Regulation of Formation of Factor XIIIa by Its Fibrin Substrates†

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ABSTRACT: Thrombin-catalyzed release of activation peptide (AP) from plasma factor XIII was studied to characterize the regulation of this initial step in the activation of factor XIII zymogen (fibrin-stabilizing factor). High-performance liquid chromatography was used to monitor the kinetics of release of AP. Non-cross-linked polymeric fibrins I and II (polymerized des-A- and des-A,B-fibrinogens), physiological substrates of factor XIIIa, were shown to be potent promoters of thrombin-catalyzed release of activation peptide from factor XIII. These promoters are proposed to act by complexing factor XIII and reducing the apparent K_m for thrombin-catalyzed release of AP. Since thrombin-catalyzed release of AP is inefficient in the absence of polymerized fibrin, this mode of regulation should minimize formation of factor XIIIa prior to the formation of its fibrin substrates. The promoting activity of polymeric fibrin was rapidly lost when catalytically competent factor XIIIa was allowed to form. This observation suggested the possibility that factor XIIIa catalyzed cross-linking of fibrin inactivates fibrin as a promoter for the thrombin-catalyzed release of AP from factor XIII. Consistent with this view, the thiol reagent *S*-methyl methanethiosulfonate inactivated factor XIIIa, blocked cross-linking of fibrin, and protected against loss of its promoter activity. This mode of feedback regulation of the activation process by catalytically active factor XIIIa may serve to ensure against continued generation of factor XIIIa after its fibrin substrates have been cross-linked.

The transamidase factor XIIIa catalyzes formation of γ-glutamyl-ε-lysyl peptide cross-links in fibrin that increase the chemical stability and mechanical strength of blood clots. Factor XIIIa is generated from its zymogen factor XIII during the last stages of the blood-clotting cascade. [For a recent review, see Lorand et al. (1980)].

Activation of plasma factor XIII (a_2b_2) proceeds via the multistep reaction depicted in eq 1. In the initial proteolytic

$$a_2b_2 \xrightarrow[\text{AP}]{\text{thrombin}} a_2'b_2 \xrightarrow[b_2]{Ca^{2+}} a_2' \xrightarrow{Ca^{2+}} a_2^* \qquad (1)$$

step (Lorand & Konishi, 1964), thrombin catalyzes hydrolysis at Arg-a36 to release the 36 amino acid activation peptide (AP)[1] from the N-terminus of the a chains (Takagi & Doolittle, 1974). Subsequent to this proteolytic event, calcium-dependent reactions occur in which the b chains dissociate and the remaining a_2' ensemble undergoes a conformational change to a_2^* (factor XIIIa), which unmasks the active center thiol group (Lorand et al., 1974; Curtis et al., 1974).

We recently showed that the initial proteolytic event is very inefficient unless fibrinogen is present (Janus et al., 1983). Now we present evidence that indicates that non-cross-linked polymeric fibrins I and II (polymerized des-A-fibrinogen and des-A,B-fibrinogen), the substrates for factor XIIIa, are the true promoters for the thrombin-catalyzed release of AP from factor XIII. Thus, when fibrinogen and factor XIII are both present, thrombin catalyzes proteolysis at Arg-Aα16 in fibrinogen to release FPA and produce fibrin I, which then undergoes end to end polymerization to form fibrin I polymer, an immediate promoter of thrombin-catalyzed hydrolysis at Arg-a36 in factor XIII. Evidence is also presented that suggests that catalytically competent factor XIIIa, the final product of the activation pathway, inactivates polymeric fibrin as a promoter of the thrombin-catalyzed release of AP.

Experimental Procedures

Previously described procedures were used to prepare fibrinogen (Lewis & Shafer, 1984) and factor XIII (Lorand et al., 1981). The resulting fibrinogen was chromatographed on an immobilized phenylmercuri derivative according to the procedure of McDonagh et al. (1976) to remove any contaminating factor XIII. Fibrin I was prepared by allowing 5 mL of a solution of fibrinogen (0.6 mg/mL) in 0.14 M NaCl–9.5 mM phosphate buffer, pH 7.4, to react at 37 °C for 4 h with 0.01 mL of a solution of reptilase R, which was obtained by addition of 1 mL of water to a vial containing 34 mg of lyophilized reptilase R from Abbott Laboratories. After the reaction mixture was cooled in an ice bath, the resulting clot was collected on a glass rod, rinsed with water, and dissolved in 0.4 mL of 0.02 M acetic acid. An $E^{1\%}_{280}$ of 14.0 and an M_r of 340 000 were used to calculate the concentration of fibrin I and fibrin II monomers in 0.02 M acetic acid. Pure human α-thrombin with a specific activity of 24 TU/μg [using the method of Lewis & Shafer (1984)] was generously supplied by Dr. John Fenton II. Fibrin II was prepared in a manner similar to that used for the preparation of fibrin I, except that thrombin (0.3 TU/mL) was used in place of reptilase. Additionally, the solution of the fibrin II clot in 0.4 mL of 0.02 M acetic acid was diluted with 0.6 mL of H_2O and mixed with 1 mL of 19 mM sodium phosphate buffer (initially at pH 7.4),

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[1] Abbreviations: AP, activation peptide; DTT, dithiothreitol; FPA, fibrinopeptide A; FPB, fibrinopeptide B; HPLC, high-performance liquid chromatography; GPRP, glycyl-L-prolyl-L-arginyl-L-proline; MMTS, *S*-methyl methanethiosulfonate; PEG, poly(ethylene glycol) 6000; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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containing sufficient hirudin to neutralize 1.0 TU of thrombin. The resulting clot was collected, rinsed with water, and redissolved in 0.2 mL of 0.02 M acetic acid.

Reaction Kinetics. The time dependence of the release of fibrinopeptides and activation peptide at pH 7.4, 37 °C, was monitored by HPLC as previously described (Higgins et al., 1981; Janus et al., 1983). The reaction buffer was either 10 mM Tris or 9.5 mM sodium phosphate. Control experiments (in the absence of added Ca^{2+}) indicated that the time dependencies of the release of fibrinopeptides and activation peptide were essentially the same in the Tris and phosphate buffer system. The Tris buffer system was employed in studies of the effects of Ca^{2+} on the reaction kinetics so as to avoid formation of a precipitate (calcium phosphate) that occurred in the phosphate buffer at the higher concentrations of Ca^{2+}. Because both calcium and phosphate are present in plasma, several kinetic studies were done (as indicated in the text) in the phosphate buffer system in the presence of a level of Ca^{2+} (1–1.4 mM) close to that found in plasma. To prevent precipitation of calcium phosphate, a solution of 274 mM NaCl, 5 mM KCl, and 0.2% PEG containing twice the desired Ca^{2+} and protein concentrations was mixed with stirring with an equal volume of 19 mM sodium phosphate, adjusted to yield a final pH of 7.4. This solution of buffer (pH 7.4) did not have any visible precipitate. In most kinetic runs, a reaction solution for each time point was incubated at 37 °C in a 1.5-mL microfuge tube. After thermal equilibration, reaction was initiated by addition of thrombin with rapid mixing. At the specified times, samples were quenched with 0.1 mL of 3 M $HClO_4$ and analyzed as described previously (Janus et al., 1983). When fibrin I or fibrin II was present, the fibrin (in 0.02 M acetic acid) was mixed rapidly in a microfuge tube with buffer containing the desired concentration of factor XIII and sufficient base to neutralize the acetic acid (and bring the pH to 7.4). After allowing the solution to equilibrate at 37 °C for 20 min to allow the fibrin to polymerize, thrombin was added with mixing.

In kinetic runs where MMTS (Aldrich) or GPRP (Vega Biochemicals) was present, freshly prepared solutions of the compounds in water were added to the reaction mixture a few seconds before addition of thrombin. Kinetics of γ-dimer formation were followed in reaction mixtures containing 1.1 μM fibrinogen and 0.10 μM factor XIII, which were equilibrated at 37 °C in the presence of 1 mM $CaCl_2$. The reactions were initiated by addition of thrombin (final concentration 0.29 nM). Prior to clotting, 50-μL aliquots of the reaction mixture were transferred to separate tubes equilibrated at 37 °C. At the specified times, 50 μL of 10 M urea, 2% SDS, and 50 mM DTT with 0.002% bromophenol blue was added followed by heating at 95 °C for 5 min. Samples (50 μL) of this solution were applied to a 6% polyacrylamide gel, prepared according to the method of Laemmli (1970) with a acrylamide:bis-(acrylamide) ratio at 30:0.8. The gels were stained with Coomassie blue, destained, and dried. γ dimer was quantified from the integrated density of the bands as determined with a Zenith Model 5L-TRFF soft laser scanning densitometer. After reaction times of 15 min, the area approached a constant value that was independent of the thrombin concentration and proportional to the initial fibrinogen concentration. The ratio of the density of the γ-dimer band at a given time to its final density was equated to the fraction of γ-dimer formation.

Results and Discussion

In our initial report of the promotion by fibrinogen of the thrombin-catalyzed release of AP from factor XIII, we pointed out a lag in the appearance of AP in the presence of fibrinogen

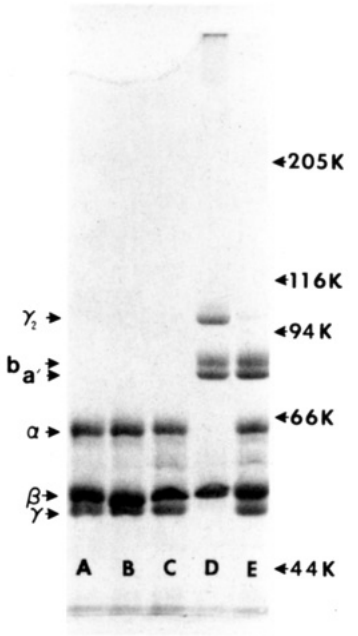


FIGURE 1: Electrophoretic determination of cross-links in fibrin I and fibrin II. The indicated solutions containing 9.5 mM phosphate, 0.14 M NaCl, and 0.1% PEG were incubated at 37 °C, pH 7.4. After 30 min, reaction mixtures were combined with an equal volume of an urea–SDS solution and subjected to SDS–PAGE as described under Experimental Procedures. The reaction mixtures were as follows: A, 1.0 μM fibrin I; B, 1.0 μM fibrin II; C, 1.0 μM fibrin II, 1 mM $CaCl_2$, and 18 nM α-thrombin; D, 1.0 μM fibrin II, 1 mM $CaCl_2$, 18 nM α-thrombin, and 0.2 μM factor XIII; E, 1.0 μM fibrin II, 1 mM $CaCl_2$, 18 nM α-thrombin, 0.2 μM factor XIII, and 1 mM MMTS. The migration positions of γ dimer, of b and a' chains of factor XIII, and of the α, β, and γ chains of fibrin are indicated on the left. Indicated on the right is the migration position of the molecular weight standards myosin (205K), β-galactosidase (116 K), phosphorylase *b* (94K), bovine serum albumin (66K), and ovalbumin (44K).

(Janus et al., 1983). This observation together with the finding that FPA was not a promoter suggested the possibility that the immediate promoter of the release of AP was not fibrinogen but rather an intermediate or product of the thrombin-catalyzed conversion of fibrinogen to fibrin. To assess the possibility that fibrin I or fibrin II (or both) was the true promoter, these materials were prepared and studied. Fibrin I was prepared by treating fibrinogen with reptilase R, an enzyme that selectively releases FPA from fibrinogen (Blomback, 1958). The resulting clot was washed and dissolved in 0.02 M acetic acid. Fibrin II was prepared by treating fibrinogen with thrombin so as to release FPA and FPB. The resulting clot was dissolved in 0.02 M acetic acid and treated with a buffered solution of hirudin (pH 7.4) to reduce contamination by thrombin. The clotted material was collected, washed, and dissolved in 0.02 M acetic acid. The observation that thrombin treatment of fibrin I yielded no detectable FPA (<5%) but yielded FPB in >90% yield suggested that this material was not contaminated by fibrinogen. Additionally, treatment of the fibrin II with excess thrombin yielded no detectable FPA or FPB, indicating the absence of contaminating fibrinogen and fibrin I. The solubility of the fibrin I and fibrin II in 0.02 M acetic together with the absence of γ dimer after SDS gel electrophoresis under reducing conditions indicated that the fibrin I and fibrin II were not cross-linked (see Figure 1, lanes A and B).

In separate experiments, the solutions of fibrin I and fibrin II monomers were mixed with a buffered solution of factor XIII (final pH 7.4). After a 20-min wait to ensure complete polymerization of fibrin,[2] thrombin was added. As shown in

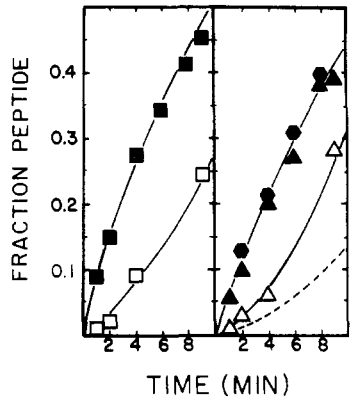


FIGURE 2: Time dependence of the release of FPB, left-hand panel, and AP, right-hand panel, in reaction mixtures containing factor XIII and either fibrinogen (□, △) or polymeric fibrin I (■, ▲) or fibrin II (●). Reaction mixtures contained 0.29 nM thrombin, 0.1 μM factor XIII, and 0.1 μM fibrinogen or 0.1 μM (with respect to fibrin monomer) polymeric fibrin. Reactions were carried out in 9.5 mM phosphate buffer, pH 7.4, 0.14 NaCl, and 0.1% PEG, 37 °C. The solid line for the release of FPB (■) was calculated from eq 2 with a value of 1.22×10^{-3} s^{-1} for $k_{cat}[T]/K_m$. The solid lines for the release of AP in the presence of 0.1 μM fibrin II was calculated from the relationship fraction AP = $1 - \exp(-k_3 t)$, with a value of 9.83×10^{-4} s^{-1} for k_3. Values of 1.22×10^{-3} s^{-1} for k_2 and 9.83×10^{-4} s^{-1} for k_3 were substituted in eq 3 and used to calculate the dashed line, which represents the expected time dependence for AP release in the presence of fibrin I, if fibrin II were the sole promoter of thrombin-catalyzed release of AP from factor XIII.

Figure 2 (right-hand panel), the lag in the release of AP seen with fibrinogen (open triangles) was absent when polymeric fibrin I or fibrin II was used to promote the release of AP (closed triangles and closed hexagons). It should also be noted that the lag in the release of FPB (Figure 2, left-hand panel) seen with fibrinogen as substrate (open squares) was abolished when fibrin I polymer (closed squares) was used. This observation is consistent with previous reports that FPB is preferentially released from fibrin I polymer [e.g. see Higgins et al. (1983) and Lewis et al. (1985)].

Elimination of factor XIII from the assay did not significantly alter the rate of release of FPA or FPB from fibrinogen, as judged by the fit of the data to the line for the release of FPB (Figure 2, left-hand panel). The line for release of FPB from fibrin I polymer was calculated by the relationship

$$\text{fraction FPB} = 1 - \exp(-k_{cat}[T]t/K_m) \qquad (2)$$

where the specificity constant, k_{cat}/K_m, was set at the value (4.2×10^6 M^{-1} s^{-1}) determined in the absence of factor XIII (Higgins et al., 1983). The time and thrombin concentration are denoted by t and [T], respectively. The line for release of FPB from fibrinogen was calculated as described previously (Higgins et al., 1983) with kinetic parameters determined in the absence of factor XIII.

The observation that no release of FPB or AP was detected unless thrombin was added to reaction mixtures containing polymeric fibrin I and factor XIII indicated that none of the release of AP observed upon addition of thrombin to fibrin I could be ascribed to a possible contamination of the fibrin I by reptilase. It is important to note, however, that a low blank rate of AP release was observed in reaction mixtures containing fibrin II and factor XIII but no added thrombin.

[2] Data presented by Hantgan & Hermans (1979) suggest that a 20-min incubation should be adequate to complete polymerization of fibrins I and II. Consistent with this conclusion, the reaction kinetics were unaltered by increases in the time (e.g. to 40 min). On the other hand, lags in the release of activation peptide appeared when the thrombin was added immediately after the addition of the acetic acid solution of fibrin.

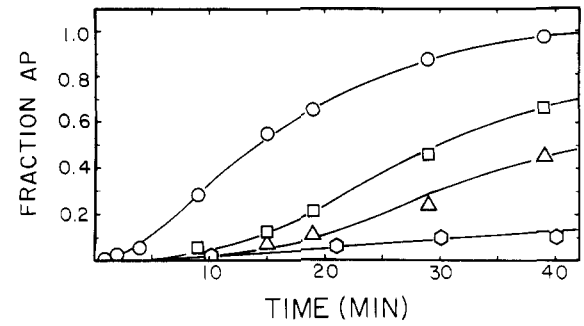


FIGURE 3: Thrombin-catalyzed release of AP from 0.10 μM factor XIII, with 0.10 μM fibrinogen added, in the absence (○) and presence of 0.10 (□), 0.21 (△), and 0.490 mM (○) GPRP. Reactions were carried out as described in Figure 2.

We attribute this blank rate to the presence of thrombin as a contaminant in the fibrin II. The amount of AP released from the blank at each time point (<20% of that observed in the presence of added thrombin) was subtracted from the values observed with added thrombin at the corresponding times to yield the plotted values (closed hexagons) in Figure 2. Thus, the time dependencies (Figure 2) for the release of AP from factor XIII in the presence of fibrin II, fibrin I, and fibrinogen should be directly comparable since they reflect the effect of equivalent amounts of thrombin. The elimination of the lag in AP release with polymeric fibrin II clearly shows that it is competent to promote the thrombin-catalyzed activation of factor XIII.

Assessment of the competence of fibrin I to promote thrombin-catalyzed release of AP from factor XIII is less straightforward, since the elimination of the lag in AP release seen with fibrin I might be ascribed entirely to the promoting activity of the fibrin II that is formed contemporaneously with AP. Quantitative analysis of the time dependence of AP release indicated, however, that fibrin I must also be competent to promote release of AP. In particular, the conversion of fibrin I to fibrin II, as reflected by the release of FPB from fibrin I (Figure 2, left-hand panel), is too slow to account for the similar rates of AP release seen in the presence of fibrin I and fibrin II in terms of a reaction pathway wherein fibrin II is the sole promoter of thrombin-catalyzed release of AP from factor XIII. The discrepancy between the observed time dependence for AP release in the presence of fibrin I and that predicted, assuming that fibrin II is the sole promoter of AP release, is illustrated by the marked deviation of the experimental data from the dashed line in Figure 2. Equation 3

$$\text{fraction AP} = \frac{[AP]}{[AP]_f} = 1 + \frac{k_3 e^{-k_2 t}}{k_2 - k_3} - \frac{k_2 e^{-k_3 t}}{k_2 - k_3} \qquad (3)$$

(dashed line in Figure 2), which describes the time dependence of appearance of the second product in a consecutive first-order reaction, was used to calculate the predicted time depenendence for the release of AP in the presence of fibrin I, assuming only fibrin II promotes AP release. In eq 3, k_2 represents the pseudo-first-order rate constant for the release of FPB (i.e., the conversion of fibrin I to fibrin II), k_3 represents the pseudo-first-order rate constant that approximates the release of AP in the presence of 0.1 μM fibrin II, and [AP] and $[AP]_f$ represent the concentration of [AP] at time t and when the reaction is complete, respectively. The deviation of the experimental data from the dashed line, together with the similar rates of AP release observed in the presence of fibrin I and fibrin II, is most consistent with the view that fibrin I and fibrin II are both competent to promote thrombin-catalyzed release of AP from factor XIII.[3]

The data in Figure 3 show that GPRP, an inhibitor of fibrin polymerization (Laudano & Doolittle, 1978, 1980), antagonizes the enhancement seen when fibrinogen is present. It is unlikely that this effect is due to inhibition of thrombin, since GPRP has no effect on the thrombin-catalyzed release of FPA from fibrinogen (Higgins et al., 1983). Assuming that the sole effect of GPRP is to inhibit polymerization, the simplest explanation of the antagonism by GPRP is that fibrin I monomer is not a promoter of the thrombin-catalyzed release of AP from factor XIII and that the ability of GPRP to act as an antagonist is due to its suppression of the polymerization of fibrin I.

In the absence of fibrinogen or fibrin, the thrombin-catalyzed release of AP from factor XIII is characterized by a K_m of $\sim$80 μM, which is much higher than the plasma concentration of factor XIII (Janus et al., 1983). Thus, thrombin-catalyzed release of AP at plasma concentrations of factor XIII is an inefficient process in the absence of polymeric fibrin. In an attempt to determine how polymeric fibrin might promote the thrombin-catalyzed release of AP from factor XIII, we studied the dependence of the rate of AP release on the factor XIII concentration. Increasing the concentration of factor XIII from 0.3 to 0.6 μM in the presence of 1 and 2 μM fibrin I polymer increased the initial rate of thrombin-catalyzed AP release by a factor of only 1.1–1.2. In the absence of fibrin I polymer, there was a 2-fold increase in rate upon doubling the factor XIII concentration from 0.3 to 0.6 μM. This doubling in rate is consistent with the high K_m for the thrombin-catalyzed release of AP in the absence of fibrin I polymer. The small dependence of the initial rate of release of AP on the factor XIII concentration seen in the presence of fibrin I polymer suggests that in the range of 0.3–0.6 μM factor XIII the thrombin was greater than 50% saturated, so that the apparent K_m for this reaction was probably below 0.3 μM, when $\geq$1 μM fibrin I polymer was present. These observations are consistent with the notion that fibrin I polymer promotes catalysis by forming a complex with factor XIII, which has a higher affinity for thrombin than free factor XIII.

It is important to note that up to this point all of our studies regarding thrombin-catalyzed release of AP from factor XIII were carried out in the absence of Ca^{2+}. Although AP release (the initial event in factor XIII activation) was measured in these studies, enzymically active factor XIIIa was not generated in the absence of Ca^{2+}, since Ca^{2+} is required for formation of catalytically competent factor XIIIa. Thus, fibrin incubated with thrombin and factor XIII in the absence of Ca^{2+} did not become cross-linked (data not shown). Since Ca^{2+}-dependent cross-linking of polymeric fibrin could well alter its activity as a promoter, the effect of Ca^{2+} on the thrombin-catalyzed release of AP in the presence of fibrinogen was studied. The plots in Figure 4A show that 1.4 mM Ca^{2+} abolishes the promotion of AP release that is produced in the presence of 0.1 μM fibrinogen.

The presence of 1.4 mM Ca^{2+} did not effect the unpromoted rate of release of AP from factor XIII observed in the absence of fibrinogen. This can be seen from the fit of the data to the

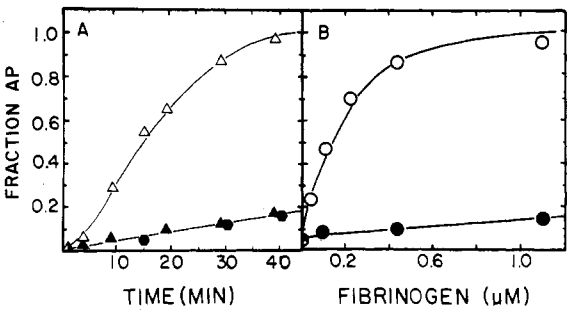


FIGURE 4: (Panel A) Effect of Ca^{2+} on the thrombin-catalyzed release of activating peptide (AP) from 0.10 μM factor XIII in the presence of 0.10 μM fibrinogen, with (▲) and without (△) 1.4 mM $CaCl_2$. Also shown is the thrombin-mediated release of AP from 0.10 μM factor XIII without fibrinogen and in the presence of 1.4 mM $CaCl_2$ (●). Reactions were carried out as described in Figure 2. (Panel B) Dependence of thrombin-catalyzed release of AP from 0.10 μM factor XIII on the fibrinogen concentration in the absence (○) and presence (●) of added 1 mM $CaCl_2$. The ordinate is the fraction of AP released in 15 min. The reaction conditions are as described in Figure 2 except the buffer contains 0.01 M Tris instead of 9.5 mM phosphate. Control experiments showed that the fibrinogen-promoted release of AP from factor XIII was the same in both buffers when Ca^{2+} was absent and that Ca^{2+} at 1 mM antagonized the effect of 0.1 μM fibrinogen to the same extent in 0.01 M Tris and 9.5 mM phosphate buffer.

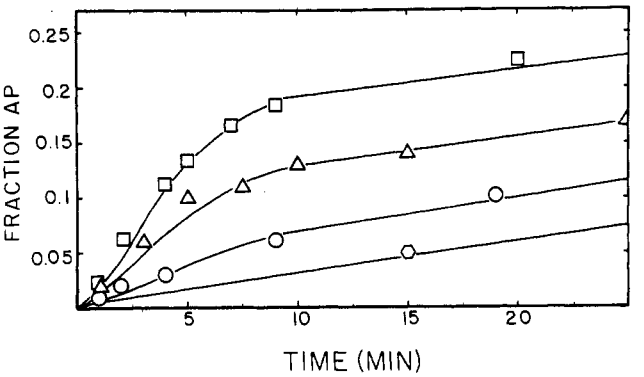


FIGURE 5: Thrombin-catalyzed release of AP from 0.10 μM factor XIII with 0.10 (○), 1.1 (△), and 6 μM (□) fibrinogen in the presence of 1.0 mM $CaCl_2$. The lowest line and (○) represent the release of AP in the absence of fibrinogen (see Figure 4A). Reaction conditions are as described in Figure 2, except that the curve for 1.1 μM fibrinogen was determined in 0.01 M Tris buffer.

lower solid line of Figure 4A. This line represents the initial rate of release of AP as calculated from

$$d(\text{fraction AP})/dt = k_{cat}[T]/K_m \quad (4)$$

with k_{cat}/K_m set at 0.14×10^6 M^{-1} s^{-1}, the previously determined value in the absence of Ca^{2+} and fibrinogen (Janus et al., 1983), and [T] being the concentration of thrombin (0.29 nM). Figure 4B depicts the inhibitory effect of 1 mM Ca^{2+} on the fraction AP released in a 15-min incubation with thrombin at several concentrations of fibrinogen. Although Ca^{2+} markedly reduces the effect of fibrinogen, the plots in Figure 5, showing the time dependence of AP release at several concentrations of fibrinogen, nevertheless indicate that fibrinogen concentrations higher than 1 μM produce substantial enhancements in the rate of release of AP. The fact that fibrinogen at a concentration of 6 μM, which is in the range of that in plasma (6–9 μM), is effective as a promoter (Figure 5) in the presence of plasma levels of Ca^{2+} suggests that promotion by fibrinogen of thrombin-catalyzed release of AP is probably physiologically important.

Close examination of Figure 5 reveals that in the presence of Ca^{2+} only a fraction of the AP is released at an enhanced rate. For example, in the presence of 6 μM fibrinogen and

[3] In the derivation of eq 3, the combination of fibrin II with factor XIII was assumed to be fast; if this assumption were not made, the theoretical time dependence of AP release (predicted assuming fibrin II is the only promoter) would show even greater deviations from the experimental data. It also should be noted that eq 3 applies to cases wherein fibrin II promoted AP release can be represented by a first-order rate law. This assumption appears to be justified under the conditions used in Figure 2 (<40% AP release, 0.1 μM fibrin II, 0.1 μM factor XIII) as judged by the fit to a first-order rate law of the time dependence of AP release in the presence of fibrin II.

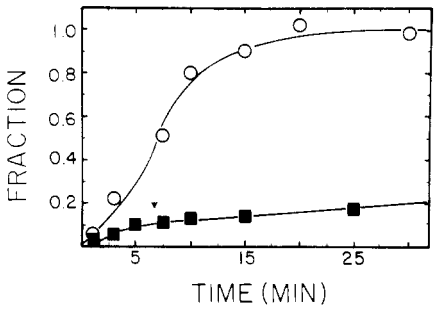
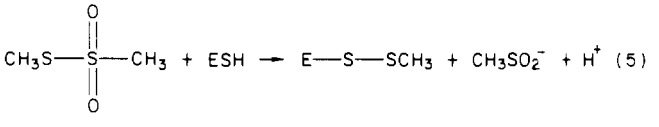


FIGURE 6: Comparison of the time dependence of γ-dimer (○) formation and AP release (■). Reaction mixtures contained 0.10 μM factor XIII, 1.1 μM fibrinogen in 0.29 nM thrombin, and 1 mM $CaCl_2$. The reaction conditions are as described in Figure 2 except 0.01 M Tris was used as the buffer. The method used for quantification of γ-dimer formation is described under Experimental Procedures. The arrow indicates the approximate point in time where the rate of AP release falls to the rate seen in the absence of fibrinogen.

1 mM Ca^{2+}, after 20% of the AP is released, the rate falls to the level seen in the absence of fibrinogen. With no Ca^{2+} present, 6 μM fibrinogen causes an enhancement in the rate of AP release until more than 90% of the AP is liberated (data not shown).[4] To determine whether the time-dependent loss of promoting activity might be due to cross-linking of fibrin by the activated factor XIIIa, the time dependencies of cross-linking and AP release were compared. Factor XIIIa catalyzed cross-linking of polymeric fibrin initially occurs between γ chains on adjacent monomeric units of fibrin and results in the formation of γ dimers (Schwartz et al., 1971). This can be quantified by SDS–PAGE after reduction. Figure 6 illustrates the time dependence of γ-dimer formation and AP release when 0.1 μM factor XIII was treated with 0.29 nM thrombin in the presence of 1 mM Ca^{2+} and 1.1 μM fibrinogen. A comparison of the plots for γ-dimer formation and AP release reveals that the rate of release of AP falls to the unenhanced rate at a time when γ-dimer formation is about 40% complete. The fact that the rate of AP release falls to the rate seen in the absence of fibrinogen prior to complete cross-linking suggests that if cross-linking is a determinant of inactivation of the promoter, less than 50% cross-linking in a polymeric unit is sufficient to inactivate it as a promoter.

To test further the notion that the cross-linking activity of factor XIIIa is responsible for the loss in the promoter activity of fibrin I polymer, we studied the effect of the thiol reagent MMTS on the thrombin-catalyzed release of AP from factor XIII. This reagent, which reacts rapidly and specifically with thiol groups (eq 5; Smith et al., 1975), would be expected to



react with the active site thiol group of factor XIIIa and inactivate the factor XIIIa before it could cross-link fibrin. Control experiments such as those depicted in Figure 1 showed that, in the presence of Ca^{2+}, cross-links (γ dimer) formed when MMTS was absent but little or no cross-links formed when MMTS was present. The plots depicted in Figures 7 and 8 show that MMTS diminished the effectiveness of Ca^{2+} as an inhibitor. The ability of MMTS to block cross-linking of fibrin together with the ability of MMTS to block substantially the inhibitory effects of Ca^{2+} is consistent with the

[4] The maintenance in the absence of Ca^{2+} of enhanced rates of AP release until AP release is nearly complete is illustrated in Figure 2 for a lower concentration of fibrinogen.

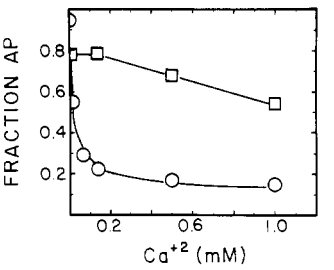


FIGURE 7: Thrombin-catalyzed calcium-dependent release of AP from 0.10 μM factor XIII and 1.1 μM fibrinogen in the presence (□) and absence (○) of 1.2 mM MMTS. Reaction conditions are as described in Figure 2 except for the use of 0.01 M Tris buffer instead of phosphate. The ordinate represents the fraction of AP release after a 15-min incubation with 0.29 nM thrombin.

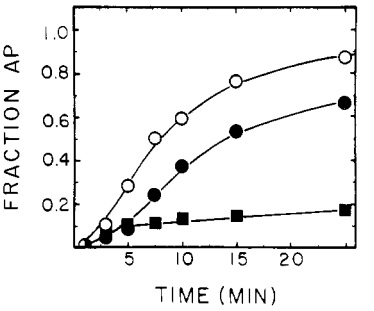


FIGURE 8: Ability of MMTS to block the inhibitory effects of Ca^{2+} on thrombin-catalyzed release of AP from factor XIII. Reaction mixtures contained 0.29 nM thrombin, 1.1 μM fibrinogen, and 0.1 μM factor XIII and (■) no MMTS and 1 mM Ca^{2+}, (●) 1.2 mM MMTS and 1 mM Ca^{2+}, or (○) 1.2 mM MMTS and no added Ca^{2+}. Reaction conditions are as described in Figure 2 except the buffer species was 0.01 M Tris.

view that Ca^{2+}-dependent cross-linking by factor XIIIa inactivates fibrin as a promoter of the thrombin-catalyzed release of AP from factor XIII.

The failure of MMTS to block completely the Ca^{2+} inhibition of AP release is not understood. The observation that 0.05 mM and 1.2 mM MMTS were equally effective in blocking Ca^{2+} inhibition (data not shown) makes it unlikely that inefficient trapping of the factor XIIIa is responsible for the inability of MMTS to render calcium completely ineffective as an inhibitor. Perhaps Ca^{2+} has secondary effects on this system. For example, Ca^{2+} might alter the strength or geometry of the interaction between polymeric fibrin and factor XIII, thereby altering the efficiency of proteolysis. Further studies will be necessary to characterize such secondary effects of Ca^{2+}.

It is interesting to note that although fibrinogen binds factor XIII very tightly (Greenberg & Shuman, 1982),[5] it does not appear to be an efficient promoter of thrombin-catalyzed release of AP from factor XIII. It follows from this observation that factor XIII interacts differently with fibrinogen and polymeric fibrins I and II. The observation that catalytically active factor XIII inactivates the promoter activity of polymeric fibrin suggests that the factor XIII zymogen also interacts differently with non-cross-linked and cross-linked polymeric fibrin. The identities of the structural determinants of the unique ability of non-cross-linked polymeric fibrin to function as an efficient promoter of thrombin-catalyzed release

[5] The value of 10^{-8} M for the equilibrium constant for dissociation of fibrinogen from factor XIII, which has been reported by Greenberg & Shuman (1982), indicates that >90% of factor XIII should be complexed with fibrinogen when excess fibrinogen is present at concentrations > 0.1 μM.

of AP from factor XIII remain to be established.

Promotion of the initial step in the activation of factor XIII by polymeric fibrin may serve to ensure that significant amounts of factor XIII are not activated until its physiological substrate polymeric fibrin is present. This activation scheme would be expected to minimize both wasteful premature activation of factor XIII and deleterious cross-linking of other serum proteins. Additionally, the system appears to be regulated so as to avoid overproduction of catalytically active factor XIIIa, since enhancement of AP release dramatically falls, as the product of activation, factor XIIIa, begins to cross-link its fibrin substrates.

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Structural Evidence for Leucine at the Reactive Site of Heparin Cofactor II†

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ABSTRACT: The reaction products formed during the enzymatic inactivation of heparin cofactor II (HCII) by a proteinase isolated from *Echis carinatus* were analyzed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis and by reverse-phase high-performance liquid chromatography. By $NaDodSO_4$–polyacrylamide gel electrophoresis, limited proteolysis of HCII was observed, which resulted in a decrease in the apparent molecular weight of the protein from ~68 000 to ~53 000. By reverse-phase high-performance liquid chromatography, at least 20 peptides were observed. Primary structure analysis of these peptides indicated that significant proteolysis had occurred in the NH_2-terminal region of the protein. HCII inactivation, however, coincided with the appearance of a peptide from the COOH-terminal region of the protein. The peptide differed from the previously identified reactive site peptide [Griffith, M. J., Noyes, C. M., & Church, F. C. (1985) *J. Biol. Chem. 260*, 2218–2225] by only one residue: a leucyl residue at the NH_2-terminal of the peptide. We conclude that leucine, as opposed to the expected arginine, is at the reactive site of HCII.

Several studies have been reported during the past few years in which the structural and functional properties of heparin cofactor II[1] (HCII)[2] have been investigated (Tollefsen et al., 1982; Wunderwald et al., 1982; Witt et al., 1983; Griffith et al., 1983, 1985; Griffith, 1983; Hurst et al., 1983; Griffith &

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[1] The term heparin cofactor II (Tollefsen et al., 1982), used in this study, corresponds to the second antithrombin heparin cofactor identified in human plasma. The activity corresponding to heparin cofactor II has been referred to in other work as heparin cofactor A (Briginshaw & Shanberge, 1974a,b; Griffith et al., 1983) and as antithrombin BM (Wunderwald et al., 1982; Witt et al., 1983).

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