Effect of Active Site-Modified Thrombin on the Hydrolysis of Platelet-Associated Glycoprotein V by Native Thrombin

By Charles L. Knupp and Gilbert C. White II

To determine the relationship between equilibrium binding of thrombin to sites on the platelet surface and the cleavage of membrane glycoprotein V (GPV) by thrombin, we examined the effect of active site-modified thrombin (1-chloro-3-tosylamido-7-amino-l-2-heptanone thrombin, ToslysCH2-thrombin) on the binding of native thrombin to platelets and on the hydrolysis of GPV by native thrombin. ToslysCH2-thrombin inhibited binding of native thrombin to high affinity sites on the platelet surface. In contrast, hydrolysis of GPV by native thrombin, even at threshold thrombin concentrations, was not inhibited by pretreat-

ment with ToslysCH2-thrombin at concentrations up to 210 nmol/L. ToslysCH2-thrombin also had no appreciable effect on platelet aggregation or release of 14C-serotonin induced by native thrombin. Because ToslysCH2-thrombin does not inhibit platelet release, aggregation, or GPV hydrolysis by native thrombin but does inhibit high affinity surface binding by native thrombin, these results indicate that thrombin binding and hydrolysis of GPV are separate and unrelated events.

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MATERIALS AND METHODS

Materials

Vibrio cholerae neuraminidase, 500 U/mL, was purchased from Schwarz Mann, Cambridge, Mass. Galactose oxidase was obtained from United States Biochemical Corp., Cleveland, and was purified and assayed for activity by the method of Hatton and Regoeczi.12 Tritiated sodium borohydride (NaBH4), 5 to 15 Ci/mmol was purchased from Research Products International (Mt Prospect, Ill)

*The nomenclature used in this article is based upon that of Phillips and Agin.13 Others have used a nomenclature based on Mr by PAGE,14 ie, GPV is termed GPV 80,000.

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GPV HYDROLYSIS BY THROMBIN

Thrombin was modified with toslysCH₂Cl according to the method of Glover and Shaw except that 1×10⁻² mol/L sodium phosphate, pH 7.0, was radiiodinated, using either Enzymobeads or lodobeads as recommended by the supplier. Following iodination, thrombin was dialyzed against 0.05 mol/L sodium phosphate, pH 8.0. The radiolabeled thrombin had a specific activity of 115,000 to 180,000 cpm per unit and retained greater than 95% clotting activity after the iodination and dialysis procedures.

Thrombin was modified with toslysCH₂Cl according to the method of Glover and Shaw except that 10⁻⁴ mol/L sodium phosphate, pH 7.0, was incubated with thrombin for two hours at room temperature followed by 24 hours at 4 °C. The toslysCH₂-thrombin was then extensively dialyzed at 4 °C for 24 hours against Ca²⁺-free Tyrode’s solution, pH 7.4, to remove residual toslysCH₂Cl. Protein determination was made by the ninhydrin reaction after alkaline hydrolysis, using bovine serum albumin as a standard. Fibrinogen clotting activity was performed as previously described.

Platelet preparation. Platelets were obtained from normal healthy donors who denied recent alcohol or aspirin ingestion, as approved by the Committee on the Protection of the Rights of Human Subjects, School of Medicine, University of North Carolina at Chapel Hill. Six parts of venous blood were collected into plastic syringes containing one part acid–citrate–dextrose, pH 6.8. Platelet-rich plasma (PRP) was prepared by centrifugation of the anticoagulated blood at 100 × g for 20 minutes at room temperature. For studies of platelet aggregation, the PRP was incubated with ⁴C-serotonin (0.1 μCi/mL of platelet-rich plasma) at 37 °C for 30 minutes and gel-filtered in a plastic column of Sepharose CL-2B equilibrated with Ca²⁺-free Tyrode’s solution, pH 7.4, containing 0.0001 mol/L EDTA to separate platelets from plasma and unincorporated ⁴C-serotonin. For studies of GPV hydrolysis, PRP was gel-filtered on Sepharose CL-2B equilibrated with Ca²⁺-free Tyrode’s solution, pH 7.4, containing 0.0001 mol/L EDTA and labeled with neuraminidase, galactose oxidase, and H⁺-borohydride as described previously.

Binding assay. Thrombin binding under equilibrium conditions was measured as previously described, using washed platelet suspensions of 0.5 to 7.0 × 10⁴ platelets/mL in Ca²⁺-free Tyrode solution, pH 7.4, containing 0.0001 mol/L EDTA, with fatty acid free bovine serum albumin at a final concentration of 5 mg/mL. Two changes from the originally described method were made. First, platelets with bound ligand were separated from unbound ligand using a silicone oil barrier technique. Second, nonspecific binding was determined as thrombin bound in the presence of a 100-fold molar excess of unlabeled thrombin. Competition studies were performed by incubating unlabeled toslysCH₂-thrombin with washed platelets for three minutes prior to the addition of ¹²⁵I-thrombin.

**RESULTS**

**Binding Studies**

Iodo-[¹²³I]-thrombin and iodo-[¹²⁵I]-toslysCH₂-thrombin I125 bound to gel-filtered platelets in a similar fashion. Binding was biphasic, demonstrating both high and low affinities. Using Scatchard analysis, the number of binding sites and dissociation constants were similar for both radiolabeled thrombins. Iodo-[¹²³I]-thrombin and iodo-[¹²⁵I]-toslysCH₂-thrombin also bound to gel-filtered platelets that had been treated with neuraminidase, galactose oxidase, and cold NaBH₄. Binding was qualitatively similar to that to unmodified platelets, but the total binding and number of binding sites were reduced as compared to the unmodified platelets.
1. Binding of iodo-[^125I]-thrombin to neuraminidase–galactose oxidase–NaBH₄-treated platelets and competition by toslysCH₂-thrombin thrombin. Binding of iodo-[^125I]-thrombin to gel-filtered platelets (250 µL at 6.0 x 10⁶/mL) treated with neuraminidase, galactose oxidase, and NaBH₄, and suspended in Ca²⁺-free Tyrode solution containing 10 mg/mL fatty acid free bovine serum albumin was determined as described in Materials and Methods in the presence and absence of 370 nmol/L toslysCH₂-thrombin. Thrombin bound (T₁) is plotted as a function of the concentration of thrombin added (TT) for the lowest thrombin concentrations to illustrate high-affinity binding. (Inset) Expanded scale showing T₁ vs TT at higher thrombin concentrations. Binding of iodo-[^125I]-thrombin to platelets (solid circle); binding of iodo[^125I]-thrombin in the presence of toslysCH₂-thrombin (open circle). The results are representative of two experiments.

When neuraminidase-, galactose oxidase-, and NaBH₄-treated platelets were pretreated with unlabelled toslysCH₂-thrombin and then incubated with serial dilutions of iodo-[^125I]-thrombin, there was inhibition of high-affinity binding of iodo-[^125I]-thrombin as compared to the control platelets that had not been pretreated with toslysCH₂-thrombin (Fig 1, Table 1).

GPV Hydrolysis

Intact tritium-labeled platelets in buffer containing EDTA were treated with native thrombin for three minutes and centrifuged; the platelet pellet and corresponding supernatant fractions were analyzed by NaDodSO₄-PAGE under reducing conditions. Gels stained for protein showed several changes in the samples treated with native thrombin. There was disappearance from the platelet pellet of bands corresponding to thrombospondin (Mr 190,000) and the α-chain (Mr 67,000), β-chain (Mr 55,000), and γ-chain (Mr 50,000) of fibrinogen on reduced gels, and the appearance in the supernatant fractions of bands with identical mol wt. In addition, at higher thrombin concentrations, a 92,000 Mr band was seen in reaction supernatants that migrated in the region of the γ-γ dimer of fibrin.

 Autoradiograms of the tritium-labeled thrombin-treated platelets compared with those of controls showed decreased radioactivity associated with GPV (Mr 69,000). The supernatant fraction from the thrombin-treated platelets showed increased radioactivity in GPV₁₁ (Mr 58,000). In addition, both control and thrombin-treated supernatants contained radio-labeled bands that corresponded to glycosaminoglycan (GC) and GPV. The amounts of these two proteins varied somewhat from experiment to experiment, usually in association with the platelet counts of the reaction mixtures. However, for a given experiment, the areas of each of these bands in the supernatants remained nearly constant for three-minute incubation periods, based on the densitometric tracings of the autoradiographs, except at higher thrombin concentrations in which soluble GPV was hydrolyzed.

At three minutes, the amount of GPV₁₁ in the supernatant correlated with thrombin concentration. Supernatant GPV₁₁ was maximal at thrombin concentrations of 10 nmol/L or greater, whereas at lower thrombin concentrations less GPV₁₁ was liberated (Table 2).

ToslysCH₂-thrombin did not hydrolyze platelet-associated GPV or result in the appearance of the hydrolytic product, even at concentrations as high as 210 nmol/L (Fig 2, lane 2). Neither was there any release of thrombospondin or fibrinogen.

When labeled, gel-filtered platelets were incubated with toslysCH₂-thrombin at concentrations between 2.1 and 210 nmol/L for three minutes followed by native thrombin at a concentration of 21 nmol/L, equivalent amounts of GPV₁₁ were present in each of the supernatants and were identical to the amount of

Table 1. Effect of TosLysCH₂-Thrombin on Binding of Native Thrombin to NGB-Treated Platelets

<table>
<thead>
<tr>
<th>Sites/Platelet</th>
<th>High Affinity</th>
<th>Low Affinity</th>
<th>High Affinity</th>
<th>Low Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ila</td>
<td>1,344</td>
<td>15,850</td>
<td>2.20 x 10⁸</td>
<td>6.70 x 10⁶</td>
</tr>
<tr>
<td>Ila/toslys-CH₂-lla</td>
<td>—</td>
<td>4,700</td>
<td>6.50 x 10⁴</td>
<td>—</td>
</tr>
</tbody>
</table>

Washed platelets treated with neuraminidase, galactose oxidase, and borohydrate (NGB) were pretreated with buffer (Ila samples) or toslysCH₂-thrombin (Ila/toslysCH₂-lla samples), then incubated with different concentrations of native thrombin as described in Materials and Methods. The numbers of binding sites per platelet and association constants (Ka) were calculated from Scatchard plots of the binding data in Fig 1.
GPV HYDROLYSIS BY THROMBIN

Table 2. Effect of TosLysCH2-Thrombin on GPV Hydrolysis by Native Thrombin

<table>
<thead>
<tr>
<th>Native Thrombin Concentration</th>
<th>GPVf/GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>TosLysCH2-thrombin 210 nmol/L</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin 2.1 nmol/L</td>
<td>0.004</td>
</tr>
<tr>
<td>TosLysCH2-thrombin 210 nmol/L/thrombin 2.1 nmol/L</td>
<td>0.003</td>
</tr>
<tr>
<td>Thrombin 5.2 nmol/L</td>
<td>0.050</td>
</tr>
<tr>
<td>TosLysCH2-thrombin 210 nmol/L/thrombin 5.2 nmol/L</td>
<td>0.040</td>
</tr>
<tr>
<td>Thrombin 10.5 nmol/L</td>
<td>0.130</td>
</tr>
<tr>
<td>TosLysCH2-thrombin 210 nmol/L/thrombin 10.5 nmol/L</td>
<td>0.120</td>
</tr>
</tbody>
</table>

TosLysCH2-thrombin and buffer for three minutes followed by native thrombin concentrations of 2.1, 5.2, or 10.5 nmol/L. After an additional three minutes, the samples were centrifuged at 7,000 g for five minutes. The resulting supernatants and pellets were prepared for NaDodSO4-PAGE and radioautography as described in Methods. The results are expressed as the ratio of the area of the GPVf peak in the supernatant to the area of the GC peak in the supernatant.

GPVf in a sample pretreated with buffer (Fig 2). When the concentration of tosLysCH2-thrombin was held constant at 210 nmol/L, and native thrombin was added at concentrations of 2.1 nmol/L, 5.2 nmol/L, and 10.5 nmol/L for three minutes, GPVf appeared in the supernatant samples in proportion to the concentration of native thrombin and was similar to the amount of GPVf that appeared in supernatant samples that had been pretreated with buffer for three minutes, followed by native thrombin at the same concentrations (Table 2).

At all concentrations of native thrombin that hydrolyzed GPV, thrombospondin and fibrinogen were increased in the supernatants. There was no difference between the samples that were pretreated with tosLysCH2-thrombin and those samples pretreated with buffer.

Platelet Release and Aggregation Studies

Native thrombin induced the release of 14C-serotonin at concentrations from 0.42 to 210 nmol/L, with 50% release occurring at 1.5 to 3.5 nmol/L (range of two experiments). TosLysCH2-thrombin, in concentrations from 2.1 to 210 nmol/L, did not induce 14C-serotonin release from gel-filtered platelets. When gel-filtered platelets were pretreated for three minutes with concentrations of tosLysCH2-thrombin ranging from 4.2 to 210 nmol/L, followed by native thrombin at various concentrations for three minutes, the 14C-serotonin release curves were no different from those of platelets which had been pretreated with buffer.

Platelet aggregation results were similar to the 14C-serotonin release results. Native thrombin produced aggregation with threshold concentrations of 0.42 to 2.1 nmol/L (range of two experiments). Maximal aggregation occurred at 2.1 to 4.2 nmol/L. Pretreatment with tosLysCH2-thrombin did not alter the aggregation response of platelets to native thrombin, even with tosLysCH2-thrombin concentrations of 210 nmol/L.

DISCUSSION

Previous studies have shown specific binding of native thrombin to the surface of human platelets and have demonstrated two classes of binding sites.1,5-7 Thrombin modified at the enzyme active site binds to platelets in a similar fashion and competes with native thrombin for these binding sites, suggesting that active site-modified thrombin and native thrombin bind to the same sites on platelet surfaces. Thrombin modified at the active site also inhibits membrane depolarization in response to native thrombin.21 In contrast, active site-modified thrombin does not compete with native thrombin for thrombin-induced platelet activation and in some instances potentiates the action of native thrombin.3,22 The present study confirms these observations and extends the competition studies to the hydrolysis of GPV, showing that the
hydrolysis of platelet-associated GPV by native thrombin is not inhibited by active site-modified thrombin, whereas binding is inhibited under similar conditions. Because cleavage of GPV was not inhibited under conditions in which binding was inhibited, these data suggest that high-affinity binding of thrombin to platelets and hydrolysis of surface-associated GPV are separate and unrelated events. Thus, binding does not appear to orient the thrombin molecule for the cleavage of GPV.

Although hydrolysis of GPV by thrombin does not appear to occur through currently defined binding sites, the role that GPV cleavage plays in platelet activation by thrombin is still uncertain. Several observations are consistent with the hypothesis that cleavage and activation are related. First, GPV is the only surface substrate for thrombin reproducibly identified by currently used labeling procedures. Second, GPV is cleaved and GPV₈ is generated by other thrombin-like proteases that activate platelets, but not by proteases that do not activate platelets. Third, platelets from patients with Bernard-Soulier syndrome, which have no detectable surface-oriented GPV, demonstrate an abnormal response to thrombin, although this reduced responsiveness could be related to the absence of glycoprotein Ib (GPIb) rather than to the absence of GPV. Fourth, dose-response studies indicate a close relationship between platelet activation and GPV hydrolysis by thrombin.

In summary, the molecular events relating to the activation of platelets by thrombin are complicated, and the precise relationship between thrombin binding, cleavage of GPV, and cell activation are uncertain. Although the results of this study indicate that high-affinity thrombin binding and GPV cleavage by thrombin are unrelated events, GPV remains the only substrate for thrombin that has been identified on the platelet surface. Further studies are required to determine how GPV functions in platelet activation.

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Effect of active site-modified thrombin on the hydrolysis of platelet-associated glycoprotein V by native thrombin

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