of a receptor is that the observed binding of a ligand must be saturable, reversible, and specific, and must lead to a physiological event (Kahn, 1976). We have recently shown (Harmon & Jamieson, 1986a) that the activation of intact platelets by thrombin proceeds via a receptor-dependent mechanism as shown by the fact that D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin (PPACK-thrombin)[1] binds to the high-affinity binding site for α-thrombin (K_d = 0.3 nM) and competes with it in platelet activation, while TLCK-thrombin does not bind to the high-affinity site accessible to α-thrombin and hence cannot compete with it in platelet activation (Harmon & Jamieson, 1986a). These results have resolved long-standing questions regarding the correlation of equilibrium binding of thrombin with its ability to activate platelets [for a review, see Phillips (1985)].

Intact platelets also contain a moderate-affinity binding site (~1700 sites/platelet; K_d = 11 nM) (Harmon & Jamieson, 1986a), and in contradistinction to the high-affinity site, both PPACK-thrombin and TLCK-thrombin compete with α-thrombin in binding to the moderate-affinity site. However, the question of whether this moderate-affinity binding site is a classical receptor, capable of fully activating platelets in the absence of the high-affinity receptor, cannot be answered with intact platelets since these are fully activated by α-thrombin at concentrations well below those at which antagonism by TLCK-thrombin at the moderate-affinity binding site would be observed. The low-affinity binding of α-thrombin to platelets is not thought to be of physiological relevance since its dissociation constant (K_d ~ 3 μM) is far above thrombin concentrations that are likely to be encountered physiologically.

In the present studies, we have removed the high-affinity thrombin receptor by treating platelets with *Serratia marcescens* protease and have shown that moderate-affinity binding identifies a receptor fully capable of activating platelets

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[1] Abbreviations: TLCK-thrombin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone treated thrombin; PPACK-thrombin, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GPIb, glycoprotein Ib; Tris, tris(hydroxymethyl)aminomethane.

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