Functional Characterization of Thrombin Salakta: An Abnormal Thrombin Derived From a Human Prothrombin Variant

By Annie Bezeaud, Jacques Elion, and Marie-Claude Guillou

The genetic variant prothrombin Salakta has been described in a patient presenting with a normal level of prothrombin antigen but reduced prothrombin activity. Initial studies indicated that factor Xa–catalyzed cleavages proceed normally but lead to the production of a thrombin molecule with an altered enzymatic activity. To characterize the functional abnormality of thrombin Salakta more precisely, it was purified by chromatography on heparin-Sepharose and diethylaminoethyl–Sephadex. The purified variant does not differ from normal thrombin by size, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and is 93.1% ± 7.6% active by titration with p-nitrophenyl-p'-guanidinobenzoate. Its activity, however, is altered to various extents toward the following substrates: H-D-phenylalanyl-L-piperoclyl-L-arginine para-trioanilide (S 2238), fibrinogen, factor V, protein C, and antithrombin III. The Michaelis constant ($K_m$) of thrombin Salakta for $S\text{2238}$ is higher (12.2 ± 3.3 μmol/L) than normal (2.8 ± 0.7 μmol/L), whereas the turnover number ($k_{cat}$) is normal (84.4 ± 6.6 s⁻¹ v 85.9 ± 14.0 s⁻¹) for normal thrombin. The interaction of thrombin Salakta with benzamidine is also altered as evidenced by an increased inhibition constant ($K_i = 3.5$ mmol/L v 0.28 mmol/L for normal thrombin). The inability of fibrinogen to act as a competitor in the inactivation of thrombin Salakta by diisopropylfluorophosphate clearly indicates that fibrinogen binding to the fibrinopeptide groove is drastically impaired. In contrast, interactions involving sites remote from the active site such as those with fibrin and thrombomodulin are only slightly impaired. These results indicate that thrombin Salakta exhibits a specific pattern of functional alterations different from those reported for other variants. The structural defect seems to affect essentially the primary substrate binding site and to a lesser extent recognition site(s) remote from the catalytic site such as those for fibrin and thrombomodulin.

EXPERIMENTAL PROCEDURES

Materials. Diisopropylfluorophosphate (DFP), 4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid (HEPES), p-nitrophenyl-p'-guanidinobenzoate HCl (NPGB), N-tosyl-L-lysine chloromethylketone (TLCK), 2-(N-morpholino) ethanesulfonic acid, poly(ethylene glycol) 8000 (PEG), Tris base, and benzamidine hydrochloride were purchased from Sigma Chemical Co, St Louis. Purified human fibrinogen and H-D-phenylalanyl-L-piperoclyl-L-arginine para-trioanilide (S 2238) were from Kabi Diagnostics, Stockholm; diethyl aminoethyl (DEAE)-Sephadex A 50 and CNBr-activated Sephadex were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Normal thrombin and thrombin Salakta were purified as previously described.⁶ All the preparations used throughout this study were more than 97% $\alpha$-thrombin, as judged by sodium dodecyl sulphate (SDS) gel electrophoresis.⁷ Human AT isolated according to McKay⁸ was further purified by DEAE-Sephadex chromatography.⁹ Rabbit lung thrombomodulin,¹⁰ bovine protein C,¹¹ and bovine factor V¹² were isolated according to the published methods. Molecular weights and extinction coefficients (ml x mg⁻¹ x cm⁻¹) used for the calculations of protein concentrations were, respectively, thrombin, 35,600 and 1.83²; AT, 58,000 and 0.6¹¹; protein C, 62,000

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Submitted June 18, 1987; accepted September 21, 1987.

Supported by grants from Institut National de la Recherche Médicale (CRE 825011) and from Faculté Xavier Bichat, Université Paris VII.

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and 1.45; and fibrinogen, 340,000 and 1.52. The rabbit thrombomodulin concentration was determined by titration with human α-thrombin. The factor V concentration was measured by a one-stage assay.

**Thrombin enzymatic activity assays.** Active site titration of thrombin was performed with NPGB by using a modification of the procedure of Chase and Shaw in which 0.1 mol/L HEPES, pH 8.3, and 0.1 mol/L NaCl were used in place of the barbitral buffer. Amidolytic activity was measured by using S 2238. Assays were performed in polystyrene cuvettes at 25°C in 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% PEG, pH 7.8. The release of p-nitroaniline (pNA) upon the addition of thrombin was followed by measuring the increase in absorbance at 405 nm with a DW 2A Aminco spectrophotometer (Travenol, Silverspring, MD). The amount of product formed was calculated by using a molar absorptivity of 9,920 mol/L × cm⁻¹ for pNA at 405 nm, and the concentration of S 2238 was determined spectrophotometrically at 316 nm by using an absorption coefficient of 1.3 × 10⁴ mol/L × cm⁻¹. Kinetic parameters were obtained from duplicate determinations of the initial velocity for at least six substrate concentrations ranging from 0.5 to ten times the Michaelis constant (Kₘ). In four separate experiments for thrombin Salakta, and in five experiments for normal thrombin, Kₘ was determined from Lineweaver-Burk plots and the turnover number (k₉ₐ) from the ratios of initial velocities to enzyme concentrations.

Clotting activity was determined by using purified human fibrinogen and, as a reference, the thrombin lot B-3 (kindly provided by Dr D.L. Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, MD).

Thrombin activation of bovine factor V was studied at 37°C in 20 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 10 mmol/L CaCl₂. At timed intervals, aliquots were diluted 1:40 in chilled buffer, and factor V was immediately assayed.

The rate of protein C activation was measured either in the absence or in the presence of thrombomodulin as previously described. Less-than-saturating concentrations of protein C were used to conserve material, and concentrations of thrombin or the thrombin-thrombomodulin complex were adjusted so that initial rate conditions were achieved over the time of the experiment.

**Thrombin inhibition.** The rate of thrombin inactivation by DFP or TLCK was determined at 25°C in 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% PEG, pH 7.8. Thrombin (15 to 44 mmol/L) was incubated with various concentrations of DFP (0.1 to 1 mmol/L or TLCK (2.5 to 20 mmol/L). At timed intervals, the aliquots were assayed for residual thrombin activity by using S 2238 (0.125 mmol/L). A plot of the logarithm of the percentage of residual activity v as a function of reaction time was drawn, and the second-order rate constant (k₂) was determined by using the equation ln (T/T₀) = -k₂t, where [I] is the concentration of DFP or TLCK, T₀ the initial thrombin activity, and T the residual thrombin activity at time t.

The rate of thrombin inhibition by AT was determined at 37°C under pseudo-first-order conditions as previously described. AT being in a 5 to 50 molar excess relative to thrombin.

Thrombin inhibition by benzamidine was measured in the same buffer and temperature conditions as before. The kinetics of S 2238 hydrolysis by thrombin were determined in the absence and presence of benzamidine. A Lineweaver-Burk plot was constructed in the absence of inhibitor and at a fixed concentration of benzamidine (0.4 mmol/L and 10 mmol/L for normal thrombin and thrombin Salakta, respectively), and the value of the inhibition constant (Kᵢ) was determined by using the equation Kᵢ = Kᵢ /[I] + Kᵢ where [I] is the concentration of benzamidine and Kᵢ and Kᵢ are the Michaelis constant in the absence and presence of benzamidine, respectively.

**Thrombin interaction with thrombomodulin, fibrinogen, and fibrin.** The thrombin-thrombomodulin interaction was studied as described previously: briefly, the clotting time of fibrinogen by an equimolar complex (28 mmol/L) of active thrombin and thrombomodulin was recorded either in the presence of buffer alone or with increasing amounts of TLCK-thrombin. The thrombin-thrombomodulin complex gave a clotting time of 140 seconds, and the displacement of active thrombin by inactivated thrombin was evidenced by a decrease in the clotting times. A control was included in which active thrombin was diluted to yield a clotting time of 140 seconds in the absence of thrombomodulin; the addition of TLCK-thrombin (2- to 100-fold molar excess) did not modify the clotting time.

TLCK-thrombins were prepared by adding TLCK (15 mmol/L, final concentration) to thrombin (28 mmol/L) in 20 mmol/L Tris, pH 7.5, and 0.5 mol/L NaCl. After 15 minutes of incubation at room temperature, the solution was brought to 30 mmol/L TLCK and the incubation continued overnight at 4°C. TLCK-thrombins were extensively dialyzed against 20 mmol/L Tris and 150 mmol/L NaCl, pH 7.5.

The interaction of fibrinogen with thrombin was evaluated as described by Lundblad et al by studying the influence of fibrinogen on the rate of thrombin inactivation by DFP. Thrombin was diluted (0.30 to 0.34 mmol/L) in 20 mmol/L Tris, 20 mmol/L sodium pyrophosphate, 150 mmol/L NaCl, pH 7.5, and 1% bovine serum albumin (BSA) containing 11.8 mmol/L fibrinogen. An aliquot was immediately removed to determine the initial thrombin activity. DFP was then added to the solution at final concentrations of 0.15 and 0.30 mmol/L for normal thrombin and thrombin Salakta, respectively. At intervals, aliquots were removed and assayed for residual thrombin activity by using S 2238. The interaction of thrombin with fibrin was investigated by using fibrin-Sepharose columns prepared as described by Berliner et al. A 10-μl sample of thrombin (1 mg/mL) was applied to the resin equilibrated in 50 mmol/L Tris, pH 7.5, and the column (0.9 x 2 cm) was washed with the equilibrating buffer. Bound thrombin was then eluted with a linear NaCl gradient (0 to 0.3 mol/L) in 50 mmol/L Tris, pH 7.5, at 22°C. Each fraction was assayed for thrombin activity by using S 2238.

**RESULTS**

**Catalytic properties of thrombin Salakta.** Thrombin Salakta was 93.1% ± 7.6% (m ± SD) active when titrated with NPGB + 95.4% ± 6.6% for normal thrombin.

Table 1 summarizes the kinetic parameters of S 2238 hydrolysis. Thrombin Salakta is characterized by a normal kcat but an increased Kᵢ, which results in a decreased catalytic efficiency (kcat/Kᵢ).

**Thrombin inhibition.** As expected for irreversible inhibitors, the time-dependent loss of thrombin catalytic activity in the presence of DFP (Fig 1A), TLCK (Fig 1B), and AT (Fig 1C) followed pseudo-first-order kinetics. This is illustrated

<table>
<thead>
<tr>
<th>Table 1. Hydrolysis of S 2238</th>
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<tr>
<td></td>
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<tr>
<td><strong>Thrombin</strong></td>
</tr>
<tr>
<td>Salakta</td>
</tr>
<tr>
<td>(n = 4)</td>
</tr>
<tr>
<td><strong>Normal</strong></td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>(n = 5)</td>
</tr>
<tr>
<td>Kᵢ (μmol/L)</td>
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<tr>
<td>k₉ₐ (s⁻¹)</td>
</tr>
<tr>
<td>k₉ₐ/Kᵢ (10² M⁻¹ s⁻¹)</td>
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</table>

Values are indicated as means ± SD. Hydrolysis was carried out with 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% PEG, pH 7.8, at 25°C.
by the linearity of the plots of the ln of thrombin activity vs time. The $k_2$ values are given in Table 2. The rate constant for thrombin Salakta inhibition by AT is 17.5-fold lower than that observed for normal thrombin. The rate constants for thrombin Salakta inhibition by DFP and TLCK are also reduced, although to a lesser extent (2.5-fold and 3.2-fold, respectively).

Benzamidine behaves as a competitive inhibitor of thrombin. Consequently, hydrolysis of S 2238 in the presence of benzamidine (Fig 2) results in an increase in the apparent $K_m$ but does not affect $V_{max}$. A Lineweaver-Burk plot was obtained from velocity data in the absence and at a fixed concentration of the inhibitor. $K_c$, calculated as in Methods, was 3.5 mmol/L for thrombin Salakta vs 0.28 mmol/L for normal thrombin, thus indicating that the affinity of benzamidine for thrombin Salakta is reduced.

Factor V activation. Figure 3 shows the relative initial rates of bovine factor V activation upon the addition of thrombin. A comparison of the slopes indicates that factor V activation is 4.4-fold slower with thrombin Salakta as compared with normal thrombin.

Protein C activation and thrombomodulin interaction. Activation of protein C by thrombin Salakta is three fold slower than normal, either in the absence (Fig 4A) or in the presence of thrombomodulin (Fig 4B). The apparent enhancement of protein C activation by rabbit thrombomodulin, calculated after correction for the different concentrations of thrombin used in the two experiments, was 400-fold for both normal thrombin and thrombin Salakta. However, a study of the displacement of normal active thrombin from the thrombin-thrombomodulin complex by TLCK-thrombin Salakta (Fig 5) indicated a slight decrease in the affinity of thrombin Salakta for thrombomodulin when compared with normal thrombin. This abnormality had been masked in the former experiment (Fig 4), presumably because a molar excess of thrombomodulin over thrombin was used.

Thrombin interaction with fibrinogen and fibrin. The clotting activity of thrombin Salakta was 535 U/mg vs 3,200

![Diagram](image)

**Table 2. Kinetics of Thrombin Inhibition by AT, DFP, or TLCK**

<table>
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<th>Inhibitor</th>
<th>$k_2, \text{M}^{-1} \times \text{s}^{-1}$</th>
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<tr>
<td>AT</td>
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<tr>
<td>DFP</td>
<td>$4.70 \pm 0.37$</td>
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<tr>
<td>TLCK</td>
<td>$0.19 \pm 0.01$</td>
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</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_2, \text{M}^{-1} \times \text{s}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$11,000 \pm 1,700$</td>
</tr>
<tr>
<td>Salakta</td>
<td>$11.73 \pm 1.42$</td>
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</table>

Values are indicated as means ± SD.

Fig 1. Kinetics of inactivation of thrombin Salakta by DFP (A), TLCK (B), and AT (C). (Panel A) Thrombin Salakta (35 nmol/L) was incubated in 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% PEG, pH 7.8, at 25°C with various concentrations of DFP: O, 0.1 mmol/L; ●, 0.25 mmol/L; △, 0.5 mmol/L; ▲, 0.75 mmol/L; □, 1 mmol/L. (Panel B) Thrombin Salakta (33 nmol/L) was incubated in 10 mmol/L HEPES, 10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% PEG, pH 7.8, at 25°C with various concentrations of DFP: O, 0.5 mmol/L; ●, 0.7 mmol/L; ●, 1.0 mmol/L; △, 1.5 mmol/L; □, 2.0 mmol/L. (Panel C) Thrombin Salakta (30 nmol/L) was incubated in 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, and 0.1% PEG at 37°C with various concentrations of AT: O, 0.97 μmol/L; ●, 2.84 μmol/L; △, 4.87 μmol/L; ▲, 7.8 μmol/L; □, 9.7 μmol/L. In the three experiments (A, B, and C) residual thrombin activity was assayed at timed intervals by using S 2238 (0.125 mmol/L). The insets show the plots of pseudo-first-order rate constants vs inhibitor concentrations. The slope of the line obtained by linear regression represents $k_2$. 

NaCl, and 0.1% PEG, pH 7.8, at 25°C with various concentrations of DFP: O, 0.5 mmol/L; ●, 0.7 mmol/L; △, 1.0 mmol/L; ▲, 1.5 mmol/L; □, 2.0 mmol/L. In the three experiments (A, B, and C) residual thrombin activity was assayed at timed intervals by using S 2238 (0.125 mmol/L). The insets show the plots of pseudo-first-order rate constants vs inhibitor concentrations. The slope of the line obtained by linear regression represents $k_2$. 

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U/mg for normal thrombin. The reduced ability of thrombin Salakta to clot fibrinogen was further explored by studying the affinity of the variant for either fibrinogen or fibrin.

Human fibrinogen was used as a competitor in the inactivation of thrombin by DFP; it was assumed that thrombin bound to fibrinogen will not react with DFP. Results obtained in an experiment with normal thrombin are shown in Fig 6A. The presence of 11.8 μmol/L fibrinogen clearly prevented thrombin inhibition by DFP. In contrast (Fig 6B), the rate of inhibition of thrombin Salakta by DFP was only minimally reduced in the presence of an identical fibrinogen concentration. This result indicates that the affinity of thrombin Salakta for fibrinogen is reduced.

Binding of thrombin to fibrin was studied by using fibrin monomers covalently linked to Sepharose. When using a...
all the enzyme was bound to the column. Normal thrombin activity was measured by using S 2238 (0.3 mmol/L thrombin and thrombin Salakta by DFP. Thrombin was incubated at 25°C in 20 mmol/L Tris, 20 mmol/L sodium pyrophosphate, 150 mmol/L NaCl, pH 7.5, and 1% BSA containing DFP, either in the absence (A) or in the presence (C, O) of fibrinogen (11.8 μmol/L). Aliquots were removed at the indicated times, and the residual thrombin activity was measured by using S 2238 (0.3 mmol/L). ---, 0.30 mmol/L normal thrombin and 0.15 mmol/L DFP; --.., 0.34 mmol/L thrombin Salakta and 0.30 mmol/L DFP.

Fig 6. The effect of fibrinogen on the inactivation rate of normal thrombin or thrombin Salakta by DFP. Thrombin was incubated at 25°C in 20 mmol/L Tris, 20 mmol/L sodium pyrophosphate, 150 mmol/L NaCl, pH 7.5, and 1% BSA containing DFP, either in the absence (A) or in the presence (C, O) of fibrinogen (11.8 μmol/L). Aliquots were removed at the indicated times, and the residual thrombin activity was measured by using S 2238 (0.3 mmol/L). ---, 0.30 mmol/L normal thrombin and 0.15 mmol/L DFP; --.., 0.34 mmol/L thrombin Salakta and 0.30 mmol/L DFP.

Fig 7. Elution profiles of normal thrombin (O) and thrombin Salakta (O) from fibrin-Sepharose. The fibrin-Sepharose column (0.9 x 2 cm) was equilibrated in 50 mmol/L Tris, pH 7.5. Thrombin (10 μg) was applied and eluted upon application of a linear gradient of NaCl (0 to 0.3 mol/L) in 50 mmol/L Tris, pH 7.5, at 22°C. Each fraction was assayed for thrombin activity by using S 2238 (0.2 mmol/L).

DISCUSSION

In a previous report6 we have shown that the abnormal activity of prothrombin Salakta is not related to the impairment of one of the factor Xa-catalyzed cleavages but rather to an altered enzymatic activity of the generated thrombin. The mutant Salakta does not differ from normal thrombin by size, as judged by SDS-polyacrylamide gel electrophoresis, and is therefore assumed to result from a single amino acid substitution. On a theoretical basis, this point mutation could affect either the catalytic triade or one of the substrate binding sites. The abnormal enzymatic properties of thrombin Salakta are evidenced by the values of the kinetic parameters determined on S 2238 and the altered reactivity toward all the macromolecular substrates or chemical inhibitors examined in this study.

The kcat value of thrombin Salakta on S 2238 is normal, thus indicating that the limiting step in the catalytic process, ie, the deacylation of the acyl-enzyme intermediate, is preserved. On the contrary, the elevated Km value of thrombin Salakta for S 2238 is evidence that the molecular defect affects the formation of the acyl-enzyme intermediate either by altering substrate binding or by impairing the acylation step. Although the normal active site titration with NPGB is in favor of a preserved catalytic site, the rate of inhibition by the active site-directed irreversible inhibitor DFP is slightly decreased so that the possibility of subtle changes in the microenvironment of the catalytic Ser(205) cannot be totally ruled out. The abnormal interaction of thrombin Salakta with benzamidine, however, strongly suggests that the structural abnormality mainly affects the primary binding site, either directly or indirectly. Benzamidine is a cationic compound of small molecular size. Its inhibitory activity is solely related to the occupancy of the primary binding pocket through an ionic interaction with the negatively charged aspartate in position 199. No significant extension of the contact area exists beyond this pocket.24 Therefore, an alteration of the primary binding site could be the major factor accounting for a decreased binding of S 2238 to thrombin Salakta, its reduced reactivity towards TLCK, and most probably its abnormal interaction with the macromolecular substrates such as AT, protein C, factor V, and fibrinogen.

Several studies suggest that thrombin recognizes discrete domains located on both the fibrinopeptide and the fibrin monomer.25,26 We studied the affinity of thrombin Salakta for fibrinogen by measuring its rate of inhibition by DFP in the presence of fibrinogen. The experiments were performed in the presence of pyrophosphate at concentrations that prevent thrombin binding to fibrin monomers.21 Thus, under these conditions, interaction with fibrinogen should occur primarily through the fibrinopeptide groove of thrombin. The failure of fibrinogen to modify the rate of thrombin Salakta inhibition by DFP indicates a decrease in the affinity of thrombin Salakta for fibrinogen and is consistent with a defect of the primary binding pocket as proposed earlier. In contrast with the major defect in fibrinogen recognition through the fibrinopeptide groove, we observed only a slight decrease in the affinity of thrombin Salakta for either fibrin monomers or thrombomodulin. A defect in the catalytic or the primary binding site of thrombin Salakta cannot account per se for this decrease because thrombin interactions with fibrin27 or thrombomodulin28 are independent of the catalytic center. It is possible, however, that a mutation affecting predominantly the primary binding site might also alter, although to a lesser extent, binding sites remote from the catalytic center by inducing conformational changes.

Thrombin Salakta differs from all other mutants reported thus far. Thrombin Quick, which exhibits normal esterolytic and amidolytic activities in contrast to abnormal interactions
with fibrinogen, prothrombin, platelets, and endothelial cells,1,2,3 is most probably characterized by a molecular defect affecting the secondary binding sites for macromolecular substances.4,5 The enzymatic properties of thrombins Metz7 and Tokushima9 are in many points comparable to those of thrombin Salakta, but these two variants present with an abnormal $k_{\text{cat}}$ value for S 2238, which is suggestive of an alteration in the catalytic site. Indeed, the structural defect of thrombin Tokushima has been recently reported to correspond to the replacement of Arg by Trp at position 98 from the amino terminus of the thrombin B chain.6 It is very likely that this substitution alters the behavior of Asp 99, one of the residues of the catalytic triade.

In conclusion, the present study indicates that thrombin Salakta exhibits a specific pattern of functional alterations. Thus, its structural defect is likely to be different from those of the other thrombin variants described to date. Our results strongly suggest that the mutation disturbs primarily the function of the substrate primary binding site.

REFERENCES

Functional characterization of thrombin Salakta: an abnormal thrombin derived from a human prothrombin variant

A Bezeaud, J Elion and MC Guillin