Thrombosis and Haemostasis – © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 59 (3) 415–420 (1988)

Modification of Human Thrombin: Effect on Thrombomodulin Binding

E. Anne Thompson and Hatem H. Salem

From the Department of Medicine, Monash Medical School, Prahran, Victoria, Australia

Key words

Thrombin – Chemical modification – Thrombomodulin binding

Summary

Thrombomodulin, an endothelial cell protein, binds thrombin with high affinity and alters thrombin from a procoagulant to an anticoagulant molecule. In this study, chemical and/or proteolytic modification of thrombin was carried out to identify the essential components required for its interaction with thrombomodulin.

Modification of thrombin at the catalytic site serine and histidine residues, with Diisopropylfluorophosphate and Tosyl-L-lysine chloromethyl ketone, resulted in loss of clotting and amidolytic activity. Both Diisopropyl phosphoryl-thrombin and Tosyl-L-chloromethyl ketone-thrombin inhibited native-thrombin:thrombomodulin catalyzed protein C activation with K_i values of 5 nM and 6 nM respectively indicating no loss of affinity for thrombomodulin.

Oxidation of tryptophan residues with N-bromosuccinimide or iodination of tyrosine residues of thrombin led to reduced clotting and amidolytic activity as well as a reduced ability to interact with thrombomodulin. Modification of arginine residues with Phenylglyoxal and 2,3,Butanedione led to loss of thrombomodulin binding affinity. Limited proteolysis of thrombin by trypsin yielded the derivative β-thrombin which had also lost its ability to interact with thrombomodulin. Deglycosylation of thrombin did not alter its binding affinity for thrombomodulin.

These results indicate that one or more tryptophan, arginine and tyrosine residues are essential for the recognition of thrombin by thrombomodulin whilst the carbohydrate side chain and the active site residues of the thrombin molecule are not involved in thrombomodulin binding.

Introduction

Thrombin plays an important central role in haemostasis, interacting with a wide variety of plasma components such as fibrinogen and clotting factors V, VIII and XIII and with most cell types so far examined with the exception of the mammalian red cell (1). Although thrombin's catalytic mechanism is similar to that of other serine proteases, it exhibits a remarkable degree of specificity cleaving only a limited number of peptide bonds (2, 3).

The thrombin molecule is comprised of an A chain linked by a disulphide bond to the B chain. The B chain contains the active site residues (SER, ASP, HIS). Thrombin also contains an exosite (completely independent of the catalytic site) involved in fibrinogen and platelet binding. Although, several amino acids, namely TYR, TRP, ARG and LYS have been shown to be essential components of the binding reaction, the exact location of this macromolecular binding domain has not been identified (4–6).

Thrombomodulin, an endothelial cell surface protein, binds to thrombin with high affinity. The binding of thrombin to thrombomodulin, results in significant changes in its catalytic properties which includes: (A) 1,000 fold increase in the rate of protein C activation (7); (B) reduction in the clotting and platelet activation ability (8, 9); (C) inhibition of cleavage of protein S (10); (D) inhibition of activation of factors V and XIII (9, 11) and (E) reduced inactivation of thrombin by antithrombin III (12).

The aim of the present study was to identify components of the thrombin molecule essential for its interaction with thrombomodulin. For this purpose, thrombin was chemically and proteolytically modified and the ability of the modified thrombin to bind thrombomodulin examined.

Materials and Methods

Materials

Chemicals were purchased from the following suppliers: Tris, Ammonium Persulphate, Bovine Serum Albumin (BSA), N-Bromosuccinimide, Phenylglyoxal, TPCK-treated Trypsin, B-Galactose dehydrogenase, B-Galactosidase, Neuraminidase, N_α-p-Tosyl-L-Lysine chloromethyl ketone (TLCK), 2,3,Butanedione, NAD^+, 2-Mercaptoethanol, Coomassie Brilliant Blue R and Di-isopropylfluorophosphate (DFP) (Sigma Chemical Co., St Louis, USA), Sodium Chloride and Calcium Chloride (Ajax Chemicals, Melbourne, Australia), Acrylamide, Amberlite CG50 resin and Affigel-10 (Biorad Laboratories Co., Richmond, USA), Sodium Dodecyl Sulphate and Iodobeads (Pierce Chemical Co., Rockford, USA), D-Phe-pipecolyl-Arg-p-nitroanilide (S2238) (Kabi Diagnostica, Stockholm, Sweden), and Nonidet (NP40) (BDH Chemicals LTD., Poole, England).

Methods

All proteins used were of human origin. Thrombomodulin was prepared by a modification of the previously published procedure, replacing the DEAE-Sepharose chromatography by a second DFP-thrombin affinity chromatography (7).

Protein C, fibrinogen, antithrombin III and α-thrombin were prepared as indicated (13–16). α-thrombin had a specific activity of 4435 U/mg.

Protein concentrations were determined by Biorad Protein Assay according to the manufacturers instructions.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (17) using a 4% stacking gel and 12.5% acrylamide in the running gel. Samples were boiled for 5 min in 0.25 M Tris buffer, pH 6.8 containing 0.1% SDS (and 5.0% (v/v) 2-mercaptoethanol for reduced samples) prior to application onto the gel. After electrophoresis, the gel was fixed, stained with coomassie brilliant blue R and dried.

This project was supported by grants from the NHMRC (Australia). E. A. Thompson is holder of a Commonwealth Postgraduate Research Award and this study forms part of her PhD thesis.

Abbreviations:
DFP – Diisopropylfluoro phosphate, DIP – Diisopropyl phosphoryl, NBS – N-bromosuccinimide, TLCK – Tosyl-L-lysine chloromethyl ketone

Correspondence to: E. A. Thompson, Katholieke Universiteit Leuven, Departement Medische Navorsing, Campus Gasthuisberg, Onderwijs & Navorsing, Herestraat, B-3000 Leuven, Belgium

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Assays of Enzymatic Activity of Thrombins

(A) Clotting activity. The clotting activity of the control and modified thrombins was measured in a fibrinogen clotting assay. The thrombin sample (200 µl), diluted in 0.02 M Tris buffer containing 5 mg/ml BSA and 10 mM $CaCl_2$, was placed in a coagulator cup. Fibrinogen (100 µl, 200 µg/ml final concentration) was added and the clot time recorded. Thrombin activity was determined by reference to a standard thrombin preparation (the standard thrombin was a generous gift from Dr. D. Aronson, NIH, USA). Assays were performed in duplicate at two dilutions.

(B) Amidolytic activity. This was determined by measuring the rate of hydrolysis of the chromogenic substrate S2238 at 405 nm. The amidolytic reaction (600 µl) contained 0.2 mM S2238 in 20 mM Tris buffer, pH 7.4 containing 0.15 M NaCl and 5 mg/ml BSA. The concentration of thrombin present was quantitated by reference to a standard curve constructed using known amounts of thrombin.

Ability of the Thrombins to Bind Thrombomodulin

The thrombin-thrombomodulin interaction was evaluated by using DIP-thrombin or modified-DIP-thrombin as a competitive inhibitor of protein C activation by native thrombin in the presence of thrombomodulin.

In this assay, 1.0 µM protein C was incubated at 37° C for 15 min with varying concentrations of native thrombin and thrombomodulin in the presence or absence of the modified thrombins. The total volume of reaction mixture was 30 µl and the buffer used was 20 mM Tris, pH 7.4 containing 0.15 M NaCl, 5 mg/ml BSA and 2.5 mM $CaCl_2$. Protein C activation was stopped by addition of antithrombin III/heparin (0.35 mg/ml and 1.4 U/ml final concentrations respectively) and the incubation continued for a further 5 min. The amount of activated protein C generated was assayed by measuring the rate of hydrolysis of S2238. The reaction mixture contained 0.2 mM S2238 in 20 mM Tris, pH 7.4 containing 0.15 M NaCl and 5 mg/ml BSA. The concentration of activated protein C formed was determined by reference to a standard curve constructed using known amounts of activated protein C.

The data was plotted as a Dixon plot of 1/rate of protein C activation (nM/min) versus the inhibitor concentration to obtain a K_i value.

Preparation of Chemically Modified Thrombins

In all functional studies, modified thrombin was compared to native thrombin treated in an identical manner but without exposure to the modifying agent. After the modification, the thrombin was dialyzed extensively against 20 mM Tris buffer, pH 7.4 containing 0.15 M NaCl at 4° C.

(a) DIP-thrombin. DFP (1 mM final concentration) was incubated with thrombin (26.3 µM) at 37° C for 1 h.

(b) TLCK-thrombin. Thrombin (2.6 µM) was incubated with TLCK (9 mM final concentration) in 50 mM potassium phosphate, pH 7.0 for 2 h at room temperature.

(c) NBS-treated-thrombin. Thrombin (4.4 µM) and DIP-thrombin (2.6 µM) were dialyzed against 50 mM sodium acetate, pH 5.6 containing 0.15 M NaCl and then treated with different concentrations of NBS (5.5–44 µM) at room temperature for 30 min. The change in absorbance at 280 nm was monitored throughout the incubation and the number of tryptophan residues oxidized determined using the formula:

$n = \Delta OD_{280} \times 1.31 \times MW \times \text{Volume (ml)}/[\text{Mass (mg)} \times 5{,}500]$

where n is the number of tryptophan residues oxidized, 1.31 is an empirical factor to correct for absorbance at 280 nm of the oxidation product of tryptophan (oxindole), MW is the molecular weight of thrombin (38,000), Volume is the volume of the sample, Mass is the amount of thrombin in the sample (0.10 or 0.17 mg) and 5,500 is the extinction coefficient at 280 nm for tryptophan (18).

(d) Phenylglyoxal-treated-thrombin. Thrombin (2.6 µM) and DIP-thrombin (2.6 µM) were incubated for 16 h at room temperature with phenylglyoxal (1–10 mM final concentration) in 20 mM sodium tetraborate buffer, pH 8.5 containing 0.15 M NaCl.

(e) 2,3,Butanedione-treated-thrombin. Thrombin (2.6 µM) and DIP-thrombin (2.6 µM) were incubated for 16 h at room temperature with 2,3,Butanedione (25 mM final concentration) in 20 mM sodium tetraborate buffer, pH 8.5 containing 0.15 M NaCl.

(f) Iodinated-thrombin. Thrombin (2.6 µM) and DIP-thrombin (2.6 µM) were incubated for 20 min with an iodobead and potassium iodide (6–120 µM) at room temperature.

(g) Deglycosylated-thrombin. Thrombin (25 µM) and DIP-thrombin (25 µM) were incubated with Neuraminidase (0.5 U/ml final concentration) or Neuraminidase and B-galactosidase (1,000 U/ml) in 50 mM sodium acetate buffer, pH 5.6 containing 0.15 M NaCl for 5 h at room temperature. At the end of this period, aliquots were removed for assay of sialic acid, galactose residues and SDS-PAGE analysis. Free N-acetyl-neuraminic acid was measured by the thiobarbituric acid method of Warren (19) and free galactose quantitated by the extent of NAD^+ reduction after the addition of B-galactose dehydrogenase as described by Finch et al. (20).

(h) Trypsin cleaved-thrombin. TPCK-treated Trypsin (3.3 mg) was coupled to 4.5 ml Affigel-10 (according to the manufacturer's instructions) to a final concentration of 0.6 mg/ml of gel. Thrombin and DIP-thrombin (19.5 µM, 1 ml) were incubated with 0.5 ml of trypsin-Affigel-10 for 16 h at 4° C. The thrombin was separated from the beads by centrifugation, and the sample applied to an ion-exchange chromatography column (Amberlite CG 50 resin). Trypsin-cleaved-thrombin eluted at 0.3 M NaCl whilst intact thrombin eluted at 0.75 M NaCl. Samples were concentrated by lyophilization and resuspended in 20 mM tris buffer, pH 7.4 containing 0.15 M NaCl.

Results

DFP and TLCK Treated-Thrombin

The reaction of thrombin with DFP or TLCK was accompanied by a complete loss of clotting activity and >2,000 fold decrease in amidolytic activity. Fig. 1 shows the Dixon plots obtained when TLCK-thrombin, or DIP-thrombin, was added to the protein C activation assay. The K_i for TLCK-thrombin was 6 nM, similar to that of DIP-thrombin (5 nM). These values are similar to the K_m of thrombin for thrombomodulin reported by Owen and Esmon (21). These results indicate that alkylation of histidine residues or the blocking of the serine active site are not associated with any reduction in the affinity of thrombin for thrombomodulin.

NBS-Treated-Thrombin

The reagent, NBS, has been shown to specifically react with tryptophan at acid pHs (22). The reaction of thrombin with NBS (5.5 to 44 µM) was accompanied by the gradual oxidation of eight tryptophan residues and loss of both fibrinogen clotting and amidolytic activity (Fig. 2). A slight difference in the extent of loss of clotting and amidolytic activity was observed. When eight tryptophan residues were oxidized, there was complete loss of clotting activity although 20% of amidolytic activity remained.

Table 1 shows the results of kinetic studies using NBS-treated DIP-thrombin in a protein C activation assay. The K_i values for each sample were obtained from Dixon plots using two concentrations of thrombin : thrombomodulin complex. As the tryptophan residues were oxidized, there was a gradual reduction in the affinity of NBS-treated DIP-thrombin for thrombomodulin. This was reflected by a greater than 30 fold increase in the K_i value of the modified thrombin to inhibit protein C activation.

Phenylglyoxal-Treated-Thrombin

Phenylglyoxal hydrate reacts primarily with arginine residues although it can also deaminate free amino acids and the N-terminal amino group of peptides (23). Treatment of thrombin with phenylglyoxal (1–10 mM) representing a 300 to 3,000 fold excess of reagent to thrombin, produced a dramatic loss of clotting activity with only 1.4% of residual activity remaining using 10 mM phenylglyoxal (Fig. 3). In contrast, thrombin's

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amidolytic activity was reduced to a lesser extent, with still 31% activity present following treatment with 10 mM phenylglyoxal.

The affinity of phenylglyoxal-treated DIP-thrombin for thrombomodulin was also decreased. The control DIP-thrombin had a K_i value of 5.7 nM and following treatment with 2.0 mM phenylglyoxal, the K_i value for the DIP-thrombin increased approximately two fold. At higher phenylglyoxal concentrations, the K_i increased by 15 fold to 80.0 nM (Table 2).

2,3,Butanedione-Treated-Thrombin

This reagent causes modification of arginine residues and to a lesser extent lysine residues (24). Following treatment with 25 mM 2,3,butanedione, thrombin's clotting activity decreased by 99% whilst the amidolytic activity dropped to 51% of the control value. The K_i of 2,3,butanedione-treated-DIP-thrombin for the inhibition of protein C activation was 175 nM indicating a 30 fold drop in the affinity of the modified thrombin for thrombomodulin.

Iodinated-Thrombin

Iodination of thrombin with 6 μM potassium iodide did not alter the clotting or amidolytic activity of thrombin (Table 3). As the concentration of potassium iodide was increased, the clotting and amidolytic activity of thrombin progressively decreased. The affinity of DIP-thrombin iodinated with 36 μM potassium iodide for thrombomodulin, as measured by inhibition of protein C activation, was reduced four fold that of control DIP-thrombin and DIP-thrombin iodinated with 6 μM potassium iodide. At higher potassium iodide concentrations (120 μM), a further reduction in affinity for thrombomodulin was observed (K_i = 84.1 nM).

Deglycosylated-Thrombin

After five hours incubation with neuraminidase and B-galactosidase, 2 moles of sialic acid residues and 2 moles of galactose were released per mole of thrombin. Deglycosylation was evident as a 2,500 molecular weight reduction on SDS-PAGE (Fig. 4) and caused no change in the amidolytic or clotting activity of thrombin.

When deglycosylated DIP-thrombin was compared to DIP-thrombin in its ability to interact with thrombomodulin, similar K_i values were obtained.

Trypsin-Cleaved Thrombin

Controlled incubation of thrombin and DIP-thrombin with trypsin-agarose led to cleavage of the B chain of thrombin yielding several smaller molecular weight fragments. Although we have not determined the exact localization of the trypsin cleavage site, Boissel et al. showed that trypsin cleaved thrombin at two sites in the region of residues ARG 62 to ARG 73 of the B chain to form the derivative β-thrombin. β-thrombin consisted of the A chain (6 kDa) linked to a B chain fragment (B2) which had a molecular weight of 21 kDa (25).

In our experiments, trypsin cleavage resulted in the formation of a major thrombin derivative of 23 kDa under reducing conditions which is similar to the molecular weight of the B2 fragment described by Boissel et al. (25). Some uncleaved thrombin and several smaller molecular weight peptides were also observed (Fig. 5, lane 3). β-thrombin was separated from the other molecules by ion exchange chromatography and lane 4 shows the reduced SDS-PAGE of the purified material.

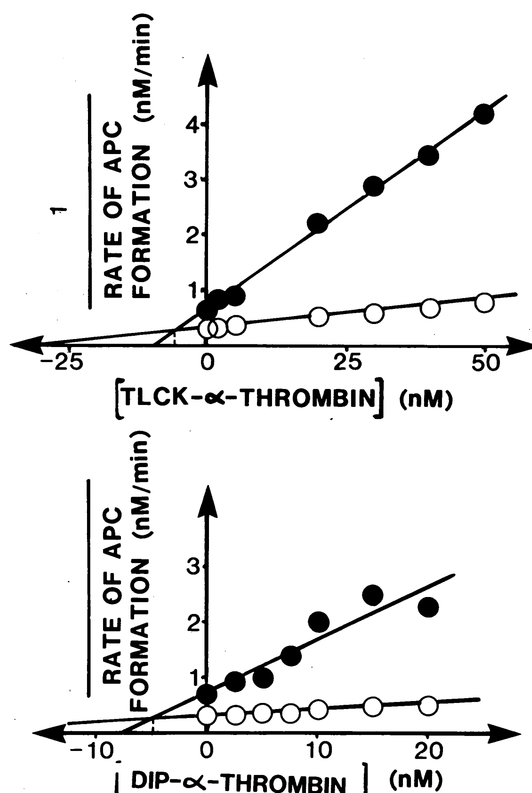

Fig. 1 The binding of TLCK-thrombin and DIP-thrombin to thrombomodulin. The interaction of TLCK-thrombin and DIP-thrombin with thrombomodulin was assessed using a competitive inhibitor assay system (see Methods section). TLCK-thrombin (0–50 nM) and DIP-thrombin (0–20 nM) were added to an incubation mixture containing either 2.5 nM ● or 5.0 nM ○ native thrombin:thrombomodulin complex. Protein C (1 μM) was added and incubated for 15 min at 37° C. The amount of activated protein C generated was assayed and the data plotted as a Dixon plot. The dotted line from the intercept of the two lines meets the x-axis at the -K_i value

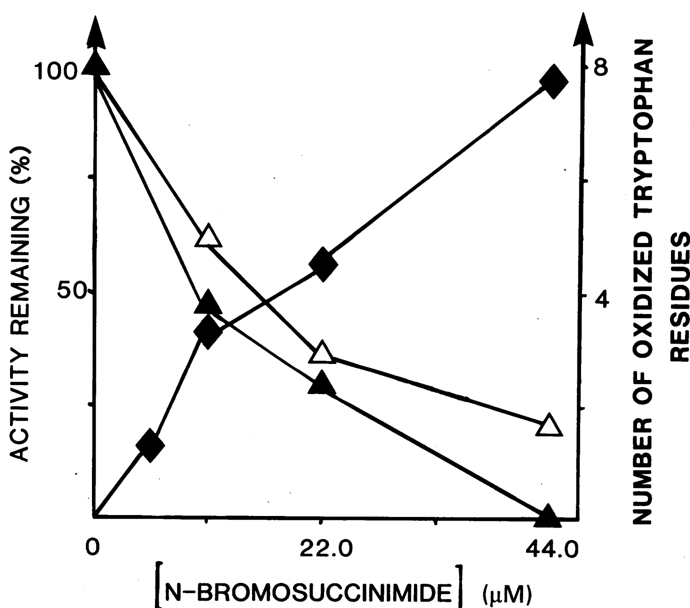

Fig. 2 Effect of NBS on thrombin activity. Thrombin (4.4 μM) was treated with NBS (0–44 μM) at room temperature for 30 min and then dialyzed extensively. The number of tryptophan residues oxidized ◆ and the residual clotting ▲ and amidolytic △ activity remaining in the dialyzed NBS-treated sample were measured as described in the Methods section

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Table 1 Effect of tryptophan oxidation of DIP-thrombin by NBS on its ability to interact with thrombomodulin

Concentration of NBS (μM)	No. of tryptophan residues oxidized/mole DIP-thrombin	K_i (nM)
0.0	0.0	7.0
11.0	3.3	33.0
22.0	4.6	62.5
44.0	7.9	200.0

DIP-thrombin (2.6 μM) was modified by NBS (0–44 μM) for 30 min at room temperature. The number of tryptophan residues oxidized and the effect of modification on thrombomodulin binding affinity was determined as described in the methods section.

Table 2 Effect of phenylglyoxal treatment of DIP-thrombin on its ability to interact with thrombomodulin

Concentration of phenylglyoxal (mM)	K_i (nM)
0.0	5.7
1.0	13.7
2.0	10.0
5.0	77.5
10.0	80.0

DIP-thrombin (2.6 μM) was incubated with phenylglyoxal (0–10 mM) for 16 h at room temperature and following dialysis, the effect of this treatment on DIP-thrombin's ability to interact with thrombomodulin was assessed as described in the methods section.

In comparison to α-thrombin, β-thrombin exhibited a 4% increase in amidolytic activity but had no detectable clotting activity. β-thrombin prepared from DIP-thrombin demonstrated significant loss in binding affinity to thrombomodulin with a K_i of 146 nM (Fig. 6).

Discussion

Thrombin plays a central bioregulatory role in haemostasis. Besides catalyzing the conversion of fibrinogen to a fibrin clot, thrombin also interacts with several other clotting factors, platelets and endothelial cells (2).

Thrombomodulin, an endothelial cell surface protein, binds to thrombin causing an alteration in its conformation and its biological functions (7–12). Thrombin bound to thrombomodulin is a potent protein C activator, but shows great reduction in its affinity for fibrinogen and platelets. Previous studies have documented that chemical modification of TYR, TRP, ARG and LYS inhibits the interaction of thrombin with fibrinogen and platelets (4–6). Based on these observations we can postulate that modification of one or all of these amino acids might block the recognition of thrombin by thrombomodulin.

This paper describes studies performed to identify components of the thrombin molecule essential for its interaction with thrombomodulin.

Chemical modification of the active site SER and HIS residues did not alter recognition of thrombin by thrombomodulin. Modification of TRP residues with N-bromosuccinimide, of ARG residues with phenylglyoxal and 2,3,butanedione and TYR

Table 3 Effect of iodination on thrombin's clotting and amidolytic activity and the ability of iodinated DIP-thrombin to interact with thrombomodulin

Potassium iodide concentration (μM)	% clotting activity remaining	% amidolytic activity remaining	K_i (nM)
0.0	100	100	7.0
6.0	100	100	7.4
36.0	39	84	29.6
120.0	6	16	84.1

Thrombin and DIP-thrombin (2.6 μM each) were incubated with an iodobead and potassium iodide (0–120 μM) at room temperature for 20 min. Changes in thrombin's clotting and amidolytic activity following modification were determined as described in the methods section. The effect of iodination on DIP-thrombin's ability to interact with thrombomodulin was assessed as described in the methods section.

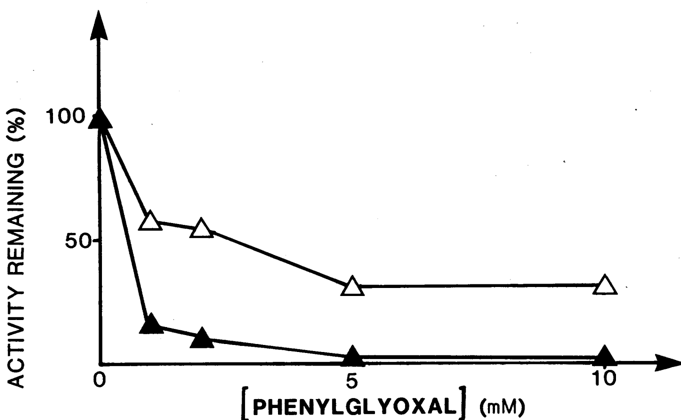

Fig. 3 Effect of phenylgloxal on thrombin activity. Thrombin (2.6 μM) was treated with Phenylglyoxal (0–10 mM) for 16 hours at room temperature and then dialyzed extensively. The percentage of clotting ▲ and amidolytic △ activity remaining in the samples was measured as described in the Methods section

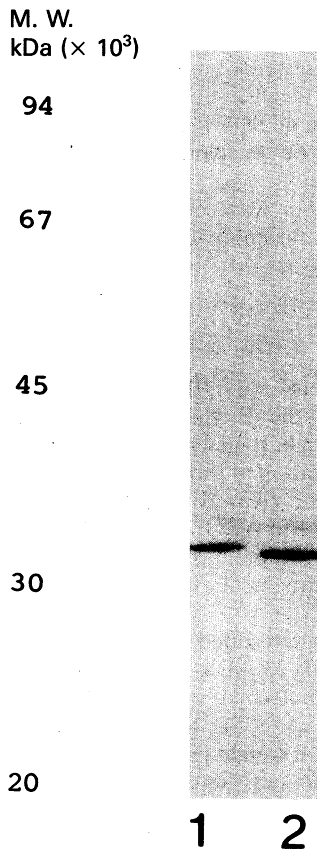

Fig. 4 Native and deglycosylated thrombin. Reduced SDS-PAGE of thrombin samples. Lane 1 is native thrombin and lane 2 is thrombin following deglycosylation with neuraminidase and B-galactosidase

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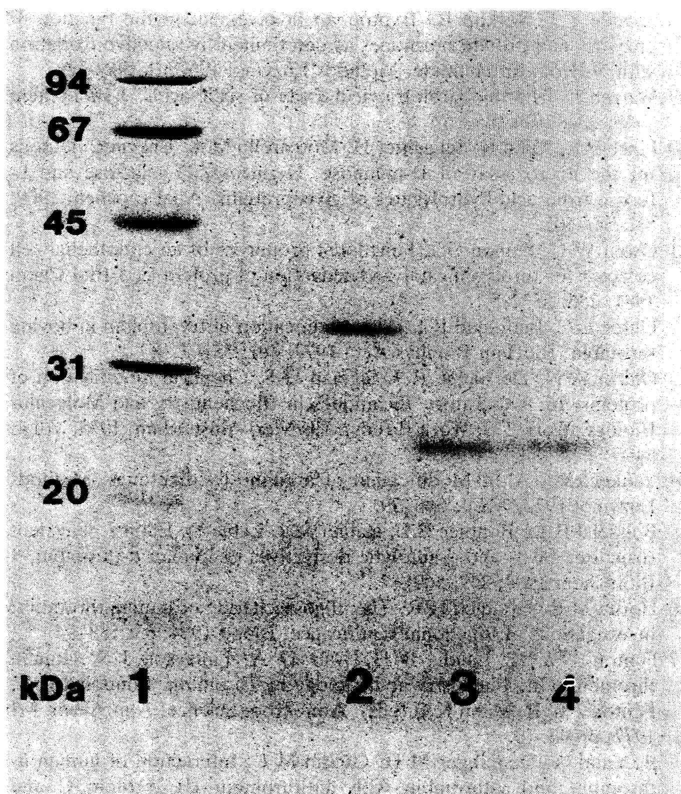

Fig. 5 Effect of trypsin cleavage on thrombin. Reduced SDS-PAGE of thrombin samples. Lane 1 is the protein standards, Lane 2 is native thrombin, lane 3 is thrombin after 16 hour incubation at 4° C with trypsin, lane 4 shows the isolated β-thrombin

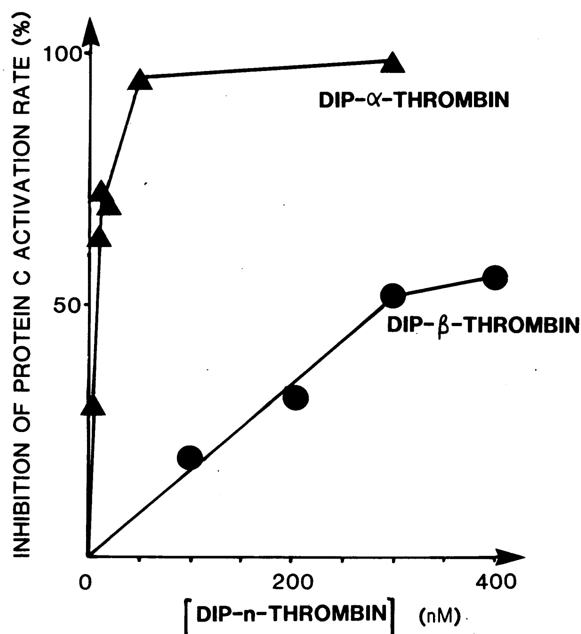

Fig. 6 Ability of DIP-α-thrombin and DIP-β-thrombin to interact with thrombomodulin. The interaction of DIP-α-thrombin and DIP-β-thrombin with thrombomodulin was assessed using a competitive inhibitor assay (see Methods section). The effect of inhibitor concentration (0–300 nM) on the protein C activation rate by native thrombin: thrombomodulin complex was expressed as a percentage of the control rate, obtained in the absence of inhibitor. DIP-α-thrombin ▲, DIP-β-thrombin ●

residues with iodine caused a loss in the ability of thrombin to bind thrombomodulin as well as reduction in clotting and amidolytic activities.

The reagent, 2,3,butanedione primarily modifies ARG but can also modify LYS residues to a lesser extent (24). To examine if modification of lysine residues was contributing to the loss of thrombomodulin recognition observed with 2,3,butanedione, a lysine specific modifier, ethylacetimidate was employed (6). Treatment of DIP-thrombin with ethylacetimidate had no effect on the recognition of thrombomodulin by DIP-thrombin (data not shown). These results suggest that the effects of 2,3,butanedione on the thrombin : thrombomodulin interaction are primarily mediated through arginine.

Previous studies have reported that deglycosylation of thrombin does not change its following activities: fibrinogen clotting, activation of factor VIII, inhibition by defibrinated plasma and stimulation of platelet release and aggregation (26). Similarly, we found that deglycosylation of thrombin did not alter its binding affinity for thrombomodulin. Thus, the carbohydrate moiety appears to be located outside the major substrate binding regions of the molecule.

Autolysis or treatment of thrombin with trypsin leads to the formation of two derivatives referred to as β- and γ-thrombin. These derivatives have reduced ability to clot fibrinogen, and activate platelets whilst their esterolytic and amidolytic activity is retained (27). In a number of respects, these features are similar to thrombin when bound to thrombomodulin (8, 9). Hence, one would have expected that the regions cleaved during the formation of these derivatives constitute the thrombomodulin binding domain. Bezeaud and coworkers showed a marked reduction in thrombomodulin binding to γ-thrombin (28). This thrombin derivative consists of three small fragments (25) but Bezeaud and coworkers did not explore whether larger fragments of thrombin due to formation of β-thrombin, precursor of γ-thrombin, retain the thrombomodulin binding site.

Our results showed that β-thrombin had lost its ability to displace thrombin from thrombomodulin in the protein C activation assay. These results suggest that the thrombomodulin recognition site resides in the amino terminal fragment (B1) of the B chain of thrombin. Further studies are required to delineate its exact localization.

These studies have outlined several important moieties in the thrombin molecule required for thrombomodulin recognition. Mutations involving any of these components could theoretically result in forms of thrombin that have reduced ability to bind thrombomodulin and hence lead to inefficient protein C activation and a propensity to thrombosis. Studies on abnormal forms of prothrombin should help clarify if such a situation does exist.

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Received October 19, 1987 Accepted after revision January 27, 1988

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