Factor XIIIa Formation Promoted by Complexing of α-Thrombin, Fibrin, and Plasma Factor XIII

By Charles S. Greenberg, Komandoor E. Achyuthan, and John W. Fenton II

Fibrin polymers (des A,B fibrinogen) reduced the concentration of α-thrombin required for 50% activation of plasma factor XIII (a₂b₂ tetramer) by approximately 100-fold. In the presence of fibrin, the amount of γ-thrombin required for activation was not affected. Catalytically inactive i-Pr2P- and d-Phe-Pro-Arg-CH₂-Cl, α-thrombin were found to inhibit over 95% of the activation by α-thrombin in the presence of fibrin. Unlike plasma factor XIII, the concentration of α-thrombin required for 50% activation of platelet factor XIII (a₂ dimer) was lower, and the activation was not enhanced by fibrin. However, when the α₂ platelet factor XIII was incubated with purified b-chains, the α- and γ-thrombin concentrations required for activation increased tenfold and reached levels similar to those required for activation of the plasma factor XIII. When fibrin was present, the α-thrombin concentrations needed for activation of the a₂b₂ complexes were reduced, and the presence of fibrin had no effect on γ-thrombin cleavage of the a₂b₂ complexes. Therefore, the b-chains must inhibit a-chain cleavage by α-thrombin in the absence of fibrin. These results imply that the formation of a cocomplex involving α-thrombin, fibrin, and plasma factor XIII causes some conformational change in plasma factor XIII such that the b-chains no longer inhibit cleavage of the a-chains.

© 1987 by Grune & Stratton, Inc.

Materials and Methods

Factor XIII–deficient plasma was purchased from George King Biomedical (Overland Park, KS) and adsorbed with 15 mg/mL of bentonite (Sigma Chemical Co, St Louis) for 30 minutes at 22 °C.® Human plasma and platelet factor XIII were purified from fresh, frozen plasma and stored at −70 °C.®° Fibrinogen and des-A,B-fibrinogen were prepared as previously described,® and d-Phe-Pro-Arg-CH₂-Cl, i-Pr₂P-F, anti–factor XIII a-chain and anti–factor XIII b-chain antibodies were purchased from Calbiochem-Behring Corp (La Jolla, CA).

Thrombin preparations. Human α-thrombin was purified from fraction III paste and stored frozen at −70 °C in 0.75 mol/L NaCl, pH 6.0. This preparation contained a final protein concentration of 69.7 μmol/L, an active site titration of 68.2 μmol/L, and fibrinogen clotting activity of 3,100 US National Institutes of Health (NIH) U/mg.®° γ-Thrombin was prepared by passing purified α-thrombin through a trypsin-agarose column®® and stored as noted before (52 μmol/L protein, 24.8 μmol/L active site titration, and 0.36 NIH U/mg activity). Inactivated i-Pr₂P-α-thrombin was prepared by repeated additions of the fluoride reagent to α-thrombin until the clotting activity declined to <2%. This was followed by extensive charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
dialysis against 0.75 mol/L NaCl at 4 °C, and the enzyme was stored frozen (52 μmol/L protein, <0.5 μmol/L active site titration, and 0.37 NIH U/mg activity). Affinity-labeled d-Phe-Pro-Arg-CH2-α-thrombin was made by incubating equimolar amounts of the chloromethyl ketone reagent and α-thrombin for one hour followed by dialysis to remove unreacted reagent. Protein concentrations were calculated from absorbance at 280 nm by using an extinction coefficient of 1.83 mg/mL/cm and a molecular weight of 36,500 for α-thrombin.19 All concentrations of reagents used in the study were based upon the absolute protein concentrations rather than the active site concentrations.

Measurement of α- and γ-thrombin cleavage of plasma and platelet factor XIII in the presence and absence of des-A,B-fibrinogen. α- or γ-thrombin at 0.01 to 1,000 nmol/L was incubated with 6 nmol/L platelet factor XIII or 9 nmol/L plasma factor XIII for five minutes at 37 °C in the presence or absence of 620 nmol/L des-A,B-fibrinogen in Tris-buffered saline (0.13 mol/L NaCl at pH 7.4 and 0.02 mol/L Tris-HCl (TBS), and 5 mmol/L CaCl2. Thrombin was inhibited by a 100-fold molar excess of d-Phe-Pro-Arg-CH2-Cl, and factor XIIIa was assayed after stabilization in a buffer containing glycerol at 4 °C.19 Preliminary experiments demonstrated that the presence of d-Phe-Pro-Arg-CH2-Cl did not interfere with the factor XIIIa assay. Less than 5% of the factor XIIIa preparation expressed activity in the absence of α-thrombin, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated over 87% cleavage of the a-chains by thrombin in the factor XIII preparations.3

Factor XIIIa assay. Plasma and platelet factor XIIIa formation was quantitated using the [3H]-putrescine incorporation assay.21 Factor XIIIa activity was expressed as nanomoles of putrescine incorporated into casein in 60 minutes at 37 °C, where this activity correlated with thrombin cleavage of the a-chains.5 Factor XIIIa activity was expressed as the percentage of total factor XIIIa determined after incubating samples with 5 mmol/L CaCl2 and 190 nmol/L α-thrombin at 37 °C for 15 minutes.

Effect of i-Pr2-P- and d-Phe-Pro-Arg-CH2-γ-thrombin on α-thrombin cleavage of plasma factor XIII in the presence or absence of fibrinogen. Concentrations of either i-Pr2-P-thrombin or d-Phe-Pro-Arg-CH2-thrombin ranging from 9 to 90 nmol/L were incubated at 22 °C for five minutes in TBS containing 9 nmol/L plasma factor XIII and the presence or absence of 620 nmol/L fibrinogen. These reaction mixtures were subsequently incubated for five minutes at 37 °C with 9 nmol/L α-thrombin followed by the addition of 1 mmol/L d-Phe-Pro-Arg-CH2-Cl. Factor XIIIa activity was measured by the [3H]-putrescine incorporation assay. Samples of the reaction mixtures that contained fibrinogen were further ultrafiltrated through an Amicon ultrafiltration cell using an XM 100A membrane (Amicon Corp. Danvers, MA) to determine whether the effect of the modified thrombins was reversible.

Effect of plasma factor XIII b-chains on α-thrombin cleavage of factor XIII a-chains. Plasma containing only factor XIII b-chains was prepared by bentonite adsorption of factor XIII-deficient plasma. The adsorbed plasma was electrophoresed through agarose gels containing either anti-a-chain or anti-b-chain antisera to identify the location of radiolabeled factor XIII a-chains bound to fibrinogen. Autoradiograms were scanned using a GS-300 scanning densitometer (Hoefer, San Francisco) to quantitate the amount of 125I-labeled a-chains that had complexed with b-chains.

Measurement of fibrin polymerization. Purified fibrinogen at 2 mg/mL was incubated with 10 nmol/L of α-thrombin in TBS containing 5 mmol/L CaCl2. The level of fibrin polymerization was measured as previously described,22 and the rate of polymerization was calculated as a function of the maximum slope of the polymerization curve.

RESULTS

Fibrin polymers generated from des A,B fibrinogen reduced the level of α-thrombin required for 50% activation of plasma factor XIII by approximately 100-fold. In contrast, the level of γ-thrombin required for 50% activation had slightly increased in the presence of fibrin (Fig 1). On a functional enzyme basis, the α-thrombin and γ-thrombin preparations were 97.8% active (μmol/L active site/μmol/L protein x 100) and 47.7% active, respectively. Therefore, α-thrombin exhibited an approximately 15-fold increase over γ-thrombin in its ability to activate the plasma factor XIII in the absence of fibrin and an increase greater than two orders of magnitude over γ-thrombin in the presence of fibrin. In control experiments, 1000 nmol/L γ-thrombin could not
inhibit activation of the plasma factor by 1.0 nmol/L \( \alpha \)-thrombin in either the presence or absence of fibrin.

Since the fibrin used in the aforementioned experiments was generated by diluting ten- to 20-fold des A,B fibrinogen in 1 mol/L NaBr, the high salt concentrations could potentially interfere with thrombin binding to fibrin(ogen). To address this issue, experiments were conducted with fibrin generated in situ from fibrinogen reacted with \( \alpha \)-thrombin. We found that a tenfold molar excess of inactivated i-Pr\(_2\)P- or D-Phe-Pro-Arg-CH\(_2\)-\( \alpha \)-thrombin inhibited over 95% of the activation of plasma factor XIII by \( \alpha \)-thrombin in the presence of fibrin(ogen) (Fig 2), but neither appreciably inhibited factor XIII activation in the absence of fibrin(ogen).

The highest concentrations of catalytically inactivated thrombins did not interfere with fibrin formation by \( \alpha \)-thrombin in the fibrinogen-containing solutions. However, when these solutions were subsequently ultrafiltered through membranes with a 100,000-dalton cutoff, they regained their ability to promote \( \alpha \)-thrombin activation of factor XIII. Dialyzing the modified-thrombin solutions did not interfere with their ability to competitively inhibit \( \alpha \)-thrombin cleavage of the plasma factor XIII with fibrin. In addition, when 1.8 \( \mu \)mol/L \( \alpha \)-thrombin was added to 9 nmol/L plasma factor XIII with 90 nmol/L i-Pr\(_2\)P-\( \alpha \)-thrombin, additional factor XIIIa was formed.

Unlike the plasma factor, platelet factor XIII required approximately tenfold less \( \alpha \)-thrombin for 50% activation, and the activity was not enhanced by the presence of fibrin (Fig 3). \( \gamma \)-Thrombin was at least an order of magnitude less active relative to \( \alpha \)-thrombin and was essentially unaffected by the presence of fibrin. However, when platelet factor XIII was incubated with either afibrinogenemic plasma containing \( \beta \)-chains or isolated \( \beta \)-chains from plasma factor XIII, the \( \alpha \)-thrombin concentration required to activate the \( \alpha \)2\( \beta \)2 complexes increased by approximately tenfold in the absence of fibrin and was decreased by approximately tenfold in the presence of fibrin (Fig 4). The presence of \( \beta \)-chains inhibited \( \gamma \)-thrombin cleavage of platelet factor XIII (Fig 5), and the presence of fibrin was not found to enhance \( \gamma \)-thrombin cleavage of the \( \alpha \)2\( \beta \)2 complexes (data not shown).

In additional experiments, platelet factor XIII \( \alpha \)-chains were radiolabeled with \( ^{125} \)I and incubated with isolated factor XIII \( \beta \)-chains. When electrophoresed according to Lorand et al., the \( \alpha \)-chains migrated as the \( \alpha \)2\( \beta \)2 complex, which comigrated with plasma factor XIII (data not shown).

**DISCUSSION**

Fibrin generated from the polymerization of des A,B fibrinogen clearly amplified the \( \alpha \)-thrombin activation of plasma factor XIII by approximately 100-fold. This form of the human enzyme exhibits high fibrinogen-clotting activity as well as all other thrombin-associated characteristics.9,20 The autoproteolytic or tryptic degradative form of thrombin, \( \gamma \)-thrombin, lacks clotting activity but retains its substrate-binding and certain other enzymic traits.20 Although this form has been shown to activate plasma factor XIII,14 such activation was not found to be enhanced by the presence of fibrin. This suggests that a fibrin(ogen) recognition site necessary for clotting must be involved in the enhancement phenomenon.

Like \( \alpha \)-thrombin, catalytically inactivated forms bind tightly to nonpolymerized fibrin immobilized in agarose and are eluted by similar salt concentrations. Both of the modified forms compete for \( \alpha \)-thrombin binding sites and were found to inhibit 95% of the activation of plasma factor XIII by the native enzyme. In contrast, the binding of \( \gamma \)-thrombin to nonpolymerized fibrin is very weak11 and was not found to compete with \( \alpha \)-thrombin in the activation of plasma factor XIII. Since the i-Pr\(_2\)P-group is an obstructive moiety conjugated to the catalytic serine residue and the D-Phe-Pro-Arg-CH\(_2\) group fills the fibrinopeptide groove adjacent to

---

**Fig 2.** Inhibition of \( \alpha \)-thrombin activation of plasma factor XIII with catalytically inactivated thrombins in the presence of fibrin. Des A,B fibrinogen and plasma factor XIII were incubated with increasing concentrations of either i-Pr\(_2\)P- (O—O) or D-Phe-Pro-Arg-CH\(_2\)-\( \alpha \)-thrombin (□—□) for 15 minutes prior to assaying factor XIIIa activity, as described in the text. Similar reactions were performed in the absence of fibrin (closed symbols).

**Fig 3.** Platelet factor XIII activation by \( \alpha \)- or \( \gamma \)-thrombin in the presence or absence of fibrin polymers. See Fig 1: \( \alpha \)-thrombin alone (O—O) or \( \alpha \)-thrombin plus des A,B fibrinogen (■—■); \( \gamma \)-thrombin alone (□—□) or \( \gamma \)-thrombin plus des A,B fibrinogen (■—■).
the catalytic site, an exosite independent of the catalytic site and adjacent regions must be required for \( \alpha \)-thrombin complexing with fibrinogen.

The presence of fibrin was not found to enhance the activation of platelet factor XIII by \( \gamma \)-thrombin. However, when the platelet factor, which has an \( \alpha_2 \)-chain structure, was incubated with \( \beta \)-chains from the plasma factor XIII, an \( \alpha_2\beta_2 \) complex was formed. The activation of this hybrid complex by \( \alpha \)-thrombin was enhanced by approximately 20-fold in the presence of fibrin. Although the isolated \( \beta \)-chains cannot complex with fibrinogen alone, yet the \( \alpha_2 \) dimers can, in vitro complexing of \( \alpha \)-chains with \( \beta \)-chains clearly inhibited \( \alpha \)- and \( \gamma \)-thrombin cleavage of the \( \alpha \)-chain when fibrin was absent. Thus, our data strongly suggest that activation of plasma factor XIII proceeds via the formation of a cocomplex involving \( \alpha \)-thrombin, the \( \alpha_2\beta_2 \) complex, and fibrin.

Earlier investigations suggested that plasma factor XIII \( \beta \)-chains inhibit \( \alpha \)-thrombin cleavage of the \( \alpha \)-chains. Our data support these findings since approximately tenfold more \( \alpha \)-thrombin or \( \gamma \)-thrombin was required to activate plasma factor XIII or the hybrid \( \alpha_2\beta_2 \) complexes relative to platelet factor XIII \( \alpha_2 \). Our data further demonstrate a previously unrecognized change in the interaction between the \( \alpha \)- and \( \beta \)-chains during the clotting event. We have found that the \( \beta \)-chains inhibit cleavage of the \( \alpha \)-chains by \( \alpha \)- and \( \gamma \)-thrombins in the absence of fibrin. However, in the presence of fibrin, the \( \beta \)-chains no longer inhibit \( \alpha \)-thrombin cleavage of the \( \alpha \)-chains. Since the \( \gamma \)-thrombin concentration required to cleave the \( \alpha \)-chains was not affected by the presence of fibrin, the binding of thrombin to fibrin must be required for thrombin activation of plasma factor XIII. We propose that the \( \beta \)-chains bind to the \( \alpha \)-chains of the \( \alpha_2\beta_2 \) complex, which protects them from thrombin binding in circulating blood. However, in the presence of fibrin, a binding of the \( \alpha_2\beta_2 \) complex to the fibrin polymer leads to a conformational change in the \( \alpha \)- and \( \beta \)-chain geometry, which promotes formation of the activation complex.

Preliminary experiments have indicated that non-cross-linked fibrin protofibrils promote \( \alpha \)-thrombin activation of plasma factor XIII, whereas other data show that soluble, cross-linked fibrin does not. Thus, the activation complex appears to occur temporarily during fibrin polymerization. This is also true in the active entrapment process whereby \( \alpha \)-thrombin becomes incorporated into fibrin clots.

The mechanism by which fibrin modifies the interaction between \( \alpha \)- and \( \beta \)-chains to promote thrombin cleavage does not appear to involve a requisite dissociation of the \( \beta \)-chains from the complex since des-A,B fibrinogen promotes thrombin cleavage of plasma factor XIII in the presence of EDTA under conditions whereby the \( \beta \)-chains do not dissociate from the \( \alpha \)-chains. The \( \beta \)-chain may actively promote a conformational change in the \( \alpha \)-chain, or the \( \alpha \)-chains may undergo a conformational change that rearranges the position of the \( \beta \)-chains within the complex.

ACKNOWLEDGMENT

The technical assistance of Charles C. Miraglia was greatly appreciated.

REFERENCES

4. Janus TJ, Lewis SD, Lorand L, Shafer JA: Promotion of
MECHANISM FOR PLASMA FACTOR XIII ACTIVATION

Factor XIIIa formation promoted by complexing of alpha-thrombin, fibrin, and plasma factor XIII

CS Greenberg, KE Achyuthan and JW 2d Fenton