Thrombin Metz: Characterization of the Dysfunctional Thrombin Derived From a Variant of Human Prothrombin

By M. J. Rabiet, M. Jandrot-Perrus, J. P. Boissel, J. Elion, and F. Josso

Thrombin Metz and normal thrombin, resulting from activation of the respective prothrombins by factor Xa in the presence of calcium, phospholipid, and factor Va, were purified by chromatography on sulfoethyl Sephadex. By physicochemical criteria, thrombin Metz is identical to normal thrombin. Its functional properties were investigated in some reactions in which thrombin is classically involved. Thrombin Metz exhibits less than 4% of fibrinogen clotting activity. Both $K_m$ and $K_{cat}$, determined on S2238, are abnormal. Titration with the high-affinity competitive inhibitor of thrombin, DAPA, shows that fluorescence enhancement of the probe is only 34% in binding to thrombin Metz when compared to that observed in binding to normal thrombin. High-performance liquid chromatography has been used to measure the simultaneous rate of release of fibrinopeptides A and B. A decreased release rate for both fibrinopeptides, more marked for fibrinopeptide B, results in a slow fibrin polymerization, as followed by absorbance at 450 nm. Thrombin Metz is less than 5% as effective as normal thrombin in inducing platelet aggregation. Interaction with antithrombin III is slower than normal when followed by SDS gel electrophoresis and inhibition of the amidolytic activity of thrombin on S2238. This abnormality is not observed in the presence of heparin. However, thrombin Metz binds less tightly to a heparin-Sepharose column, and the direct inhibition of heparin on its activity on S2238 is weaker. From these results, we can predict that the defect in thrombin Metz affects the catalytic site or its vicinity and, jointly or consequently, the region of interaction of thrombin with antithrombin III and heparin.

PROTHROMBIN DEFICIENCIES (hypoprothrombinemia or dysprothrombinemia) are rare blood coagulation disorders. Dysprothrombinemia is characterized by a decrease in the functional level of prothrombin relative to the immunologically detectable prothrombin.

Prothrombin Metz, a congenital dysprothrombin, has been described in a French family.1 The mother is heterozygous for the abnormal prothrombin, and the father is heterozygous for true hypoprothrombinemia. Three of the children are double heterozygotes for prothrombin Metz and hypoprothrombinemia and therefore bear only the abnormal molecule. This case constitutes the third observation of such an association.

Three of the prothrombin variants described up to now have been fully purified and characterized. In the case of prothrombin Barcelonà2 and prothrombin Madrid,3 the molecular 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, the defect affects the thrombin portion of the molecule.4

Prothrombin Metz has been shown to be totally adsorbed onto barium citrate and subsequently isolated from plasma by a standard technique for prothrombin purification.5 The purified material was indistinguishable from normal prothrombin by physicochemical criteria, such as molecular weight, charge, and N-terminal residue. Activation of the purified prothrombin variant by factor Xa in the presence of phospholipid and calcium generated less than 4% of the clotting activity observed with normal prothrombin. However, amidolytic activity of the activation product reached 70% of the normal maximum activity.

When prothrombin Metz was hydrolyzed by factor Xa alone, two main abnormal features were observed upon sodium dodecyl sulfate (SDS) gel electrophoresis. First, prothrombin I appears as for normal prothrombin, but remains throughout incubation with factor Xa as the major product. Second, an abnormal intermediate of 25,000 mol wt is formed, the nature of which has not been established.

In the present study, the thrombin moiety derived from the congenital dysprothrombin, prothrombin Metz, has been isolated and its function investigated in several reactions known to occur with normal thrombin: action on fibrinogen and small synthetic substrates, platelet aggregation, and interaction with anti-thrombin III and heparin. Thrombin Metz appears to be defective in most of these reactions.

MATERIALS AND METHODS

Thrombin Purification

Plasma from the dysprothrombinemic patients was collected by plasmapheresis on acid citrate dextrose anticoagulant after informed consent of the patients. Plasma was immediately frozen and stored at −80°C. A standard prothrombin isolation procedure, using barium...
citrate adsorption/elution, ammonium sulfate precipitation, and DEAE Sephadex chromatography,\textsuperscript{6,7} was employed to isolate both prothrombin Metz and normal human prothrombin. The respective prothrombins (1 mg/ml in 0.02 M Tris, 0.15 M NaCl, pH 7.4) were activated by bovine factor Xa (0.5 U/ml). The mixture was assayed for thrombin activity until it remained constant. Thrombin was obtained by chromatography on sulfopropyl Sephadex C50 in a manner similar to that described by Lundblad;\textsuperscript{8} the activation mixture was ultracentrifuged (2 hr, 38,000 rpm) in a Beckman LS 50 and applied to a 1.5 × 15 cm column, equilibrated with 0.015 M sodium phosphate, pH 6.5. The column was washed with the same buffer and the proteins eluted stepwise with 0.1 M and 0.25 M sodium phosphate buffers, pH 6.5.

Bovine factor Xa was Diagen chromatographed activated factor X from Diagnostic Reagents Ltd., Thame Oxford, England. Phospholipid source was thromboplastin from Laboratoire Stago, France. Semipurified factor V was prepared as described by Alagille et al.\textsuperscript{9}

Thrombin was detected by clotting assay on purified human fibrinogen\textsuperscript{10} and spectrophotometrically, using the synthetic substrate HD-phenylalanyl-L-pipecolyl-L-arginyl-p-nitroanilide bichloride (S2238) from Kabi, Sweden.\textsuperscript{11} For kinetic experiments, hydrolysis of this substrate by thrombin was followed at 405 nm using a Gilford 250 spectrophotometer equipped with a Servotrace recorder. Protein concentrations were determined by spectrophotometric absorption at 280 nm. Molecular weight of thrombin was determined by spectrophotofluorimetry at 570 and 12% acrylamide was performed as described by Laemmli.\textsuperscript{12} 2-Mercaptoethanol was used as a reducing agent.

Active Site Titration

Active site was titrated using the fluorescent competitive inhibitor of thrombin, dansylarginine-N (3 ethyl, 1-5 pentanediyl) amide (DAPA; gift of Dr. B. Furie). Microliter aliquots of 3.1 × 10\textsuperscript{-6} M DAPA were added sequentially to 1.02 × 10\textsuperscript{-4} M thrombin solution in 0.05 M Tris, 0.15 M NaCl, pH 7.4. Fluorescence was measured at 550 nm on an Aminco spectrophluorimeter SPF 500 (λex = 335 nm, bandpass 5 nm) at 20°C and corrected for the intrinsic fluorescence of DAPA. Total titrant volume did not exceed 1% of the total volume of the protein solution, and no correction was made for dilution.

Fibrinopeptides A and B Release

Human fibrinogen, 95% clottable, was purified by the method of Jaques.\textsuperscript{13} α-Thrombin (1,400 NIH U/mg) as well as γ-thrombin were kindly provided by Dr. J. W. Fenton II. Kinetic experiments were carried out at 20°C in 0.02 M ammonium bicarbonate, pH 7.4. Fibrinogen concentration (5 mg/ml) was determined spectrophotometrically (ε\textsubscript{280} = 12.4). Thrombin was used at a final concentration of 20 nM (1 U/ml or 0.2 U/mg of fibrinogen). Identical molar concentrations were used for α-thrombin, γ-thrombin, and thrombin Metz.

Digestion was allowed to proceed for various time periods at 20°C. Each reaction was quenched by addition of acetic acid (15% final) in order to dissolve any formed clot. All protein material was then immediately precipitated by addition of an equal volume of 20% trichloroacetic acid. Samples were centrifuged and the supernatants containing the fibrinopeptides were lyophilized and resuspended in doubly distilled water. Some 20-100 μl of the fibrinopeptide solution was analyzed by high-performance liquid chromatography (HPLC). The technique used was a modification of that reported by Martinelli and Sheraga.\textsuperscript{14} The separation was achieved under reverse phase conditions on a Lichrosorb RP 18 column (Merck), using a 0.01 M ammonium acetate, pH 5.6, buffer/acetonitrile gradient, from 0% to 15% acetonitrile. Fibrinopeptides were quantitated from the area of the corresponding elution peak (210 nm) by comparison to a calibration curve. This curve was established using known concentrations of fibrinopeptides as determined by amino acid analysis. The lower limit of sensitivity of this procedure was 0.1 nmol of fibrinopeptide. Fibrin monomer polymerization was monitored by measuring the optical density at 450 nm on separate samples treated with the same conditions.

Platelet Aggregation

Blood was collected in plastic tubes on 0.13 M trisodium citrate (1/9, v/v) containing 25 μg apyrase (Sigma, St. Louis, MO) and 20 nM PGE\textsubscript{1}, (Sigma). Platelets were washed 3 times at room temperature with modified Tyrode’s buffer that was free of calcium and contained 3.5 mg/ml bovine serum albumin, 25 μg/ml apyrase, and 20 nM PGE\textsubscript{1}. Platelets were then resuspended in Tyrode’s buffer. Aggregation was performed on 200 μl of freshly washed platelets (10\textsuperscript{9}/ml) by addition of 50 μl of thrombin solutions in a Labintec aggregometer.

Interaction With Antithrombin III and Heparin

Purified antithrombin III was obtained from Centre Regional de Transfusion Sanguine, Lille, France. Heparin was sodium salt, grade I, from porcine intestinal mucosa, Sigma, St. Louis, MO.

Kinetic experiments were performed by mixing thrombin with antithrombin III in different molar ratios in a 0.02 M Tris, 0.15 M NaCl, pH 7.4, buffer, in the absence or presence of heparin (0.5 U/ml). Solutions were incubated at room temperature, and aliquots were assayed at time intervals for thrombin activity on S2238 or analyzed by SDS gel electrophoresis.

Thrombin–heparin interaction was tested either by measuring amidolytic activity on S2238 in the conditions described above, but omitting antithrombin III in the incubation mixture, or by affinity chromatography on heparin-Sepharose. Heparin-Sepharose column was prepared by coupling 1 g of heparin in 0.5 ml of 0.1 M NaHCO\textsubscript{3}, 0.5 M NaCl, pH 8, to 12 ml of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The mixture was gently mixed overnight at 4°C. Excess ligand was washed with coupling buffer, and the remaining active groups blocked with 1 M ethanolamine, pH 9. The product was washed through 3 cycles of alternating 0.1 M sodium acetate, 1 M NaCl, pH 4, and 0.1 M Tris 0.1 M NaCl, pH 8 buffers, finally equilibrated in 0.05 M Tris, 0.1 M NaCl, pH 7.5, and stored at 4°C. Binding efficiency was 98%. Aliquots of 0.3 mg of thrombin were loaded on a 5-ml column. The column was washed with 0.05 M Tris, 0.1 M NaCl, pH 7.5, and the bound material was eluted with a 0.1–1.0 M NaCl gradient.

RESULTS

Because prothrombin Metz was shown previously to be adsorbed on barium citrate, a standard prothrombin isolation procedure was used to isolate prothrombin Metz and normal prothrombin. The respective prothrombins were activated with bovine factor Xa in the presence of factor Va, phospholipid, and calcium, and the generated thrombins were isolated by stepwise chromatography on sulfopropyl Sephadex C50. Both normal thrombin and thrombin Metz were eluted during the last step with 0.25 M sodium phosphate, pH 6.5. The eluted materials were subjected to SDS gel electrophoresis. Thrombin Metz and its heavy chain (\(B\)
DAPACONCENTRATION

Fig. 1. Analysis of thrombin Metz by SDS polyacrylamide gel electrophoresis. Polyacrylamide concentration was 12% with an acrylamide/bisacrylamide ratio of 30/1. Samples 1 and 2 are unreduced samples of α-thrombin and thrombin Metz, respectively; 3 and 4 are reduced samples of the same materials.

chain) were found to have the same molecular weights as normal thrombin and normal thrombin B chain on unreduced and reduced gels, respectively (Fig. 1). Because of its low molecular weight, the A chain was only barely visible on the gels, and no valid comparison could be made.

Table 1 summarizes the kinetic parameters pertaining to the enzymatic reaction with S2238. $K_m$ was calculated from Lineweaver-Burk plots, and $K_{cat}$ values were obtained from the ratios of maximal initial velocities to enzyme concentrations. $K_m$ value found for α-thrombin was in good agreement with the value of $0.7 \times 10^{-5} M$ given by Kabi.

DAPA, a competitive inhibitor of thrombin, has been shown to be extremely useful in titrating the active site, as the interaction with thrombin results in an increase of its intrinsic fluorescence.13 Data from the active site titration of thrombin Metz with DAPA are shown in Fig. 2 in comparison to α-thrombin. In the conditions used, maximum fluorescence enhancement was obtained for a DAPA/α-thrombin molar ratio of 1.02. When thrombin Metz was tested, the fluorescence enhancement was only 34% of the maximum fluorescence observed with normal α-thrombin and reached a DAPA/thrombin Metz molar ratio of 0.62.

Table 1. $K_m$ and $K_{cat}$ Values Determined on S2238 at 37°C in 2.5 ml of 0.05 M Tris. 0.15 M NaCl, pH 8.3

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mole/liter)</th>
<th>$K_{cat}$ (mole/min/mg)</th>
<th>$K_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal α-thrombin</td>
<td>$0.90 \times 10^{-6}$</td>
<td>$0.18 \times 10^{-6}$</td>
<td>$2.0 \times 10^2$</td>
</tr>
<tr>
<td>Thrombin Metz</td>
<td>$4.60 \times 10^{-5}$</td>
<td>$0.03 \times 10^{-6}$</td>
<td>$6.5 \times 10^4$</td>
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Clotting activity and protease activity of thrombin are not equivalent. The degraded γ-form of thrombin lacks significant fibrinogen clotting activity, but retains, to some extent, esterolytic and amidolytic activities of thrombin as well as interaction with anti-thrombin III and platelets. In this study, some activities of thrombin Metz have been compared to that of human α- and γ-thrombins.

In order to determine more precisely the action of thrombin Metz on fibrinogen, thrombin-catalyzed

Fig. 2. Titration of thrombin Metz (A) and normal α-thrombin (B) with the competitive inhibitor DAPA. Fluorescence was determined at 550 nm after excitation at 335 nm. Thrombin Metz and normal thrombin were used at the same molar concentration ($0.42 \times 10^{-6} M$). Open and filled symbols correspond to two sets of experiments.

Fig. 3. Thrombin-induced release of fibrinopeptides A and B from human fibrinogen. Fibrinogen (5 mg/ml) was incubated at 20°C with (A) α-thrombin. 0.2 U/mg of fibrinogen, and 0.002 U/mg of fibrinogen; (B) γ-thrombin ($20 \times 10^{-7} M$); and (C) thrombin Metz ($20 \times 10^{-7} M$). Samples at time intervals were processed and assayed for fibrinopeptide A and B released versus incubation time. Fibrin monomers polymerization was followed by absorbance at 450 nm (dotted lines). Arrows indicate the time when a solid gel was formed.
release of fibrinopeptides A and B was directly measured. A mixture of fibrinogen and thrombin was incubated over a time period of 4 hr at 20°C. Under the conditions used, no significant thrombin degradation was detectable. Release of fibrinopeptides A and B was measured simultaneously by high-performance liquid chromatography. As a consequence of the fibrinopeptides release, intermediate fibrin monomers are formed. Their polymerization has been followed by absorption measurement at 450 nm. The results are summarized in Fig. 3. At an identical molar concentration (20 mM), clotting time for normal α-thrombin was 20 sec, whereas it took about 30 min to get a visible clot with γ-thrombin and thrombin Metz. Under our conditions, the initial rates of release of fibrinopeptides A and B by α-thrombin (0.2 U/mg) were very rapid and roughly similar, although slightly more rapid, for fibrinopeptide A. In the case of thrombin Metz, not only was the rate of release of fibrinopeptides reduced, but this reduction was much more pronounced for fibrinopeptide B (Fig. 3C). These results are clearly different from those obtained with γ-thrombin (Fig. 3B) as well as from those found when α-thrombin was diluted 100-fold in order to reach a clotting time comparable to the one observed with thrombin Metz. In those conditions, the rate of release was equally reduced for both fibrinopeptides. A striking observation in these experiments is that similar rates of release of both fibrinopeptides A and B by α- or γ-thrombin was accompanied by polymerization of fibrin monomers, as monitored by the change in absorption at 450 nm, whereas polymerization was drastically reduced with thrombin Metz, where a discrepancy existed between the rate of release of fibrinopeptides A and B. The amino acid composition of fibrinopeptide A released by thrombin Metz was normal. There was not enough material for fibrinopeptide B analysis, but the elution pattern on HPLC showed no difference with the fibrinopeptides released by α-thrombin.

In addition to the conversion of fibrinogen to fibrin, thrombin also has a modulating influence on many of the reactions involved in blood coagulation. The ability of thrombin Metz to induce platelet aggregation and to interact with antithrombin III was also investigated. Figure 4 shows the results obtained in the platelet aggregation experiments. At a concentration of 0.1 U/ml of α-thrombin (2 x 10⁻⁷ M), rapid and complete aggregation was observed. Thrombin Metz at an equivalent molar concentration has no effect. As a comparison, in our conditions, α-thrombin at a concentration as low as 0.005 U/ml still induces aggregation.

Antithrombin III is a plasma protease inhibitor known to inhibit, at varying rates, most of the serine proteases of the coagulation cascade. The inhibition of thrombin is accelerated by heparin and involves the formation of an equimolar complex, possibly covalent between the inhibitor and the serine protease. Interaction of antithrombin III with α-thrombin and thrombin Metz was examined at different thrombin/antithrombin III molar ratios (2/1, 1/1, 1/2) by SDS gel electrophoresis. In all cases, a delay in the formation of the thrombin–antithrombin III complex was observed for thrombin Metz when compared to normal thrombin. As an example, Fig. 5A shows the results obtained with a 2/1 molar ratio. Within 10 min of incubation, a complex (CT₁, mol wt 100,000) was formed between α-thrombin and antithrombin III, but no complex of that kind was seen between thrombin Metz and antithrombin III. However, after 30 min of incubation, a complex was finally formed. The existence of degraded forms of the antithrombin III–thrombin complex (CT₂ and CT₃) indicates that thrombin Metz as α-thrombin is able to proteolyse the complex. The thrombin–antithrombin reaction was also studied by following the disappearance of thrombin activity as a function of time. Thrombin Metz and α-thrombin were incubated with antithrombin III at 25°C at equimolar concentration (10⁻⁶ M). The time course of thrombin inhibition was studied by measuring spectrophotometrically the
Fig. 5. Inhibition of thrombin by antithrombin III. Antithrombin III complex formation with α-thrombin and thrombin Metz was monitored by SDS gel electrophoresis (A). Thrombin and antithrombin III were used at a 2/1 molar ratio. Acrylamide concentration was 7.5% with a 30/1 acrylamide/bisacrylamide ratio. All the samples were unreduced. The uppermost band (CT, ) on the gel is antithrombin III-thrombin complex. The bands below (CT, and CT,) have been attributed to partially degraded complexes.

A-Thrombin (Δ) or thrombin Metz (Δ) were incubated with antithrombin III in equimolar ratio (B) or at a 2/1 molar ratio in the presence of 0.5 U/ml heparin (C). Remaining thrombin activity was measured spectrophotometrically, using S2238 as chromogenic substrate. Results are given in percentage of remaining thrombin activity versus incubation time.

Residual thrombin activity on S2238. In both cases, a time-dependent inhibition of thrombin activity was observed (Fig. 5B). Thrombin Metz was more slowly inhibited by antithrombin III than α-thrombin, but this inhibition was complete after 30 min of incubation. In the absence of antithrombin III and under identical conditions, activities of the two thrombins were stable for more than an hour.

There is a general agreement that heparin acts by accelerating the slow rate at which antithrombin III normally inactivates thrombin. In the presence of heparin, thrombin Metz and α-thrombin were both immediately inhibited by antithrombin III (Fig. 5C). In this experiment, the molar concentration of antithrombin III was half that of thrombin in order to slow down the inhibition kinetics. This explains the final incomplete inhibition. Even then, the reaction was so rapid that a difference between the behavior of thrombin Metz and α-thrombin could hardly be noticed.

The precise mechanism by which heparin enhances the rate of formation of the thrombin–antithrombin III complex is not clearly understood. Heparin can bind selectively to either antithrombin III or thrombin, suggesting that specific binding sites for heparin exist on both molecules. Furthermore, heparin Sepharose chromatography is commonly used for thrombin purification. Direct interaction between heparin and thrombin Metz was studied by two approaches. First, binding of α-thrombin and thrombin Metz to a heparin column was compared. Results are shown in Fig. 6. Under identical conditions, thrombin Metz was clearly eluted earlier than α-thrombin in the NaCl gradient (0.47 M versus 0.61 M NaCl). Second, the effect of heparin on amidolytic activity was determined by incubating thrombin with heparin for different periods of time. Residual activity of thrombin Metz and α-thrombin was then measured on S2238. Under our conditions, heparin alone at this concentration (0.5 U/ml) has no effect on this substrate. Results presented in Fig. 7 show that for both α-thrombin and thrombin Metz, heparin induced a decrease of 50% of the amidolytic activity. However, the inhibition seemed to be more progressive in the case of thrombin Metz. As a control, no effect of heparin on the amidolytic activity of trypsin was observed.

**DISCUSSION**

Prothrombin is the zymogen of the serine protease thrombin. The activation reaction, catalyzed by the serine protease, factor Xa, is a very complex one. Factor Xa cleaves normal prothrombin to release the N-terminal “pro” part and the two-chain thrombin molecule. In addition, the thrombin produced upon activation can cleave human prothrombin, giving rise
to fragment 1, fragment 2, and a 13 amino acid fragment, fragment 3, liberated from the N-terminal end of the thrombin part of the molecule. The "pro" part plays an important role in enhancing the rate of activation through the binding to phospholipid via calcium ions (fragment 1) and to factor Va (fragment 2).

Because of the complex nature of the prothrombin activation process, different functional alterations can be observed in the case of abnormal prothrombins, depending on the localization of the structural defect within the molecule: in the "pro" part region, as for dicoumarol-induced descarboxyprothrombin, at a residue being part of, or masking a cleavage site (as in the case of prothrombins Barcelona and Madrid) or in the thrombin portion of the molecule (as demonstrated for prothrombin Quick).

In two previous reports, prothrombin Metz has been studied both in plasma and in purified systems. In both cases, its activation has been characterized as giving rise to a low thrombin generation much more pronounced for clotting activity than for amidolytic activity. A specific impairment of the interaction of prothrombin Metz with calcium ions does not seem to be responsible for an abnormal activation, as normal immunoelectrophoretic patterns of prothrombin in plasma have been reported both in the presence and absence of calcium. Furthermore, prothrombin Metz is normally adsorbed on barium citrate. Abnormal thrombin generation does not result from the absence of one of the factor Xa cleavages, as is the case for prothrombins Barcelona and Madrid, because a normal immunoelectrophoretic pattern of the activation products of prothrombin Metz in serum has been described, as well as formation of thrombin evidenced by SDS polyacrylamide gel electrophoresis of the activation products obtained from the purified molecule. In the last experiment, however, where prothrombin Metz had been activated by factor Xa in the presence of calcium, occurrence of an abnormal activation product mol wt 25,000 has been observed.

These observations suggest that, rather than affecting the activation process itself, the defect of prothrombin Metz may result from altered catalytic properties of the generated thrombin. This hypothesis is supported by the observation that prothrombin assay in plasma, using the procoagulant fraction of Echis carinatus venom as activating agent, generates only a small percentage of normal clotting activity.
The purpose of the present study was to isolate thrombin produced upon activation of purified prothrombin Metz and to further characterize its catalytic properties in purified systems. Prothrombin Metz has been activated by factor Xa in the presence of all the components of the prothrombinase complex (factor Va, calcium, and phospholipid). The resultant enzyme, thrombin Metz, has been isolated. It behaves as an homogeneous protein and seems to have the same physicochemical properties as normal thrombin, as far as charge and molecular weight are concerned. SDS gel electrophoresis shows the integrity of the B chain. Human thrombin is known to differ from bovine thrombin by the absence, at the aminoterminal end of the A chain, of a 13 amino acid peptide that is autcatalytically removed. The effect of this peptide on thrombin activity is not known. Because of its low molecular weight, the A chain is only barely visible on the gels, and it is difficult to determine if the 13 amino acid peptide is still present in thrombin Metz. The N-terminal, either way, would be a threonine. Our thrombin Metz preparation does not contain a product identical to the one observed when prothrombin was activated by factor Xa alone with an apparent molecular weight of 25,000. Purified thrombin Metz is therefore indistinguishable from normal thrombin by the physicochemical criteria we studied, and its abnormal catalytic behavior, described in some detail in this paper, is not due to a rapid conversion into degraded forms, as has been previously hypothesized.

Abnormal catalytic properties of thrombin Metz are evidenced by values of the kinetic parameters, $K_m$ and $K_{cat}$, determined on S2238, active site titration with DAPA, decreased and dissociated rates of fibrinopeptides A and B release, and inability to induce platelet aggregation.

$K_m$ and $K_{cat}$ of thrombin Metz for S2238 are both abnormal, indicating that thrombin Metz differs from $\alpha$-thrombin not only by its affinity for the substrate but also by its hydrolytic behavior. From the $K_{cat}$ values here reported for purified $\alpha$-thrombin and thrombin Metz, one would predict that, even at saturating substrate concentration, thrombin Metz would convert S2238, at best, 17% as fast as $\alpha$-thrombin. This figure is not consistent with the previous finding that, upon activation of prothrombin Metz by factor Xa in the presence of calcium and phospholipid, a plateau of amidolytic activity on S2238 is reached for a value representing 70% of normal. One possible explanation for this discrepancy is that thrombin Metz was not in the same environment in both experiments. Presence of the elements of the activation mixture and/or of the prothrombin profragments might have altered its activity on S2238. As an example, prothrombin fragment 2 has been shown to enhance thrombin activity toward tosyl-arginyl-$\alpha$-methylester in the bovine system.

In the active site titration of thrombin Metz by DAPA, both fluorescence enhancement and binding stoichiometry are decreased, assuming a similar extinction coefficient for normal thrombin and thrombin Metz. These results are very similar to those obtained with thrombin Quick. In the latter case, the existence of two different molecular species in the preparation has been proposed, one having an active site titrated with DAPA and the other one being unable to bind DAPA. For thrombin Metz, the mode of inheritance deduced from the familial studies makes such a situation highly unlikely. Our thrombin Metz preparation behaves as an homogeneous protein and does not contain prothrombin 2, as evidenced by SDS gel electrophoresis after reduction. This of course does not exclude the possibility of contamination by an inactive molecule. However, if this was the case, all the catalytic properties should be reduced to a similar extent, and $K_m$ toward S2238 should not be affected.

In the conditions used to study fibrinopeptide release, thrombin Metz differs from both $\alpha$- and $\gamma$-thrombins in that not only the rate of release of fibrinopeptides is reduced, but also, that this reduction is more marked for fibrinopeptide B. Fibrinopeptide B, however, is present at the early stage of incubation. Consequently, the fibrin monomers formed should still retain a nonnegligible proportion of fibrinopeptide B. The results we obtained following absorbance at 450 nm could be explained by the fact that the polymerization of such monomers is not as good as the polymerization of monomers lacking as much fibrinopeptide B as fibrinopeptide A. The fibrinopeptides released from normal human fibrinogen by either thrombin Metz or $\alpha$-thrombin are, until further proof, identical, as evidenced by the amino acid composition of fibrinopeptide A and the elution volumes of both fibrinopeptides A and B on HPLC.

Thrombin Metz also presents altered interactions with antithrombin III and heparin. Both kinetics of inhibition of thrombin Metz by antithrombin III in the absence of heparin and SDS gel analysis indicate that formation of an antithrombin III–thrombin Metz complex is slower than normal. The fact that degraded forms of the complex are present suggests that the free form of thrombin Metz still retains proteolytic activity toward the complex.

Inhibition of amidolytic activity of thrombin Metz by heparin is only slightly delayed when compared to $\alpha$-thrombin, and the maximum inhibition is identical
in both cases. This inhibition must result from direct interaction between heparin and thrombin, and not with S2238, since it increases with the time of incubation of thrombin with heparin, and no effect at all is observed in the case of trypsin.

Abnormal interaction of thrombin Metz with heparin is further evidenced by its early elution from the heparin-Sepharose column. This behavior should not be explained by a charge difference, as thrombin Metz has been shown to have the same mobility as normal thrombin by double cross-immunelectrophoresis in the absence of heparin. Furthermore, in the same system, but in the presence of heparin, thrombin Metz migrates more slowly toward the anode than normal thrombin.

In conclusion, activation of thrombin Metz gives rise to a thrombin molecule that is clearly abnormal in its catalytic behavior, showing unambiguously that the structural defect is located in the thrombin part of the molecule. The fact that all the studied reactions are abnormal suggests that the molecular defect disturbs the function of the catalytic site itself and not the specific interaction with a given substrate. Precise study of thrombin Metz may therefore provide some new insights concerning the catalytic mechanism of thrombin action.

REFERENCES


Thrombin Metz: characterization of the dysfunctional thrombin derived from a variant of human prothrombin

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