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Characterization of the Kinetic Pathway for Fibrin Promotion of α-Thrombin-Catalyzed Activation of Plasma Factor XIII†

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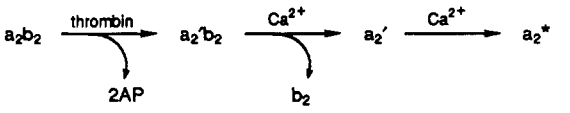
ABSTRACT: Kinetic and thermodynamic studies are presented showing that the cofactor activity of fibrin I (polymerized des-A fibrinogen) in the α-thrombin-catalyzed proteolysis of activation peptide (AP) from plasma factor XIII can be attributed to formation of a fibrin I–plasma factor XIII complex (K_d = 65 nM), which is processed by α-thrombin more efficiently ($k_{cat}/K_m = 1.2 \times 10^7$ M^{-1} s^{-1}) than free, uncomplexed plasma factor XIII ($k_{cat}/K_m = 1.4 \times 10^5$ M^{-1} s^{-1}). The increase in the specificity constant (k_{cat}/K_m) is shown to be largely due to an increase in the apparent affinity of α-thrombin for the complex of plasma factor XIII and fibrin I, as reflected by the 30-fold decrease in the Michaelis constant observed for fibrin I bound plasma factor XIII relative to that for uncomplexed plasma factor XIII. Analysis of the initial rates of α-thrombin-catalyzed hydrolysis of fibrinopeptide B (FPB) from fibrin I polymer in the presence of plasma factor XIII indicated that α-thrombin bound to fibrin I in the ternary complex of α-thrombin, plasma factor XIII, and fibrin I polymer is competent to catalyze cleavage of both FPB from fibrin I and AP from plasma factor XIII. This observation is consistent with the view that α-thrombin within the ternary complex is anchored to fibrin I polymer through a binding site distinct from the active site (an exosite) and that the active site is alternatively complexed with the AP moiety of plasma factor XIII or the FPB moiety of fibrin I. This conclusion is supported by the observation that a 12-residue peptide, which binds to an exosite of α-thrombin and blocks the interaction of α-thrombin with fibrinogen and fibrin, competitively inhibits α-thrombin-catalyzed release of both FPB and AP from the fibrin I–plasma factor XIII complex.

In the final step of the blood coagulation cascade, the transglutaminase $FXIII_a$ catalyzes formation of γ-glutamyl-ε-lysyl peptide cross-links between adjacent fibrin units of the blood clot [for a review, see Lorand et al. (1980)]. These cross-links increase the mechanical strength of the blood clot (Roberts et al., 1974; Gerth et al., 1974). $FXIII_a$ also catalyzes the cross-linking of α_2-antiplasmin to fibrin, thereby increasing the resistance of the clot to dissolution by plasmin and increasing the lifetime of the clot in plasma (Sakata & Aoki, 1980, 1982; Jansen et al., 1987).

Scheme I



Plasma factor XIII circulates in blood plasma as a zymogen composed of two a subunits and two b subunits. Activation of plasma factor XIII is accomplished by the successive actions of α-thrombin and calcium as shown in Scheme I, wherein α-thrombin catalyzes cleavage of a 37-residue activation peptide (AP)[1] from the N-terminus of each of the a subunits

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of factor XIII (Lorand & Konishi, 1964; Takagi & Doolittle, 1974; Ichinose et al., 1986). In the presence of calcium ions the b subunits dissociate (Curtis et al., 1974; Cooke & Holbrook, 1974; Chung et al., 1974) and the active-site thiol group is exposed to produce the enzymically active a_2^* dimer (Curtis et al., 1973, 1974; Cooke & Holbrook, 1974; Chung et al., 1974). $FXIII_a$ can also be generated via the actions of α-thrombin and calcium on the a_2 dimer. The dimeric a_2 form of factor XIII zymogen is found in platelets and placental tissue.

Fibrin I polymer (polymerized des-A fibrinogen) behaves as a cofactor for the activation of plasma factor XIII by accelerating the α-thrombin-catalyzed cleavage of AP from plasma factor XIII (Janus et al., 1983; Lewis et al., 1985a). Plasma factor XIII and α-thrombin both bind to fibrin (Greenberg et al., 1985; Liu et al., 1979; Kaminski et al., 1983), intimating formation of a termolecular complex comprised of α-thrombin, plasma factor XIII, and fibrin I polymer, which accelerates α-thrombin-catalyzed cleavage of the scissile bond at Arg-37 in the a subunit of factor XIII. In addition to acting as a promoter of α-thrombin-catalyzed cleavage of plasma factor XIII, fibrin I polymer is also cleaved by α-thrombin to yield fibrin II polymer (polymerized des-A, -B fibrinogen) and fibrinopeptide B (FPB) (Lewis et al., 1985b). Generally, an alternative substrate for α-thrombin, such as fibrin I, would be expected to bind to the active site of α-thrombin and inhibit the action of α-thrombin on plasma factor XIII. This behavior is observed with fibrinogen but not fibrin I. α-Thrombin appears to bind fibrin I polymer in orientations wherein the active site of α-thrombin is alternatively occupied by the FPB moiety of fibrin I or unoccupied and accessible for reaction with other small chromogenic substrates (Naski & Shafer, 1990). Thus, α-thrombin within a ternary complex of α-thrombin, plasma factor XIII, and fibrin I might have alternative binding modes that permit α-thrombin to catalyze cleavage of both factor XIII and fibrin I. On the other hand, α-thrombin in the ternary complex might only be oriented for cleavage of AP from plasma factor XIII. The kinetic behavior of the putative complex has not been reported, however.

We present in this study a kinetic pathway and the parameters that account for the cofactor activity of fibrin I in the α-thrombin-catalyzed release of AP from plasma factor XIII. In this pathway fibrin I, which binds both plasma factor XIII and α-thrombin, serves to deliver factor XIII to the active site of α-thrombin. Consistent with this view of the function of fibrin I, the apparent Michaelis constant observed for α-thrombin-catalyzed cleavage of AP from plasma factor XIII complexed with fibrin I is 30-fold smaller than that observed for cleavage of AP from uncomplexed plasma factor XIII. Additionally, we present evidence that the α-thrombin in the α-thrombin–fibrin I–factor XIII complex is anchored to fibrin I polymer through a binding site distinct from the active site and that the active site of α-thrombin is alternatively occupied by the FPB moiety of fibrin I or the AP moiety of plasma factor XIII.

Materials and Methods

Materials. Fibrinogen was purified by repeated precipitation with β-alanine from outdated human plasma (Lewis & Shafer, 1984). The final precipitate was dissolved in and dialyzed against 0.05 M sodium phosphate and 0.3 M NaCl, pH 7.5, and then chromatographed on lysine–Sepharose (Sigma) and gelatin–agarose (Sigma) to remove plasmin (Deutsch & Mertz, 1970) and fibronectin (Engvall et al., 1978). Fibrinogen concentrations were determined by using an $E_{280}^{1\%}$ of 15.1 in 0.3 M NaCl and an M_r of 340000 (Mihalyi, 1968). Plasma factor XIII tetramer and plasma factor XIII b subunits were purified from outdated human plasma according to procedures previously described (Lorand et al., 1981). Platelet factor XIII was purified from human platelets as described previously (Hornyak et al., 1989). Plasma factor XIII and platelet factor XIII concentrations were determined by using an $E_{280}^{1\%}$ of 13.8 and M_r's of 320000 and 160000, respectively (Schwartz et al., 1973). Fibrin I was prepared as described previously (Lewis et al., 1985a) except that fibrin I dissolved in 0.02 M acetic acid was reprecipitated twice in 80 mM sodium phosphate, pH 6.3. The fibrin I concentration was determined by using an $E_{280}^{1\%}$ of 14.0 and an M_r of 340000 (Lewis et al., 1985a). Protomer concentrations (both factor XIII and fibrin I protomer) are twice the factor XIII or fibrin I concentrations. Human α-thrombin was generously supplied by Dr. John W. Fenton II, New York State Department of Health. The concentration of α-thrombin was determined from measurements of the pseudo-first-order rate constant (k_{obs}) for α-thrombin-catalyzed release of FPA from fibrinogen at a fibrinogen concentration less than $K_m/10$ as described previously (Lewis & Shafer, 1984). Under these conditions the α-thrombin concentration is related to the measured value of k_{obs} by the equation

$$[\alpha\text{-thrombin}] = k_{obs}/(11.6 \times 10^6\ M^{-1}\ s^{-1}) \qquad (1)$$

where $11.6 \times 10^6\ M^{-1}\ s^{-1}$ is the specificity constant (k_{cat}/K_m) for release of FPA from fibrinogen (Higgins et al., 1983). Hirugen [Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3)-Leu] was a gift from Dr. John M. Maraganore, Biogen Inc., Cambridge, MA.

Binding of Factor XIII to Fibrin I Polymer. The binding of factor XIII zymogen to fibrin I was measured at 37 °C in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.139 M NaCl, 5 mM acetate, 0.1% PEG, and 20 μg/mL aprotinin (Sigma), pH 7.4. Fibrin I dissolved in 0.02 M acetic acid was added with mixing to a buffered solution of factor XIII equilibrated at 37 °C to give a final volume of 0.5 mL in microfuge tubes coated with PEG 20000. Fibrin I protomer (2 μM) and 0.088–1.2 μM plasma factor XIII promoter or 0.4–2.0 μM platelet factor XIII promoter were incubated at 37 °C for 3 h, then centrifuged for 3 min, and filtered through a 0.5-μm Teflon filter (Millipore). The free (unbound) factor XIII concentration was determined from an assay of an aliquot of the filtrate measuring the initial rate of factor $XIII_a$ catalyzed incorporation of dansylcadaverine (Sigma) into *N*,*N*′-dimethylcasein (Hornyak et al., 1989; Curtis & Lorand, 1976). The concentration of factor XIII was determined by comparison of the measured initial rate to a standard curve produced by measuring the initial rates of factor $XIII_a$ catalyzed activity at known factor XIII concentrations. An aliquot of the filtrate was subjected to nonreducing SDS–PAGE (4% gels) to determine the amount of fibrin I present in the filtrate. The concentration of fibrin I in the filtrate was less than 40 nM and was judged to be insignificant.

Reaction Kinetics. Kinetic measurements were done under the followig standard conditions: 37 °C, $\Gamma/2$ 0.17, 0.04 M sodium phosphate, 0.084 M NaCl, 5 mM acetate, pH 7.4, and 0.1% PEG. All initial rate measurements were complete before depletion of 10% of the initial concentrations of the substrates for α-thrombin (fibrin I or plasma factor XIII) that were present.

[1] Abbreviations: AP and AP′, major and minor activation peptides released upon treatment of factor XIII with α-thrombin; FPA, fibrinopeptide A; FPB, fibrinopeptide B; PEG, poly(ethylene glycol) 6000; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Initial Rates of α-Thrombin-Catalyzed Release of FPB and AP. The initial rates of the α-thrombin-catalyzed cleavage of FPB and AP were measured under standard conditions. A buffered solution of factor XIII was equilibrated at 37 °C, whereupon aliquots of α-thrombin and fibrin I were added rapidly in succession with mixing to yield the final concentrations of 0.8–5.68 μM plasma factor XIII protomer, 1.06–17.4 μM fibrin I protomer, and 8.5–13.0 pM α-thrombin in 0.5 mL. The samples were quenched at the indicated times (prior to release of 10% of the initial concentrations of either AP or FPB) with 75 μL of 14% $HClO_4$. The precipitated protein was removed by centrifugation, and the supernatant solution was filtered through a 0.45-μm membrane. The filtrate was analyzed for peptides by high-performance liquid chromatography (Janus et al., 1983). The initial rates of liberation of FPB and AP were calculated from the slopes of the plots of the area of the peptide peaks versus the time of incubation with α-thrombin. The sum of the areas of both the AP and AP′ peaks and their respective shoulders (resulting from an N–O acetyl shift in the *N*-acetylserine residue of the activation peptide) was used to determine the initial rate of AP release (Janus et al., 1983; Shafer et al., 1986). When hirugen was present, the hirugen (0–3.64 μM) was premixed with plasma factor XIII, and the experiment was performed at 1.4 μM fibrin I protomer, 0.35 μM plasma factor XIII protomer, and 3.6 pM α-thrombin. To determine the effect of hirugen on the rate of α-thrombin-catalyzed release of AP from plasma factor XIII in the absence of fibrin I, 0.45 μM plasma factor XIII protomer and 3.3 nM α-thrombin were combined in the presence and absence of 2.73 μM hirugen and the pseudo-first-order rate of release of AP from plasma factor XIII was measured as described previously (Janus et al., 1983).

Fits to Experimental Data. Parameters and standard deviations were obtained from nonlinear least-squares fits to the equations described in the text by using a computer program (BMDX85) from the Health Science Computing Facility at the University of California, Los Angeles.

Results and Discussion

We have previously demonstrated that fibrin I polymer (des-A fibrinogen) acts to accelerate α-thrombin-catalyzed cleavage of plasma factor XIII zymogen (Lewis et al., 1985a). Other work has demonstrated that both plasma factor XIII and α-thrombin bind to fibrin (Greenberg et al., 1985; Liu et al., 1979; Kaminski et al., 1983; Triantaphyllopoulos, 1973). Accordingly, the acceleration of the cleavage of plasma factor XIII by α-thrombin in the presence of fibrin polymer is assumed to result from the formation of a ternary complex of α-thrombin, plasma factor XIII, and fibrin I polymer that facilitates the cleavage of the scissile bond at Arg-37 in the a subunit of plasma factor XIII. Formation of the ternary complex and α-thrombin-catalyzed cleavage of plasma factor XIII is most simply represented by the reactions of Scheme II, which builds upon the known interactions of α-thrombin (E) with fibrin I polymer (represented in protomer units, $\alpha B\beta\gamma$) and plasma factor XIII protomer (ab). In this scheme $K_{m'}^{F}$ and $K_{m'}^{FX}$ are the apparent Michaelis constants[2] for the dissociation of α-thrombin from its complex with fibrin I

Scheme II

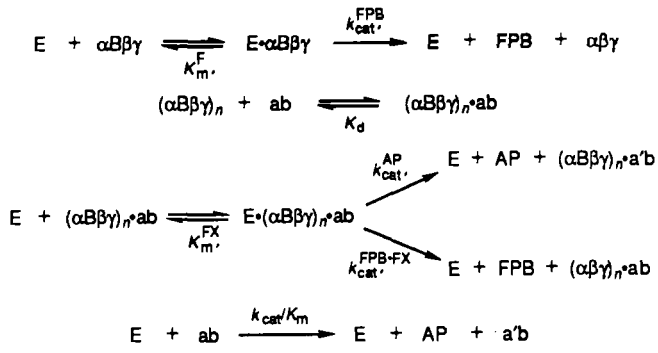

Table I: Kinetic Parameters for α-Thrombin-Catalyzed Release of FPB and AP at 37 °C, Γ/2 0.17, and pH 7.4

reaction complex or reactants	dissociation or Michaelis constant (K_d or K_m)[a] (μM)	turnover no. (k_{cat})[a] (s^{-1})	specificity constant (k_{cat}/K_m) × 10^{-6} (M^{-1} s^{-1})
E•αBβγ	7.5	37	4.5
$(\alpha B\beta\gamma)_n$•ab	0.065		
E + ab			0.14
E•$(\alpha B\beta\gamma)_n$•ab	2.5	29,[b] 21[c]	12,[b] 8.2[c]

[a] Values of K_m or K_d are based on protomer units of fibrin I ($\alpha B\beta\gamma$) and plasma factor XIII (ab). The values for the kinetic parameters, k_{cat} and K_m, are apparent values due to the existence of alternative binding modes for both α-thrombin bound to fibrin I and α-thrombin bound to the factor XIII–fibrin I complex.[2] [b] Kinetic parameter for α-thrombin (E) catalysis of the release of AP from fibrin I bound plasma factor XIII. [c] Kinetic parameter for α-thrombin-catalyzed release of FPB from the fibrin I–plasma factor XIII complex.

polymer and the plasma factor XIII–fibrin I complex, respectively. These Michaelis constants define the unit of substrate bound by α-thrombin: either a fibrin I protomer ($K_{m'}^{F}$) or the complex of factor XIII protomer with *n* fibrin I protomers ($K_{m'}^{FX}$). K_d is the dissociation constant for the fibrin I–factor XIII complex where $(\alpha B\beta\gamma)_n$ represents the unit of fibrin I polymer bound by one plasma factor XIII promoter (ab). $k_{cat'}^{AP}$ is the apparent turnover number for α-thrombin-catalyzed cleavage of AP in the ternary complex, and $k_{cat'}^{FPB\cdot FX}$ is an average apparent turnover number for α-thrombin-catalyzed release of FPB from the ternary complex containing *n* fibrin promoters. $k_{cat'}^{FPB}$ is the apparent turnover number for release of FPB from fibrin I polymer. k_{cat}/K_m is the specificity constant for the reaction of free α-thrombin with free plasma factor XIII. The values of k_{cat}/K_m and $K_{m'}^{F}$ have been determined previously (Janus et al., 1983; Naski & Shafer, 1990) (see Table I for a summary of the kinetic parameters).

As mentioned, binding of plasma factor XIII to fibrin I polymer has been observed previously (Greenberg et al., 1985). However, the dissociation constant for this interaction, K_d, was not determined. Before analyzing the kinetics of α-thrombin-catalyzed release of AP from factor XIII within the ternary complex, we needed to determine the value of the dissociation constant (K_d) for the plasma factor XIII–fibrin I polymer complex. Fibrin I was prepared by selective removal of FPA from fibrinogen by using the snake venom enzyme batroxobin, and the resulting fibrin I was dissolved in dilute acetic acid. The solubilized fibrin I rapidly polymerized upon addition with mixing to a buffered solution (final pH = 7.4) of plasma factor XIII to form an insoluble polymer. Following incubation at 37 °C, the fibrin I polymer and plasma factor XIII bound to the polymer were removed by centrifugation and filtration, and the free factor XIII in the filtrate was measured by using an assay that measures factor $XIII_a$ catalyzed incorporation of dansylcadaverine into *N*,*N*′-dimethylcasein. Figure 1 shows

[2] Due to the presence of alternative binding modes for both α-thrombin bound to fibrin I and α-thrombin bound to the fibrin I–plasma factor XIII complex, the values for the Michaelis–Menten kinetic parameters (k_{cat} and K_m) reported in this study are apparent values. The effect of alternative (or nonproductive) binding modes on the Michaelis–Menten kinetic parameters has been discussed previously (Naski & Shafer, 1990; Fersht, 1985).

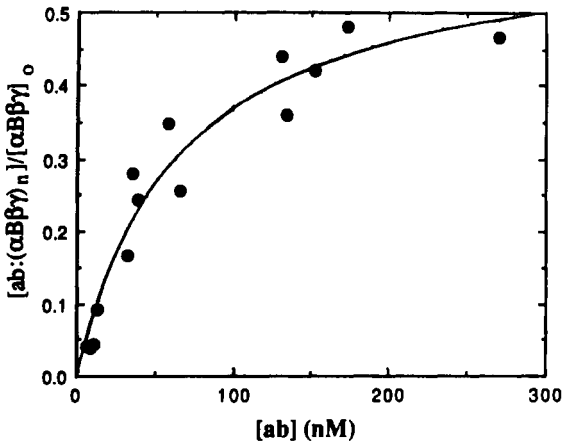

FIGURE 1: Binding of plasma factor XIII to fibrin I polymer at 37 °C, Γ/2 0.17, and pH 7.4. Plasma factor XIII ranging from 0.088 to 1.2 μM (based on the protomer unit ab) was incubated with 2 μM fibrin I polymer (based on protomer units). The concentration of free plasma factor XIII ([ab]) was determined as described under Materials and Methods. [ab·$(\alpha B\beta\gamma)_n$], $(\alpha B\beta\gamma)_n$, and $[\alpha B\beta\gamma]_o$ represent the concentration of factor XIII bound to fibrin I polymer, the unit of fibrin I polymer bound by plasma factor XIII protomer, and the total fibrin I protomer concentration, respectively. The curve shown represents the results of a nonlinear least-squares fit of the data to eq 2. The best fit yielded values for K_d and n of 65 ± 15 nM and 1.6 ± 0.2, respectively.

the results of binding experiments wherein 0.088–1.2 μM plasma factor XIII protomer (ab) was mixed with 2 μM fibrin I protomer ($\alpha B\beta\gamma$). The binding data are fit to the equation

$$\frac{[ab\cdot(\alpha B\beta\gamma)_n]}{[\alpha B\beta\gamma]_o} = \frac{[ab]}{n(K_d + [ab])} \quad (2)$$

which assumes that plasma factor XIII binds to a single class of noninteracting sites on fibrin I polymer. [ab] and [ab·$(\alpha B\beta\gamma)_n$] represent the concentrations of free and bound plasma factor XIII, and n represents the number of fibrin I protomers bound per factor XIII protomer. A nonlinear least-squares fit of the data to eq 2 yielded values of 65 ± 16 nM and 1.6 ± 0.2 for K_d, the equilibrium constant for the dissociation of factor XIII from fibrin I, and n, the average number of fibrin I units in the complex of factor XIII and fibrin I, respectively. The affinity of plasma factor XIII for fibrin polymer is similar to the affinity (K_d = 10 nM, 20 nM based on protomer units) of plasma factor XIII for fibrinogen bound to latex beads (Greenberg & Shuman, 1982). In contrast, platelet factor XIII (subunit structure a_2), which has been reported to have the same affinity as plasma factor XIII for fibrinogen-coated beads (Greenberg & Shuman, 1982), was found by us to bind much more weakly (K_d > 3.6 μM) to fibrin I polymer. For example, with 2 μM platelet factor XIII protomer, we found that only 0.2 μM protomer bound to 2 μM fibrin I polymer. Weak binding of platelet factor XIII to fibrin polymer is consistent with the absence of acceleration of the α-thrombin-catalyzed cleavage of AP from platelet factor XIII in the presence of fibrin (Greenberg et al., 1986, 1987). Since plasma factor XIII zymogen differs from platelet factor XIII zymogen in that is contains the b subunits that are absent in platelet factor XIII zymogen, we tested whether the b subunits alone could bind to fibrin I polymer and compete with the binding of plasma factor XIII to fibrin I polymer. We incubated fibrin-bound plasma factor XIII with excess b subunits and found that the binding of 88 nM plasma factor XIII protomer to 2 μM fibrin I polymer was not inhibited by 2.0 μM b subunits (a 15% decrease would have been detected), indicating that the b chains alone are not responsible for the affinity of plasma factor XIII for fibrin I polymer.

Having determined the dissociation constant for the interaction of plasma factor XIII with fibrin I polymer, we were in a position to determine the ability of Scheme II to account for the cofactor activity of fibrin I in the α-thrombin-catalyzed release of AP. The expression for the initial rate (V) of α-thrombin-catalyzed release of AP according to the reactions in Scheme II is given by

$$\frac{V}{[E]_o} = \frac{\frac{k_{cat}}{K_m}[ab] + \frac{k_{cat'}^{AP}}{K_{m'}^{FX}}[(\alpha B\beta\gamma)_n\cdot ab]}{1 + [\alpha B\beta\gamma]/K_{m'}^{F} + [(\alpha B\beta\gamma)_n\cdot ab]/K_{m'}^{FX}} \quad (3)$$

where $[E]_o$ represents the total concentration of α-thrombin. Because plasma factor XIII binds tightly to fibrin I polymer, it was not possible to assume that the free concentrations of fibrin I and plasma factor XIII were equal to their initial concentrations. Therefore, the concentrations of fibrin I bound factor XIII ([$(\alpha B\beta\gamma)_n$·ab]) and free fibrin I ([$\alpha B\beta\gamma$]) were calculated from the quadratic equations

$$[\alpha B\beta\gamma] = \{-(n[ab]_o - [\alpha B\beta\gamma]_o + K_d) + [(n[ab]_o - [\alpha B\beta\gamma]_o + K_d)^2 + 4K_d[\alpha B\beta\gamma]_o]^{0.5}\}/2 \quad (4)$$

$$[ab\cdot(\alpha B\beta\gamma)_n] = \{[\alpha B\beta\gamma]_o/n + [ab]_o + K_d - [([\alpha B\beta\gamma]_o/n + [ab]_o + K_d)^2 - 4[\alpha B\beta\gamma]_o[ab]_o/n]^{0.5}\}/2 \quad (5)$$

where $[ab]_o$ and $[\alpha B\beta\gamma]_o$ are the total plasma factor XIII protomer and fibrin I protomer concentrations, respectively. Equation 5 results from rearrangement of eq 2 using the substitution $[ab] = [ab]_o - [ab\cdot(\alpha B\beta\gamma)_n]$. Equation 4 results from rearrangement of the converse of eq 2, where fibrin I rather than factor XIII is considered to be the ligand: $([\alpha B\beta\gamma]_o - [\alpha B\beta\gamma])/[ab]_o = n[\alpha B\beta\gamma]/(K_d + [\alpha B\beta\gamma])$. The concentration of free plasma factor XIII ([ab]) was simply the total plasma factor XIII concentration minus the bound factor XIII concentration ($[ab] = [ab]_o - [ab\cdot(\alpha B\beta\gamma)_n]$).

Initial rates of α-thrombin-catalyzed cleavage were determined from reaction mixtures of α-thrombin, factor XIII, and fibrin I polymer. α-Thrombin and fibrin I solubilized in dilute acetic acid were rapidly added to a buffered solution of plasma factor XIII. Polymerization of the fibrin I units and binding of plasma factor XIII to fibrin I polymer were assumed to be rapid so that the concentrations of uncomplexed fibrin I and bound plasma factor XIII would be predicted by the equilibrium expressions, eqs 4 and 5. The dependence of the initial rate of α-thrombin-catalyzed release of AP on the initial concentration of fibrin I protomer at several plasma factor XIII concentrations is shown in Figure 2. The lines shown in Figure 2 result from a nonlinear least-squares fit of the data to eq 3, where the concentrations of unbound fibrin I protomer ([$\alpha B\beta\gamma$]) and fibrin I bound plasma factor XIII ([$(\alpha B\beta\gamma)_n$·ab]) were determined from the initial concentrations of fibrin I protomer and plasma factor XIII protomer indicated in the figure by using eqs 4 and 5. When the values of k_{cat}/K_m and $K_{m'}^{F}$ were set to the previously determined values of 1.4 × 10^5 M^{-1} s^{-1} and 7.5 μM (Table I), the best fit of the data to eq 3 yielded values for $k_{cat'}^{AP}/K_{m'}^{FX}$ and $K_{m'}^{FX}$ of (1.2 ± 0.1) × 10^7 M^{-1} s^{-1} and 2.5 ± 0.5 μM, respectively. The turnover number ($k_{cat'}^{AP}$) is calculated to be 29 ± 6 s^{-1}. Comparison of the specificity constants for α-thrombin-catalyzed release of AP from unbound factor XIII (k_{cat}/K_m = 1.4 × 10^5 M^{-1} s^{-1}) and fibrin I bound factor XIII ($k_{cat'}^{AP}/K_{m'}^{FX}$ = 1.2 × 10^7 M^{-1} s^{-1}) reveals an 80-fold increase in the specificity constant for release of AP when plasma factor XIII is bound to fibrin I polymer. The pronounced cofactor activity of fibrin I in the activation of factor XIII suggests that formation of factor $XIII_a$ is tightly coupled to the production of fibrin. The similarity of the

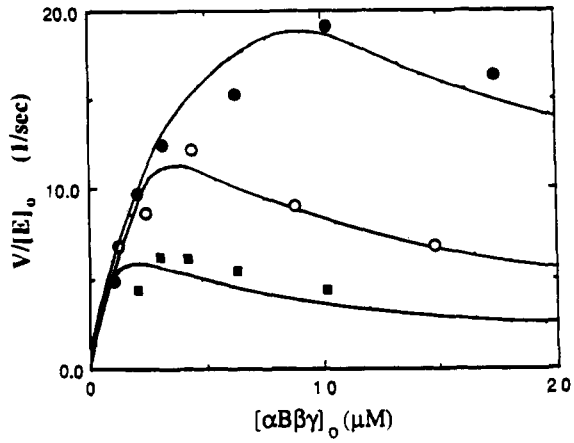

FIGURE 2: Dependence of the initial rate (V) of α-thrombin-catalyzed release of AP from plasma factor XIII on the total concentration of fibrin I protomer ($[\alpha B\beta\gamma]_o$). The lines shown represent the results of a nonlinear least-squares fit of the data to eq 3 at 5.68 (●), 1.96 (○), and 0.8 μM (■) plasma factor XIII protomer. The concentrations of free fibrin I protomer ($[\alpha B\beta\gamma]$) and bound plasma factor XIII protomer ($[\alpha B\beta\gamma)_n$·ab]), required for the fit to eq 3, were determined from eqs 4 and 5 by using the total concentrations of plasma factor XIII and fibrin I indicated in the figure. With the values of k_{cat}/K_m and $K^F_{m'}$ set equal to the previously determined values of 1.4×10^5 M^{-1} s^{-1} and 7.5 μM, respectively, the best fit of the data to eq 3 yielded values for $k^{AP}_{cat'}/K^{FX}_{m'}$ and $K^{FX}_{m'}$ of $(1.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and 2.5 ± 0.5 μM, respectively. $[E]_o$ represents the total concentration of α-thrombin.

specificity constants for α-thrombin-catalyzed release of FPA from fibrinogen (1.2×10^7 M^{-1} s^{-1}; Higgins et al., 1983) and AP from fibrin I bound plasma factor XIII (1.2×10^7 M^{-1} s^{-1}) indicates that once the substrate (fibrin) for factor $XIII_a$ is formed, the α-thrombin-catalyzed cleavage of factor XIII proceeds as efficiently as the production of additional fibrin. The coupling of the activation of plasma factor XIII to the presence of its substrate is similar to the activation of plasminogen by tissue plasminogen activator in the presence of fibrin. Both tissue plasminogen activator (Higgins & Vehar, 1987) and plasminogen (Lucas et al., 1983; Bok & Mangel, 1985) bind to fibrin, and these interactions result in a 600–900-fold increase in the specificity constant for activation of plasminogen (Hoylaerts et al., 1982). Thus, activation of both plasminogen and plasma factor XIII proceeds much more efficiently when the substrate (fibrin) of the enzyme being formed is present because the substrate acts as a cofactor for activation. The cofactor activity of the substrate ensures that conversion of zymogen to enzyme occurs most rapidly in the vicinity of the substrate and conserves the zymogen when the substrate is not available. The enhancement in the rate of α-thrombin-catalyzed release of AP from plasma factor XIII appears to be largely due to an approximately 30-fold increase in the apparent affinity of α-thrombin for the complex of plasma factor XIII and fibrin I polymer as compared to the apparent affinity of α-thrombin for uncomplexed factor XIII. In the absence of fibrin the apparent dissociation constant (as approximated by the Michaelis constant) for the interaction of α-thrombin with plasma factor XIII is ~84 μM (Janus et al., 1983), whereas the apparent dissociation constant (as approximated by the Michaelis constant) for the interaction of α-thrombin with plasma factor XIII bound to fibrin I polymer is 2.5 μM. Heparin catalysis of the inactivation of α-thrombin by antithrombin III shows a similar kinetic effect whereby heparin increases 300-fold the affinity of α-thrombin for antithrombin III (Olson, 1988).

The decrease in the rate of α-thrombin-catalyzed activation of plasma factor XIII at high fibrin I polymer concentrations (Figure 2) is predicted by eq 3 when $[\alpha B\beta\gamma] \gg K^F_{m'}$ and $[\alpha B\beta\gamma]/K^F_{m'} \gg [(\alpha B\beta\gamma)_n$·ab$]/K^{FX}_{m'}$. The decrease in the rate results from the binding of plasma factor XIII and α-thrombin to distinct fibrin I molecules rather than the formation of a ternary complex. Similar to the inhibition of α-thrombin-catalyzed activation of plasma factor XIII in the presence of excess fibrin I polymer, heparin catalysis of the inactivation of α-thrombin by antithrombin III also displays diminution of the accelerated rate when the concentration of heparin exceeds the dissociation constants for both the heparin–antithrombin III and heparin–α-thrombin complexes (Olson, 1988; Griffith, 1979). Thus, the high concentrations of heparin result in the formation of separate heparin–α-thrombin and heparin–antithrombin III binary complexes that react slower than do α-thrombin and antithrombin III when they are bound to the same heparin molecule.

Fibrin I polymer is cleaved by α-thrombin at Arg-14 in the Bβ chain of fibrin I to produce FPB and fibrin II polymer (des-A, -B fibrinogen). We previously characterized the interaction of α-thrombin with fibrin I polymer in the absence of factor XIII and showed that α-thrombin has alternative modes of binding to fibrin I polymer (Naski & Shafer, 1990). We found that α-thrombin bound to fibrin I polymer was not only competent to process the FPB moiety of the Bβ chain of fibrin I polymer but also competent to process other small amide substrates and antithrombin III. We now wished to determine whether α-thrombin bound in the ternary α-thrombin–fibrin I–factor XIII complex also had alternative binding modes. In other words, is α-thrombin bound in the ternary complex competent to release FPB from fibrin I as well as AP from plasma factor XIII?

The reaction scheme for α-thrombin-catalyzed release of FPB from fibrin I polymer in the presence of plasma factor XIII is shown in Scheme II. In this scheme $k^{FPB}_{cat'}$ and $k^{FPB\cdot FX}_{cat'}$ represent the apparent turnover numbers for α-thrombin-catalyzed release of FPB from the fibrin I polymer–α-thrombin complex and the ternary complex, respectively. Equation 6

$$\frac{V}{[E]_o} = \frac{\frac{k^{FPB}_{cat'}}{K^F_{m'}}[\alpha B\beta\gamma] + \frac{k^{FPB\cdot FX}_{cat'}}{K^{FX}_{m'}}[(\alpha B\beta\gamma)_n\cdot ab]}{1 + [\alpha B\beta\gamma]/K^F_{m'} + [(\alpha B\beta\gamma)_n\cdot ab]/K^{FX}_{m'}} \quad (6)$$

describes the initial rate of FPB release from fibrin I according to Scheme II. Contemporaneously with the measurements of the release of AP depicted in Figure 2, we also measured the initial rate of α-thrombin-catalyzed release of FPB from fibrin I in the presence of plasma factor XIII (Figure 3). The concentrations of uncomplexed fibrin I protomer and plasma factor XIII bound to fibrin I were calculated by using eqs 4 and 5. A nonlinear least-squares fit of the data to eq 6 (Figure 3, solid lines), where $K^{FX}_{m'}$ was set equal to the value (2.5 μM) determined in Figure 2 and $K^F_{m'}$ was set equal to the previously determined value (7.5 μM), yielded values for $k^{FPB}_{cat'}/K^F_{m'}$ and $k^{FPB\cdot FX}_{cat'}/K^{FX}_{m'}$ of $(4.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} and $(8.2 \pm 0.4) \times 10^6$ M^{-1} s^{-1}, respectively. The nonzero value for $k^{FPB\cdot FX}_{cat'}/K^{FX}_{m'}$ indicates that α-thrombin within the ternary complex is indeed competent to catalyze release of FPB. This result implies that α-thrombin within the ternary complex of α-thrombin, plasma factor XIII, and fibrin I polymer can catalyze cleavage of both AP from plasma factor XIII and FPB from fibrin I polymer. The specificity constant ($k^{FPB}_{cat'}/K^F_{m'}$) for release of FPB from fibrin I not complexed with factor XIII agrees with previously reported values of $(4.2–6.5) \times 10^6$ M^{-1} s^{-1} for the specificity constant for the release of FPB from fibrin I in the absence of factor XIII (Lewis et al., 1985b; Hofsteenge et al., 1986; Naski & Shafer, 1990).

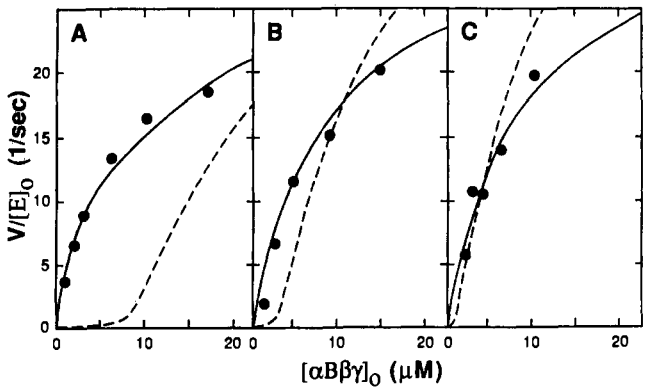

FIGURE 3: Initial rates (V) of α-thrombin-catalyzed release of FPB from fibrin I polymer in the presence of plasma factor XIII. The lines shown represent the results of a nonlinear least-squares fit of the data in all three panels to eq 6 (solid lines) or eq 7 (broken lines) where the concentrations of free fibrin I protomer ($[\alpha B\beta\gamma]$) and factor XIII bound to fibrin I polymer ($[(\alpha B\beta\gamma)_n$·ab]) were determined from eqs 4 and 5 by using the total concentrations of plasma factor XIII protomer and fibrin I protomer ($[\alpha B\beta\gamma]_o$) indicated in the figure. The total concentrations of plasma factor XIII protomer were 5.68 (panel A), 1.96 (panel B), and 0.8 μM (panel C). With the values of $K_{m'}^{FX}$ and $K_{m'}^{F}$ set equal to their previously determined values of 2.5 μM and 7.5 μM, respectively, the best fit (solid lines) of the data to eq 6 yielded values for $k_{cat'}^{FPB}/K_{m'}^{F}$ and $k_{cat'}^{FPB\cdot FX}/K_{m'}^{FX}$ of $(4.5 \pm 0.2) \times 10^6$ M^{-1} s^{-1} and $(8.2 \pm 0.4) \times 10^6$ M^{-1} s^{-1}, respectively. The best fit of the data to eq 7 yielded a value for $k_{cat'}^{FPB}/K_{m'}^{F}$ of $(6.6 \pm 0.9) \times 10^6$ M^{-1} s^{-1}. $[E]_o$ represents the total concentration of α-thrombin.

If α-thrombin bound in the ternary complex were only to catalyze release of AP and not FPB, then $k_{cat'}^{FPB\cdot FX} = 0$ and eq 6 would reduce to eq 7. With the values of $K_{m'}^{F}$ and $K_{m'}^{FX}$ set

$$\frac{V}{[E]_o} = \frac{\dfrac{k_{cat'}^{FPB}}{K_{m'}^{F}}[\alpha B\beta\gamma]}{1 + [\alpha B\beta\gamma]/K_{m'}^{F} + [(\alpha B\beta\gamma)_n\cdot ab]/K_{m'}^{FX}} \quad (7)$$

equal to the previously determined values of 7.5 and 2.5 μM, respectively, a nonlinear least-squares fit of the data in Figure 3 to eq 7 yielded a value for $k_{cat'}^{FPB}/K_{m'}^{F}$ of $(6.6 \pm 0.9) \times 10^6$ M^{-1} s^{-1}. The fit of the data to eq 7 (Figure 3, broken lines), however, is substantially worse than that observed for the fit of the data to eq 6 (Figure 3, solid lines). Thus, the fits to the data indicate that α-thrombin within the ternary complex can catalyze release of FPB from fibrin I and hence imply alternative binding modes. It appears that in one orientation α-thrombin within the ternary complex is competent to release AP from plasma factor XIII and in a second orientation α-thrombin is competent to release FPB from fibrin I.

We have previously attributed the alternative binding modes of α-thrombin to result from the binding of α-thrombin to fibrin I through a site distinct from the active site (an exosite) wherein the active site of α-thrombin is alternatively occupied by Arg-14 of the Bβ chain of fibrin I or unoccupied and free to process other substrates (Naski & Shafer, 1990). In the ternary α-thrombin–fibrin I–factor XIII complex we suggest a similar form of alternative binding wherein α-thrombin is anchored to fibrin I through the fibrin-binding exosite and the active site of α-thrombin is alternatively occupied by Arg-14 of the Bβ chain of fibrin I or Arg-37 of the a subunit of plasma factor XIII.

It is well documented that α-thrombin interacts with the substrate fibrinogen through interactions both within and outside the active site, and it is apparent that the interactions outside the active site of α-thrombin contribute a major portion of the free energy for the binding of α-thrombin to fibrinogen (Berliner et al., 1985; Fenton et al., 1988; Lewis et al., 1987; Naski et al., 1900; Noë et al., 1988; Sonder & Fenton, 1986).

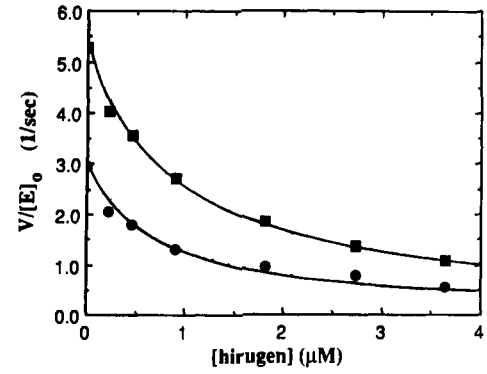

FIGURE 4: Effect of hirugen on the initial rates (V) of α-thrombin-catalyzed release of AP (●) and FPB (■) from mixtures of 1.4 μM fibrin I protomer, 0.35 μM plasma factor XIII protomer, 3.6 pM α-thrombin ($[E]_o$), and the plotted hirugen concentrations. The lines shown represent the results of nonlinear least-squares fits of the data to eq 8 (●) or eq 9 (■) where the values of $k_{cat'}^{AP}/K_{m'}^{FX}$, $k_{cat'}^{FPB}/K_{m'}^{F}$, $k_{cat'}^{FPB\cdot FX}/K_{m'}^{FX}$, $K_{m'}^{FX}$, and $K_{m'}^{F}$ were set to the previously determined values of 1.2×10^7 M^{-1} s^{-1}, 4.5×10^6 M^{-1} s^{-1}, 8.2×10^6 M^{-1} s^{-1}, 2.5 μM, and 7.5 μM, respectively. The best fits yielded values for K_H of 0.59 ± 0.05 μM (●) and 0.75 ± 0.03 (■).

Recently we have characterized hirugen, a 12-residue peptide corresponding in primary sequence to the COOH terminus of hirudin (a potent inhibitor of α-thrombin found in the saliva of certain leeches), which competitively inhibits the interaction of α-thrombin with fibrinogen (Naski et al., 1990). This peptide was shown to be an exosite-directed competitive inhibitor of the action of α-thrombin on fibrinogen that does not inhibit the α-thrombin-catalyzed hydrolysis of small amide substrates (Naski et al., 1990). To establish whether interactions at the same exosite of α-thrombin necessary for α-thrombin-catalyzed cleavage of fibrinogen are also necessary for the promotion of α-thrombin-catalyzed release of AP from plasma factor XIII by fibrin I polymer, we measured the effect of hirugen on the initial rate of AP release from plasma factor XIII in the presence of fibrin I polymer. Maintaining constant concentrations of fibrin I, plasma factor XIII, and α-thrombin, we measured the hirugen dependence of the initial rate of AP release (Figure 4). The data of Figure 4 (circles) were fit to eq 8, which assumes that binding of hirugen (H) to α-

$$\frac{V}{[E]_o} = \frac{\dfrac{k_{cat'}^{AP}}{K_{m'}^{FX}}[(\alpha B\beta\gamma)_n\cdot ab]}{1 + [\alpha B\beta\gamma]/K_{m'}^{F} + [(\alpha B\beta\gamma)_n\cdot ab]/K_{m'}^{FX} + [H]/K_H} \quad (8)$$

thrombin competitively inhibits the interaction of α-thrombin with fibrin I bound plasma factor XIII. Equation 8 is very similar to eq 3; however, there is an additional term in the denominator, $[H]/K_H$, accounting for the α-thrombin–hirugen complex, governed by the dissociation constant K_H.[3] Setting

[3] The term in the numerator of eq 3, $(k_{cat}/K_m)[ab]$, that accounts for α-thrombin-catalyzed hydrolysis of free, uncomplexed plasma factor XIII is not included in eq 8 because under the conditions of the experiment only 6% of the total plasma factor XIII is not bound to fibrin I polymer and this amount of free plasma factor XIII would maximally contribute 0.07% of the measured initial rate. In separate control experiments (data not shown) the presence of hirugen had little effect both on the α-thrombin-catalyzed hydrolysis of plasma factor XIII in the absence of fibrin I and on the binding of plasma factor XIII to fibrin I polymer. For example, 2.73 μM hirugen slightly increased (by less than 10%) the specificity constant (k_{cat}/K_m) for α-thrombin-catalyzed hydrolysis of plasma factor XIII in the absence of fibrin and had no detectable effect on the binding of 0.96 μM plasma factor XIII protomer to 2 μM fibrin I protomer (a 15% decrease would have been detected).

the values of $k_{cat'}^{AP}/K_{m'}^{FX}$, $K_{m'}^{FX}$, and $K_{m'}^{F}$ equal to the previously determined values of 1.2×10^7 M^{-1} s^{-1}, 2.5 μM, and 7.5 μM, respectively, and determining the concentrations of $\alpha B\beta\gamma$ and $ab\cdot(\alpha B\beta\gamma)_n$ by using eqs 4 and 5, we obtained a good fit of the data to eq 8 with a value for K_H, the dissociation constant for the α-thrombin–hirugen complex, equal to 0.59 ± 0.05 μM. This value for K_H agrees with previously determined values (0.54–0.64 μM) for this dissociation constant (Naski et al., 1990). These results indicate that (i) hirugen is a competitive inhibitor of the action of α-thrombin on fibrin I bound plasma factor XIII, (ii) the same exosite of α-thrombin necessary for the interaction of α-thrombin with fibrinogen is also necessary for the interaction of α-thrombin with fibrin I bound plasma factor XIII, and (iii) the major determinant of the interaction of α-thrombin with factor XIII in the ternary complex results from the binding of α-thrombin to fibrin I polymer. In accord with these conclusions is the finding that γ-thrombin, a proteolytic derivative of α-thrombin with a defective fibrinogen-binding exosite (Lewis et al., 1987), does not cleave plasma factor XIII at an accelerated rate in the presence of fibrin (Shafer et al., 1986; Lewis et al., 1987; Greenberg et al., 1986, 1987). The importance of the interaction between α-thrombin and fibrin I polymer for accelerating the rate of AP release from plasma factor XIII is also suggested by the similarity of the apparent affinity (as approximated by the Michaelis constants) of α-thrombin for fibrin I bound plasma factor XIII (K_m = 2.5 μM)[4] to the apparent affinity (as approximated by the Michaelis constants) of α-thrombin for fibrin I polymer (K_m = 7.5 μM; Naski & Shafer, 1990) and fibrinogen (K_m = 7.2–11.2 μM; Higgins et al., 1983; Hofsteenge et al., 1986).

Hirugen also behaves as a competitive inhibitor of the initial rate of α-thrombin-catalyzed release of FPB from fibrin I polymer in the presence of plasma factor XIII. Figure 4 displays the results showing the hirugen dependence of the initial rate of α-thrombin-catalyzed release of FPB from fibrin I polymer in the presence of plasma factor XIII (squares), which were obtained at the same time as those for the initial rates of AP release (circles). The curve shown represents a nonlinear least-squares fit of the data to eq 9. This equation

$$\frac{V}{[E]_o} = \frac{\frac{k_{cat'}^{FPB}}{K_{m'}^{F}}[\alpha B\beta\gamma] + \frac{k_{cat'}^{FPB\cdot FX}}{K_{m'}^{FX}}[(\alpha B\beta\gamma)_n\cdot ab]}{1 + [\alpha B\beta\gamma]/K_{m'}^{F} + [(\alpha B\beta\gamma)_n\cdot ab]/K_{m'}^{FX} + [H]/K_H} \quad (9)$$

is identical with eq 6 except for the additional term in the denominator, $[H]/K_H$, accounting for the α-thrombin–hirugen complex, which blocks the interaction of α-thrombin with fibrin I and represents the effect of hirugen as a competitive inhibitor of the initial rate of FPB release. With the values of $k_{cat'}^{FPB}/K_{m'}^{F}$, $k_{cat'}^{FPB\cdot FX}/K_{m'}^{FX}$, $K_{m'}^{F}$, and $K_{m'}^{FX}$ set equal to the previously determined values of 4.5×10^6 M^{-1} s^{-1}, 8.2×10^6 M^{-1} s^{-1}, 7.5 μM, and 2.5 μM (Table I), a nonlinear least-squares fit of the data to eq 9 yielded a value for K_H of 0.75 ± 0.03 μM, which is in reasonable agreement with previously determined values (Naski et al., 1990). The effect of hirugen as a competitive inhibitor of the α-thrombin-catalyzed release of (i) FPA from fibrinogen (Naski et al., 1990), (ii) FPB from fibrin I, and (iii) AP from fibrin I bound plasma factor XIII suggests that the interaction of α-thrombin with the substrates fibrinogen, fibrin I, and fibrin I bound factor XIII is largely determined by a single exosite of α-thrombin bound by the 12-residue peptide hirugen.

Our results showing that plasma factor XIII binds tightly to fibrin I polymer and that plasma factor XIII bound to fibrin I polymer is cleaved faster than free factor XIII by α-thrombin predict that during coagulation the major pathway for α-thrombin-catalyzed cleavage of plasma factor XIII occurs through formation of a ternary complex of α-thrombin, plasma factor XIII, and fibrin I polymer. If we assume typical plasma concentrations of plasma factor XIII protomer (40 nM) and fibrinogen protomer (17.6 μM) and that in the volume element where fibrin is generated 90% of the fibrinogen is converted to fibrin, eq 5 then predicts that 97.5% of the 40 nM plasma factor XIII protomer (in the volume element where fibrin is generated) is bound to the fibrin clot. Consequently, eq 3 predicts a 27-fold acceleration of the initial rate of α-thrombin-catalyzed cleavage of plasma factor XIII when compared to the initial rate expected for α-thrombin-catalyzed cleavage of plasma factor XIII in the absence of fibrin.

It is interesting to note that cross-linking of fibrin I results in rapid loss of cofactor activity. Thus, the cofactor activity of non-cross-linked fibrin may serve to (i) ensure that factor $XIII_a$ is selectively generated in close proximity to its initial substrate, fibrin I, and (ii) minimize wasteful generation of factor $XIII_a$ when its initial substrate, non-cross-linked fibrin I, is depleted (Lewis et al., 1985a).

Registry No. Blood coagulation factor XIII, 9013-56-3; thrombin, 9002-04-4; hirugen, 121822-23-9.

[4] The difference between the measured K_m's for α-thrombin-catalyzed release of FPB from the fibrin I–plasma factor XIII complex and uncomplexed fibrin I polymer may in part be due to different definitions for the unit of substrate bound by α-thrombin. The interaction of α-thrombin with fibrin I polymer is based upon a unit of substrate equal to a fibrin protomer; however, the interaction of α-thrombin with plasma factor XIII bound to fibrin I is based upon a unit of fibrin substrate equal to that present in a complex containing plasma factor XIII protomer bound to 1.6 fibrin I protomers. Assuming that α-thrombin interacts with the fibrin in the fibrin I–factor XIII complex, one should multiply by 1.6 the value of K_m for α-thrombin-catalyzed release of FPB from fibrin I complexed with factor XIII to compare it with the K_m values observed for α-thrombin-catalyzed release of FPB from uncomplexed fibrin I. Kinetic factors and perturbations of the alternative modes of binding for α-thrombin bound to fibrin I may also contribute to the difference in the K_m's of α-thrombin for fibrin I and fibrin I bound plasma factor XIII. Further work is necessary to determine the cause for the different K_m's for α-thrombin-catalyzed release of FPB from fibrin I (7.5 μM) and fibrin I complexed with factor XIII (2.5 × 1.6 or 4.0 μM).

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