A Thrombin-based Peptide Corresponding to the Sequence of the Thrombomodulin-binding Site Blocks the Procoagulant Activities of Thrombin*

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Thrombomodulin, a cofactor in the thrombin-catalyzed activation of protein C, blocks the procoagulant activities of thrombin such as fibrinogen clotting, Factor V activation, and platelet activation. The binding site for thrombomodulin within human thrombin has been localized at a region comprising residues Thr147-Ser158 of the B-chain of thrombin. The dodecapeptide sequence, TWTANVGKGQPS, corresponding to these residues inhibits thrombin binding to thrombomodulin with an apparent Kt, = 94 μM (Suzuki, K., Nishioka, J., and Hayashi, T. (1990) J. Biol. Chem. 265, 13263–13267). We have found that the inhibitory effect of the dodecapeptide on the thrombin-thrombomodulin interaction is sequence-specific, and that residues Asn151, Lys154, and Glu158 are essential for thrombomodulin binding. The dodecapeptide was also found to directly block thrombin procoagulant activities, fibrinogen clotting (concentration for half-maximum inhibition, 385 μM), Factor V activation (concentration for half-maximum inhibition, 33 μM), and platelet activation (concentration for half-maximum inhibition, 645 μM). This peptide did not block thrombin inhibition by anti-thrombin III, but blocked thrombin inhibition by hirudin. These findings suggest that the binding site for thrombomodulin in thrombin is shared with the sites for fibrinogen, Factor V, platelets, and hirudin, and that, therefore, the inhibition of thrombin procoagulant activities by thrombomodulin in part results from blocking of the interaction between thrombin and the procoagulant protein substrates by thrombomodulin.

Thrombin is the most important procoagulant serine protease, converts fibrinogen to fibrin, and activates Factor V, Factor VIII, Factor XIII, and platelets (1). In binding to its receptor on the vascular endothelium, thrombomodulin, thrombin also plays a role as an anticoagulant enzyme. Thrombin bound to thrombomodulin activates protein C, which inhibits coagulation by inactivation of Factors Va and VIIIa (2, 3). Furthermore, thrombomodulin directly blocks the procoagulant activities of thrombin such as fibrinogen clotting, Factor V activation (4), and platelet activation (5).

The change in substrate specificity brought about by thrombomodulin is presumed to arise as a result of a conformational change around the active center of thrombin (6).

Noé et al. (7) suggested that a region comprising residues Arg62 to Arg72 of the B-chain of thrombin interacts with thrombomodulin as well as with fibrinogen and hirudin from evidence that a monospecific antibody raised to a peptide composed of residues Arg62 to Arg72, RICKHSRTYER, inhibits thrombin-catalyzed activation of protein C in the presence of thrombomodulin. We previously localized the thrombomodulin-binding site within thrombin to a region that corresponds to residues Thr147 to Ser158 of the B-chain, and a dodecapeptide synthesized from this region, TWTANVGGKQPS, was found to block the interaction between thrombin and thrombomodulin with an apparent Kt, = 94 μM (8). In the present study, we have examined the effects of several homologous dodecapeptides on the thrombin-thrombomodulin interaction. We also examined the effects of the peptides on the procoagulant activities of thrombin to elucidate how the binding of thrombomodulin modulates the procoagulant activities of thrombin. The results imply that thrombomodulin directly blocks the interaction between thrombin and the procoagulant protein substrates.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—All chemicals used were of the highest commercial grade available. Protein C (2), n-thrombin (2,500 units/mg) (9), Factor V (10), prothrombin (11), Factor X (11), and anti-thrombin III (12) were all purified from human plasma as described previously. Recombinant soluble thrombomodulin, which is composed of an NH2-terminal domain, six epidermal growth factor-like structural domains, and an O-glycosylation site-rich domain, was prepared as described (13). Human fibrinogen was obtained from Daichi Pure Chemicals, Tokyo. Recombinant hirudin was provided from Mitsu Toatsu Chemicals, Tokyo. The phospholipid suspension was prepared using bovine brain extract (type III) from Sigma. The chromogenic substrate for thrombin, H-β-Phe-Pip-Apg-pNA (S-2238) was obtained from Kabi, Sweden. Chemicals for peptide synthesis were obtained from Applied Biosystems.

Preparation of Peptides—Synthetic peptides FRKSPQELL, RGKHCSRTERY, TWTANVGGKQPS, DSTRIRI, EGDSSGP, and SWGEGCDRDGK, respectively corresponding to sequence residues Phe49 to Leu17, Arg62 to Arg72, Thr147 to Ser158, Asp159 to Ile164, Glu62 to Ser726 to Lys154 of the B-chain of thrombin, and SPQGKGNATAWT which is the inverted sequence between residues Thr147 and Ser158, were produced by the solid phase method using an Applied Biosystems Model 431A peptide synthesizer. Other dodecapeptides, TWTANVGGKQPS, TWTANVGGKQPS, TWTANVGGKQPS, and TWTANVGGKQPS, which were homologous to TWTANVGGKQPS, were also synthesized by the same method. α-Butoxycarbonyl-protected amino acids were converted to the 1-hydroxybenzotriazole active ester and sequentially coupled to the peptide resin (14).
Antithrombin Activity of Thrombin-based Peptides

Effects of Peptides on Thrombin-inducing Fibrinogen Clotting—We then examined the effects of the peptides, TWTANVGKQGPS, RIGKHSRTRYER, DSTRIRI, and EGDSGGP, on thrombin-induced fibrinogen clotting. As shown in Fig. 1, only EGDSGGP did not effectively prolong the clotting time. The concentration at which half-maximum inhibition occurred by the most effective peptide, TWTANVGKQGPS, for thrombin-induced fibrinogen clotting was 385 μM, which was obtained from a double reciprocal plot for the same data in Fig. 1. Inhibition constants for RIGKHSRTRYER and DSTRIRI were unobtainable, since inhibition by both peptides was unsaturable up to 1 mM.

Effects of Peptides on Thrombin-induced Factor V Activation—As shown in Fig. 2, activation of Factor V depended on the sequence of TWTANVGKQGPS inverted, on thrombin binding to thrombomodulin was examined. As shown in Table II, replacement of a neutral amino acid residue (Ala150 → Pro or Val152 → Gly) in the original dodecapeptide did not change the inhibitory activity of the peptide. However, the activity was significantly reduced when a basic amino acid residue was replaced with an acidic residue (Asn151 → Asp, Lys154 → Glu, or Gin156 → Glu). The peptide with the inverted sequence also displayed diminished activity on thrombin binding to thrombomodulin.

RESULTS

Effects of Peptides on Thrombin Binding to Thrombomodulin—Of the peptides derived from the B-chain of thrombin, FRKSPQELL, RIGKHSRTRYER, TWTANVGKQGPS, DSTRIRI, EGDSGGP, and SWGEGCDRDGK, regions corresponding to the sequences of the latter three peptides are estimated to be located near the active center pocket of thrombin in a three-dimensional model (15), only TWTANVGKQGPS inhibited thrombin binding to thrombomodulin (Table I). To elucidate the amino acids in the TWTANVGKQGPS sequence essential for thrombin-thrombomodulin interaction, the effects of five homologous synthetic peptides, TWTADVVGKQGPS, TWTANVGEGQGPS, TWTANVGKKGPS, TWTANVNGKGQGPS, and TWTANVGKQGPS, and SPQKGVNATWT, which is a peptide with the sequence of TWTANVGKQGPS inverted, on thrombin binding to thrombomodulin were determined as described previously (8). Amino acids replaced in the peptide are underlined.

| Peptide | Amino acid sequence | Inhibition constant (μM) |
|---------|----------------------|-------------------------|
| Th184 - Ser154 | TWTANVGKQGPS | 97 |
| Ala150 → Pro | TWTANVGKQGPS | 75 |
| Asn151 → Asp | TWTANVGKQGPS | >1000 |
| Val152 → Gly | TWTANVGKQGPS | 82 |
| Lys154 → Glu | TWTANVGKQGPS | >1000 |
| Gin156 → Glu | TWTANVGKQGPS | >1000 |
| SPQKGVNATWT | >1000 |

Apparent inhibition constants (KJ) of peptides derived from thrombin B-chain for thrombin binding to thrombomodulin

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C judgments by reverse-phase HPLC and amino acid analysis, which was coupled to the α-amino deprotected resin. The side chain protection used was: Asp(OBzI), Lys(C12), Ser(Bzl), Trp(CH0), Tyr(BrZ), and Glu(OBzI). Tp deprotection was performed using 1 M piperidine in dimethylformamide for 90 min at 0 °C, and the peptide was cleaved from the resin using a mixture of HF:anisole:diamin sulfide (10:1:1) for 2 h at rt. The peptide was purified by reverse-phase preparative HPLC on a YMC-R-ODS-S 120A (4.9 × 300 mm) column (Yamamura Chemical Laboratories, Tokyo) using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The peptides were at least 95% pure as judged by reverse-phase HPLC and amino acid analysis, which was performed after hydrolysis in HCl for 2 h at 165 °C, using the Waters Pico Tag System (Millipore). The concentrations of peptides were determined by quantitative amino acid analysis of concentrated peptide solutions prior to dilution for assay.

Assays for Effects of Peptides on Thrombin Functions—Effects of peptides on several functions of thrombin were determined by the following methods.

Binding of thrombin to thrombomodulin fixed in microwell plates and apparent inhibition constants (KJ) of peptides in the thrombin-thrombomodulin interaction were determined as described previously (8).

Thrombin-induced fibrinogen clotting time was determined using an assay using KC-10 instrument as follows. Fifty μl of thrombin (3.5 μg/ml) in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM CaCl2, was mixed with 10 μl of various concentrations of peptide. After a 2-min incubation at 37 °C, 100 μl of fibrinogen (160 μg/ml) in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 50 mM CaCl2, containing 5 mM CaCl2. Thereafter, 5 μl of Factor Xa (1.1 ng/ml) in the same buffer was added to the mixture and incubated for 3 min. The reaction was terminated by the addition of 250 mM EDTA, then 500 μl of 200 μM H-D-Phe-Pip-Arg-pNA was added and incubated at 37 °C for 10 min. p-Nitroaniline liberated by the generated thrombin was determined spectrophotometrically at 405 nm. The reaction was started with 5 μl of a mixture of Factor V (1.0 μg) and various concentrations of peptide in 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl2, incubated with 5 μl of thrombin (0.1 μg/ml) at 37 °C for 5 min. One μl of the reaction mixture was then mixed with 10 μl of a mixture of prothrombin (165 μg/ml) and bovine brain phospholipids (500 μg/ml) in 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl2. Thereafter, 5 μl of Factor Xa (1.1 ng/ml) in the same buffer was added to the mixture and incubated for 3 min. The reaction was terminated by the addition of 5 μl of 250 mM EDTA, then 500 μl of 200 μM H-D-Phe-Pip-Arg-pNA was added and incubated at 37 °C for 10 min. p-Nitroaniline liberated by the generated thrombin was determined spectrophotometrically at 405 nm. Thrombin-induced platelet aggregation was determined using a Sepharose 2B gel-filtered platelet suspension, prepared according to the method of Tangen et al. (14), in a SIENCO platelet aggregometer. Two hundred μl of platelet suspension (3.2 × 10^9 cells/μl) in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl in a cuvette was mixed with 20 μl of various concentrations of peptide. Platelet aggregation was then started by the addition of 5 μl of thrombin (5 μg/ml) in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 50 mM CaCl2, and changes in transmittance (%) were measured for 1 min after thrombin addition. Zero and 100% transmittance were assigned as the platelet suspension before and after the addition of thrombin, respectively. Inhibition of thrombin by hirudin in the presence or absence of peptide was determined as follows: to 20 μl of a mixture of thrombin (0.1 μg) and various concentrations of peptide in 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl2, 10 μl of hirudin (1.72 μg/ml) was added, and the mixture was incubated at 37 °C for 15 min. Subsequently, 200 μl of 200 μM H-D-Phe-Pip-Arg-pNA was added, and, after 10 min, liberated p-nitroaniline was determined at 405 nm. Inhibition of thrombin by antithrombin III in the presence or absence of peptide was determined with H-D-Phe-Pip-Arg-pNA using a modification of the method described previously (8).
Various concentrations of peptide (indicated as final concentrations) was added to determine the clotting time. Fifty TWTANVGKGQPS, which were obtained by plotting the reaction was terminated by the addition of 5 mM CaCl₂. Changes in transmittance (%) were measured for 1 min after thrombin addition. A calibration curve for change in transmittance (%) by platelet aggregation versus concentration of treated thrombin is shown as an inset in the figure.

Effects of Peptides—Fig. 2 shows that the increase of transmittance during platelet aggregation depended on thrombin concentration (inset), and all peptides appeared to inhibit thrombin-induced platelet aggregation in proportion to the peptide concentration. DSTRIRI was particularly effective, and RIGKHSRTRYER and EGDSGGP were more effective than TWTANVGKGQPS. Maximum inhibition for platelet aggregation by DSTRIRI, RIGKHSRTRYER, EGDSGGP, and TWTANVGKGQPS were estimated to be 27%, 22%, 17%, and 15%, respectively, under the present conditions. The concentrations for half-maximum inhibition for platelet aggregation by DSTRIRI, RIGKHSRTRYER, EGDSGGP, and TWTANVGKGQPS, which were obtained by plotting the reciprocal values of the peptide concentration in Fig. 3, were 370, 515, 550, and 645 μM, respectively.

Effects of Peptides on Inhibition of Thrombin by Antithrombin III or Hirudin—None of the peptides affected the inhibition of thrombin by antithrombin III in the presence or absence of heparin (data not shown). On the other hand, as shown in Fig. 4, the amidolytic activity of thrombin was inhibited by hirudin (inset), and two peptides, RIGKHSRTRYER and TWTANVGKGQPS, blocked the inhibition of thrombin by hirudin. The inhibition constants of

![Fig. 1. Effects of peptides on thrombin-induced fibrinogen clotting. Fifty μl of thrombin (3.3 μg/ml) was mixed with 10 μl of various concentrations of peptide (indicated as final concentrations). After a 2 min incubation at 37 °C, 100 μl of fibrinogen (160 μg/ml) was added to determine the clotting time. ●, TWTANVGKGQPS; ○, RIGKHSRTRYER; ▲, DSTRIRI; □, EGDSGGP.](image)

![Fig. 2. Effects of peptides on thrombin-induced Factor V activation. Fifteen μl of a mixture of Factor V (37 μg/ml) and various concentrations of peptide (indicated as final concentrations) were incubated with 5 μl of thrombin (0.1 μg/ml) at 37 °C for 5 min. One μl of the reaction mixture was then mixed with 10 μl of a mixture of prothrombin (150 μg/ml) and bovine brain phospholipids (500 μg/ml) containing 5 mM CaCl₂. Thereafter, 5 μl of Factor Xa (1.1 ng/ml) was added to the mixture. After a 3-min incubation at 37 °C, the reaction was terminated by the addition of 5 μl of 250 mM EDTA, and p-nitroaniline liberated by the generated thrombin was determined at 405 nm. One unit (U) of Factor V is assigned as the activity of Factor V in 1 ml of human plasma. A calibration curve for Factor V activity by various concentrations of thrombin is shown as an inset in the figure. ●, TWTANVGKGQPS; ○, RIGKHSRTRYER; ▲, DSTRIRI; □, EGDSGGP.](image)

![Fig. 3. Effects of peptides on thrombin-induced platelet aggregation. Two hundred μl of gel-filtered platelets (3.2 × 10⁸ cells/μl) in a cuvette was mixed with 50 μl of various concentrations of peptide (indicated as final concentrations). Platelet aggregation was then started by the addition of 5 μl of thrombin (5 μg/ml) containing 50 mM CaCl₂. Changes in transmittance (%) were measured for 1 min after thrombin addition. A calibration curve for change in transmittance (%) by platelet aggregation versus concentration of treated thrombin is shown as an inset in the figure. ●, TWTANVGKGQPS; ○, RIGKHSRTRYER; ▲, DSTRIRI; □, EGDSGGP.](image)

![Fig. 4. Effects of peptides on thrombin inhibition by hirudin. Twenty μl of a mixture of thrombin (0.1 μg/ml) and various concentrations of peptide (indicated as a final concentration) were mixed with 10 μl of hirudin (1.72 μg/ml). After a 15-min incubation at 37 °C, 200 μl of 200 μM H-D-Phe-Pip-Arg-pNA was added to the mixture, and p-nitroaniline liberated during the 10-min reaction was measured at 405 nm. A calibration curve for residual thrombin activity treated with various concentrations of hirudin is shown as an inset in the figure. ●, TWTANVGKGQPS; ○, RIGKHSRTRYER; ▲, DSTRIRI; □, EGDSGGP.](image)
the two peptides in blocking hirudin-induced thrombin inhibition were unobtainable.

**DISCUSSION**

The modulation of the substrate specificity of thrombin from a procoagulant to an anticoagulant has been assumed on the one hand to be due to a conformational change in the active center or substrate binding sites of thrombin (6), and, on the other, to be due to steric hindrance by thrombomodulin which prevents interaction between thrombin and procoagulant protein substrates (16). In a previous study, we localized the interaction site for thrombomodulin within human thrombin, namely Thr$^{147}$ to Ser$^{158}$ of the thrombin B-chain (8).

In the present study, we found that the inhibitory effect of the peptide TWTANVKGKQPS corresponding to residues Thr$^{147}$ to Ser$^{158}$ is sequence-specific and unrelated to the amino acid composition of the peptide, since a peptide with the sequence TWTANVKGKQPS reversed did not inhibit the thrombin-thrombomodulin interaction. We also found that residues Arg$^{166}$, Lys$^{154}$, and Gln$^{156}$ in this region presumably located in the outer loop structure on the surface of the thrombin molecule (15), are essential for binding of thrombin to thrombomodulin. As thrombin has been suggested to bind to the fifth epidermal growth factor-like structure of thrombomodulin (17, 18), acidic amino acid residues in the fifth epidermal growth factor-like structure of thrombomodulin may contribute to the interaction with thrombin.

Furthermore, the present study using synthetic peptides corresponding to residues located on the surface and near the active center of thrombin, RIKHSRTRYER, TWTANVKGKQPS, DSTRIRI, and EGDSGGP, respectively, Arg$^{166}$ to Arg$^{170}$, Thr$^{147}$ to Ser$^{158}$, Asp$^{175}$ to Ile$^{181}$, and Glu$^{202}$ to Pro$^{208}$, of the B-chain, indicated that the sequence Thr$^{147}$ to Ser$^{158}$ inhibited all the procoagulant activities of thrombin: fibrinogen clotting, Factor V activation, and platelet activation, in addition to inhibiting the thrombin-thrombomodulin interaction. These findings indicate that at least one of the binding sites on thrombin for fibrinogen, Factor V, or platelets is shared with the site for thrombomodulin. These results imply that the antithrombin activity of thrombomodulin is mainly due to steric hindrance which prevents the interaction of thrombin with procoagulant protein substrates.

We also found that other peptides in addition to TWTANVKGKQPS inhibit thrombin-induced Factor V activation and platelet activation. This suggests that there are several interaction sites for Factor V and platelets near the active center of thrombin.

Recently, two interaction sites for thrombin in hirudin were revealed: one is located in the carboxyl-terminal tail region of hirudin which interacts with the region involved in residues Arg$^{14}$ to Arg$^{20}$ of the B-chain of thrombin (19), and the other is located in the amino-terminal core region of hirudin which interacts with a distinct site in thrombin (20). In addition to the report by Noé et al. (7) in which they found the blocking by hirudin of the thrombin-thrombomodulin interaction and also that by Dodt et al. (20) in which they showed that cleavage of the peptide bond at Ala$^{150}$-Asn$^{153}$ of the B-chain of α-thrombin by pancreatic elastase results in the loss of hirudin binding to thrombin, the present study suggests that another interaction site for hirudin in thrombin is probably located at the same or a proximal site for interaction with thrombomodulin. Recent studies on the crystal structure of the thrombin-hirudin complex support these observations (21, 22).

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