Prothrombin Tokushima: Characterization of Dysfunctional Thrombin Derived From a Variant of Human Prothrombin

By Takashi Inomoto, Akira Shirakami, Shigenori Kawauchi, Toshibo Shigekiko, Shiro Saito, Kazuo Miyoshi, Takeki Morita, and Sadaaki Iwanga

A mutant prothrombin, designated prothrombin Tokushima, was purified from plasma of a proband with 12% of normal plasma clotting activity and 42% of normal prothrombin antigen. The purified preparation gave a single band with the same mobility as that of “prothrombin” by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The factor Xa-catalyzed proteolysis of prothrombin Tokushima examined by SDS-PAGE was found to be identical to that of “prothrombin.” Subsequently thrombin Tokushima was prepared by CM-Sepharose CL-6B column chromatography after prothrombin activation by factor Xa. The molecular weight of thrombin Tokushima estimated by SDS-PAGE was identical to that of “thrombin.” Thrombin Tokushima exhibited less than 22% of normal clotting activity, and the value of kcat/Km (μmol/L \textsuperscript{-1} sec\textsuperscript{-1}) was less than one tenth of that of “thrombin” when Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide was used as a substrate. However, active site titration using p-nitrophenyl-p'-guanidinobenzoate failed to detect any difference between the two. Thrombin Tokushima was 2.5% as effective as “thrombin” in inducing platelet aggregation. Interaction of thrombin Tokushima with antithrombin III was much slower than “thrombin” when followed by SDS-PAGE. Based on the residual thrombin activity, it was 33% as effective as “thrombin” in forming a complex with antithrombin III. These results indicate that the molecular defect resides in the thrombin portion of prothrombin Tokushima and that the binding sites for various substrates appear to be greatly impaired.

**CONGENITAL** dysprothrombinemia is a rare coagulation disorder. Only 16 variants have been reported, all of them being characterized by a decrease in the functional level of prothrombin relative to antigenic level of prothrombin. Five of the prothrombin variants thus far identified have been purified and characterized. In the case of prothrombin Barcelona and prothrombin Madrid, the functional defect has been shown to be a specific impairment of one of the two factor Xa-catalyzed cleavages, whereas in the case of prothrombin Quick, prothrombin Metz, and prothrombin Salakta, the defect is confined to the thrombin portion of the molecule. In 1983 we reported the first case of dysprothrombinemia in Japan as prothrombin Tokushima. The maternal and paternal sides have heterozygotes, respectively, for dysprothrombinemia and hypoprothrombinemia. The proband was a double heterozygote for prothrombin Tokushima and that the binding sites for various substrates appear to be greatly impaired.

**MATERIALS AND METHODS**

Blood was collected from the proband using plastic syringes that contained 1/10 volume of 3.8% trisodium citrate. Platelet-poor plasma of the proband with 12% of the clotting activity and 42% of “prothrombin” antigen was obtained after centrifugation and stored at \(-80^\circ\text{C} \). The proband was a double heterozygote for prothrombin Tokushima and that the binding sites for various substrates appear to be greatly impaired.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Kinetic constants.** Kinetic constants were determined using t-butylxoy-carbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) as the thrombin substrate (Peptide Institute, Osaka, Japan). The fluorescence of 7-amino-4-methylcoumarin (AMC) liberated (AMC) by the stoichiometric reaction of the catalytic site of thrombin with p-NPGB was monitored at 410 nm with a Hitachi 220A recording spectrophotometer (Tokyo). Thrombin active site concentration was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

**Activation of factor XIII.** Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII was determined using a molar extinction coefficient of 16,595 for p-nitrophenol at pH 8.3.

Blood, Vol 69, No 2 (February), 1987: pp 565-569
was measured by a modified method of the monodansylcadaverine (Sigma Chemical, St. Louis) incorporation assay.23
Platelet aggregation. Blood was collected from a normal donor into plastic tubes containing 1/10 volume of 3.8% trisodium citrate and 25 μg/mL apyrase (Sigma Chemical). Platelets were subsequently isolated by gel filtration on a Sepharose 2B column equilibrated with Tyrode's buffer containing 3.5 mg/mL bovine serum albumin. Aggregation studies were performed in an aggregometer (Rikadenki Kogyo, Tokyo) on 500 μL of gel-filtered platelets (10^6 cells/mL) following addition of 39 μL of thrombin solution at the final concentrations of 1.1 x 10^-7 mol/L to 4.0 x 10^-4 mol/L.

Interaction with antithrombin III. Purified human antithrombin III was obtained from Boehringerwerke (Marburg, West Germany). Thrombin or thrombin Tokushima was incubated at 25 °C in twofold excess relative to antithrombin III in 0.05 mol/L Tris-HCl (pH 8.0)/0.1 mol/L NaCl. Serial aliquots were removed for SDS-PAGE and for residual thrombin activity measurements. Protein concentrations were determined by absorbance measurement at 280 nm. Molecular weight (72,000) and extinction coefficient (ε = 18.3) were also assumed to be the same for "prothrombin" and thrombin Tokushima. Molecular weight (36,800) and extinction coefficient (ε = 13.8) were assumed to be the same for "prothrombin" and prothrombin Tokushima. Molecular weight (36,800) and extinction coefficient (ε = 13.8) were assumed to be the same for thrombins.

SDS-PAGE (8% polyacrylamide gel) in the presence and absence of 2-mercaptoethanol was performed by the method of Weber and Osborn.25 The gels were stained with Coomassie brilliant blue R-250.

RESULTS

Properties of the purified prothrombin Tokushima. Since prothrombin Tokushima was totally adsorbed onto barium citrate, the usual technique for prothrombin purification was employed to isolate the mutant molecule. A single peak containing prothrombin Tokushima was eluted from a DEAE-Sephadex A-50 column at the same ionic strength as that observed for "prothrombin." However, the yield of prothrombin Tokushima was about half that of "prothrombin" (data not shown). Both preparations migrated as a single band with the same mobility of SDS-PAGE, and their molecular weights were estimated as 72,000.

Activation of purified "prothrombin" by factor Xa in the presence of factor Va, phospholipids and Ca^2+ produced thrombin with a specific activity of 1300 NIH U/mg after five minutes incubation. However, as shown in Fig 1, the thrombin activity produced from prothrombin Tokushima reached a plateau with about 21% of the normal level after 10 minutes.

The cleavage patterns of prothrombin by factor Xa on SDS-PAGE indicated that the proteolysis of prothrombin Tokushima was indistinguishable with that of "prothrombin," yielding thrombin, fragment 1 and fragment 2, with the molecular weights identical to those of the normal counterparts (Fig 2). Moreover, upon reduction thrombin Tokushima yielded B chain and A chain with the same molecular weight as those found in "α-thrombin." These results suggest that the cleavage sites of prothrombin Tokushima by factor Xa are normal and that the defect apparently resides in the molecule in the thrombin portion.

Preparation of the purified thrombin Tokushima. Thrombin Tokushima was prepared by CM-Sepharose CL-6B column chromatography after prothrombin activation by factor Xa, as described in Materials and Methods. As shown in Fig 3, thrombin Tokushima eluted as single peak at the same ionic strength as that observed for "α-thrombin." Figure 4 shows the SDS-PAGE patterns of thrombins thus purified. The molecular weight of the reduced and unreduced forms of thrombin Tokushima appeared indistinguishable from that of "α-thrombin."

Enzymatic properties of thrombin Tokushima. Active site titration studies using p-NPGB revealed that thrombin Tokushima possessed the same catalytic activity as "α-thrombin." As shown in Table 1, 95.7% of the catalytic sites in thrombin Tokushima was titratable with p-NPGB. On the other hand, kinetic analysis for the hydrolysis of Boc-Val-Pro-Arg-MCA by thrombin Tokushima yielded a higher Km, a lower kcat, and a lower kcat/Km ratio than that of "α-thrombin." The fibrinogen clotting activity of thrombin Tokushima was 525 NIH U/mg corresponding to 21.5% of
normal, although it was 69.9% as effective as "α-thrombin" in the activation of factor XIII.

Figure 5 shows the thrombin-induced platelet aggregation by "α-thrombin" and thrombin Tokushima. At an enzyme concentration of $2 \times 10^{-9}$ mol/L, rapid aggregation was observed with "α-thrombin," whereas no aggregation was induced by thrombin Tokushima at comparable concentration. Based on the amount of enzyme required to give an equivalent incomplete aggregation, thrombin Tokushima was less than 3% as effective as "α-thrombin."

Figure 6A shows SDS-PAGE analysis of the thrombin-antithrombin III interaction. When an excess molar ratio of "α-thrombin" to antithrombin III was employed, the band of antithrombin III disappeared within ten minutes incubation, and a high molecular weight complex I, probably consisting of a 1:1 enzyme:inhibitor stoichiometric complex, appeared on SDS-PAGE. After incubation for 30 minutes, complex II, which has been attributed to partially degraded complex,25 was observed. When thrombin Tokushima and antithrombin III were incubated under identical conditions, only a small amount of complex I was formed after ten minutes. Following incubation for 30 minutes, the amount of complex I increased, while no complex II was observed. These results suggest a decreased rate in the formation of the thrombin-antithrombin III complex, and little, if any, degradation of this complex with thrombin Tokushima, as compared to that of "α-thrombin."

Table 1. Comparison of the Kinetic Parameters of "α-Thrombin" and Thrombin Tokushima

<table>
<thead>
<tr>
<th>Titrated with p-NPGB (%)</th>
<th>α-Thrombin</th>
<th>Thrombin Tokushima</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td></td>
<td>95.7</td>
</tr>
<tr>
<td>Km (\mu\text{mol/L})</td>
<td>12.0</td>
<td>94.5</td>
</tr>
<tr>
<td>kcat (\text{sec}^{-1})</td>
<td>93.5</td>
<td>49.4</td>
</tr>
<tr>
<td>kcat/Km (\mu\text{mol/L}^{-1} \text{sec}^{-1})</td>
<td>7.79</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Kinetic constants were determined at 37 °C in 1 mL of 0.05 mol/L Tris-HCl (pH 8.0) containing 0.15 mol/L NaCl and 1 mmol/L CaCl₂.
Platelet concentration was 10/mL in Tyrode's buffer. Thrombin quantitatively adsorbed onto barium citrate, and the immunoelectrophoretic pattern of prothrombin Tokushima by factor Xa is indistinguishable from "prothrombin," as shown in this report. However, the prothrombin assay in plasma using the procoagulant fraction of Echis carinatus venom and staphylocoagulase as activating agents demonstrated only a small percentage of activatable thrombin Tokushima (2.2 μg) for Boc-Val-Pro-Arg-MCA in 0.1 mol/L NaCI. Serial aliquots (60 μL) were removed for measuring activity at a concentration of 2 x 10⁻⁹ mol/L. However, at higher concentration of thrombin Tokushima (4 x 10⁻⁸ mol/L), incomplete aggregation of platelets was observed. In thrombin-mediated platelet activation, the catalytic site of thrombin and its binding site with platelets are both essential for this process. Concerning the thrombin-binding site in platelets, both high-affinity binding site (Kd = 1.5 x 10⁻⁹ mol/L) and low-affinity binding site (Kd = 3 x 10⁻⁷ mol/L) have been reported. The decreased activity of thrombin Tokushima in initiating platelet aggregation in all likelihood reflects its reduced affinity to the high-affinity binding site in platelet, since titration of thrombin Tokushima with p-NPGB reveals a normal catalytic site.

Thrombin Tokushima has no apparent platelet-aggregating activity at a concentration of 2 x 10⁻⁹ mol/L. However, at higher concentration of thrombin Tokushima (4 x 10⁻⁸ mol/L), incomplete aggregation of platelets was observed. In thrombin-mediated platelet activation, the catalytic site of thrombin and its binding site with platelets are both essential for this process. Concerning the thrombin-binding site in platelets, both high-affinity binding site (Kd = 1.5 x 10⁻⁹ mol/L) and low-affinity binding site (Kd = 3 x 10⁻⁷ mol/L) have been reported. The decreased activity of thrombin Tokushima in initiating platelet aggregation in all likelihood reflects its reduced affinity to the high-affinity binding site in platelet, since titration of thrombin Tokushima with p-NPGB reveals a normal catalytic site.

Complex formation between antithrombin III and thrombin Tokushima also occurs at a slow rate in comparison to "α-thrombin." In addition, degradation of this complex is significantly less, indicating an abnormality of thrombin Tokushima in its interaction with antithrombin III.

In conclusion, the activities of thrombin Tokushima on synthetic tripeptide substrate, fibrinogen, factor XIII, platelets, and antithrombin III are reduced when compared with "α-thrombin," while active site titration with p-NPGB shows a normal value. Thus, in thrombin Tokushima the catalytic triad, consisting of the residues with His-43, Asp-99, and Ser-205 of the thrombin B chain, seems to be structurally intact, but other residues in the vicinity of the catalytic site, eg, apolar binding sites, may be structurally abnormal.
ACKNOWLEDGMENT

We are indebted to Dr C. T. Esmon (Thrombosis/Hematology Research Program, Oklahoma Medical Research Foundation, Oklahoma City), for kindly providing bovine factor Va. We thank Dr Kiesel (Department of Pathology and Biochemistry, University of New Mexico, Albuquerque) for his help in preparing the English manuscript. We thank Mizuno Akiyoshi for her expert secretarial assistance.

REFERENCES


Prothrombin Tokushima: characterization of dysfunctional thrombin derived from a variant of human prothrombin

T Inomoto, A Shirakami, S Kawauchi, T Shigekiy0, S Saito, K Miyoshi, T Morita and S Iwanaga