Articles

Crosslinking Kinetics of the Human Transglutaminase, Factor XIII[A_2], Acting on Fibrin Gels and γ-Chain Peptides†

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ABSTRACT: Factor XIII is the terminal enzyme of the coagulation cascade which serves to rapidly crosslink the adjacent γ-chain C-termini of fibrin clots. *In vivo*, this process is initiated by the proteolytic action of thrombin which simultaneously converts both soluble fibrinogen to fibrin and activates zymogen FXIII; fibrin then spontaneously polymerizes to form a gel which activated FXIII stabilizes through crosslinking. Due to the kinetic complexity and the difficulty of investigating gel phase reactions, methods employing pre-activation of recombinant human Factor XIII (rFXIII[$\mathbf{A'_2}$]) were developed to effectively decouple these reactions. By utilizing these methods, the kinetic parameters of γ-chain crosslinking in fibrin gels could be determined by both initial rate and integrated rate techniques under physiologically relevant conditions. The crosslinking of the γ-chain of fibrin gels could be described by apparent Michaelis kinetics with $K_{m(app)} = 6.2\ \mu$M, $k_{cat} = 1872\ \text{min}^{-1}$, and $K_{sp} = 302\ \text{min}^{-1}\ \mu\text{M}^{-1}$ for a fibrin γ-chain monomer of $M_r = 170\,000$ Da. In contrast, both the crosslinking rates of α-chains within fibrin gels ($K_{sp} = 0.38\ \text{min}^{-1}\ \mu\text{M}^{-1}$: Bishop et al. (1993)) and the crosslinking of a soluble synthetic peptide containing the unique γ-chain fibrin crosslinking site ($K_{sp} = 0.030\ \text{min}^{-1}\ \mu\text{M}^{-1}$) could not be shown to saturate and gave apparent first-order rates with respect to rFXIII[$\mathbf{A'_2}$]. These observations coupled with the large differences in the turnover rates ($\sim 10^4$) suggest two likely mechanisms for FXIII[$\mathbf{A'_2}$]−substrate interactions: (1) random (or independent) binding of non- or weakly interacting substrate pairs imposes a high entropic barrier (i.e., $\Delta G_{binding}$) to the formation of a productive catalytic complex, e.g., for soluble γ-chain peptides and the flexible α-chains within fibrin, and (2) binding to an oriented substrate pair effectively lowers the entropic barrier to formation of a Michaelis complex and thus greatly enhances the rate of catalysis, e.g., for γ-chain pairs within the fibrin fibrils.

Factor XIII (FXIII[$\mathbf{A_2}$])[1] (EC 2.4.2.13), is a zymogen transglutaminase which is specifically activated during the final phase of blood coagulation by thrombin cleavage and Ca^{2+}. In the nascent fibrin clot, activated Factor XIII (FXIII-[$\mathbf{A'_2}$]) serves to catalyze the formation of γ-glutamyl-ϵ-lysyl isopeptide bonds (crosslinks) between adjacent molecules of fibrin (Loewy, 1972) and between fibrin and other plasma proteins (Ichinose et al., 1983; Mosher & Schad, 1979). The FXIII-induced inter-molecular fibrin crosslinking greatly enhances the mechanical strength (Shen & Lorand, 1983), stability (Shainoff & Page, 1962), and resistance to proteolysis of fibrin clots (Edwards et al., 1993; Francis & Marder, 1988; Siebenlist & Mosesson, 1994). The increase in proteolytic resistance of fibrin is also due in part to the FXIII-induced crosslinking of α_2-PI into the fibrin clot (Ichinose et al., 1983; Sakata & Aoki, 1980).

Factor XIII zymogen exists in two forms; in plasma it is found circulating exclusively as a heterotetrameric complex of **A** and **B** subunits [$\mathbf{A_2B_2}$] whereas in platelets, placenta, and monocyte/macrophages it is found as a homodimer [$\mathbf{A_2}$] (Henriksson et al., 1985; Muszbek et al., 1985; Schwartz et al., 1971). Recombinant human FXIII[$\mathbf{A_2}$] (rFXIII[$\mathbf{A_2}$]) has been expressed in yeast, purified to homogeneity (Bishop et al., 1990b), and characterized as functionally identical to platelet FXIII[$\mathbf{A_2}$] (Hornyak et al., 1989), and its crystallographic structure has been solved (Bishop et al., 1990a; Pedersen et al., 1994; Yee et al., 1994). The **A** subunit of the zymogen contains the latent transglutaminase activity (Curtis et al., 1973). Activation of both forms of FXIII is initiated by specific cleavage of a 4 kDa activation peptide from the N-terminus of the **A** subunit by α-thrombin to generate a 79 kD **A′** subunit (Ichinose et al., 1986; Takagi & Doolittle, 1974). In the case of the plasma heterotetramer, activation allows the release of the inhibitory **B** subunit by Ca^{2+} (Chung et al., 1974; Curtis et al., 1974; Lorand et al., 1974). Conversion of FXIII to the active state is facilitated by fibrinogen or fibrin as a cofactor (Credo et al., 1981; Curtis et al., 1983; Lewis et al., 1985a; Shafer et al., 1986)

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[1] Abbreviations: FXIII, Factor XIII; FXIII[$\mathbf{A_2B_2}$], plasma human Factor XIII zymogen; FXIII[$\mathbf{A_2}$], platelet human Factor XIII zymogen; FXIII[$\mathbf{A'_2}$], activated platelet human Factor XIII; rFXIII, recombinant human Factor XIII[$\mathbf{A_2}$]; MDC, monodansylcadaverine; FBGN, human fibrinogen.

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and irrespective of the source of FXIII, binding of Ca^{2+} to the **A′** homodimer (FXIII[$\mathbf{A'_2}$]) is essential for catalytic activity.

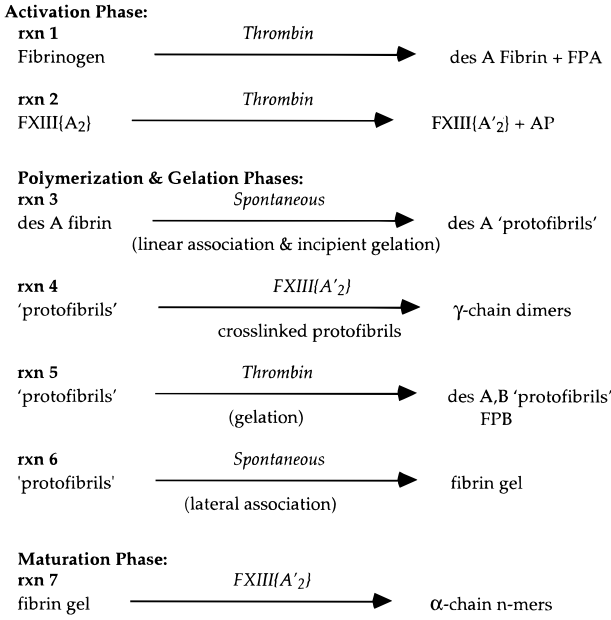
The time course of FXIII catalyzed fibrin crosslinking is sequential; during an initial phase, the bulk of fibrin γ-chains are rapidly crosslinked to form dimers, then, more slowly, the α-chains are crosslinked to high molecular weight products, and more slowly still, γ-chain trimers, tetramers, higher order *n*-mers, and α- and γ-chain hybrids are formed (Bishop et al., 1993; Francis & Marder, 1987; Mosesson et al., 1995; Shainoff et al., 1991; Siebenlist & Mosesson, 1994). The crosslinking of other, non-fibrin proteins into plasma clots is quantitatively less significant and occurs at differing rates dependent on the protein species; e.g., fibronectin, and α_2-PI. The specific formation of fibrin γ-chain dimers is characteristic of FXIII. Whereas other mammalian transglutaminases have been shown to catalyze the formation of α-chain fibrin crosslinks, significant γ-chain dimers are not observed; e.g., FXIII vis-à-vis the ubiquitous tissue transglutaminase (Murthy & Lorand, 1990; Purves et al., 1987; Shainoff et al., 1991). Rapid formation of fibrin γ-chain dimer is critical to providing mechanical integrity to a fibrin clot and thereby defines an essential role for FXIII in hemostasis (Kamykowski et al., 1981; Shen & Lorand, 1983).

The kinetics of FXIII-induced crosslinking are complex; under physiologically relevant conditions, fibrin crosslinking is initiated by the simultaneous cleavage of zymogen FXIII-[$\mathbf{A_2}$] and fibrinogen by thrombin to yield activated FXIII-[$\mathbf{A'_2}$] and fibrin, respectively. These kinetics are further complicated by the observation that the formation of gel-phase fibrin from soluble fibrin monomers is multiphasic (Carr et al., 1987; Lewis et al., 1985b; Scheraga, 1983; Weisel et al., 1993) and that the presence of polymeric fibrin accelerates the rate of thrombin activation of FXIII (Curtis et al., 1983; Greenberg et al., 1987, 1988; Lewis et al., 1985a; Shafer et al., 1986). Thus, the earliest reaction product of FXIII-induced crosslinking, γ-chain dimerization, is intimately coupled to multiple reactions involving both the formation of fibrin gel and activation of FXIII. This reaction sequence is illustrated in Scheme 1 (modified from Hantgan et al. (1983)).

In the present study, we have endeavored to decouple these reactions and to accurately determine the kinetic parameters of the γ-chain crosslinking alone. Thrombin activation of rFXIII[$\mathbf{A_2}$] was temporally dissociated from fibrin crosslinking by using a preactivated preparation of rFXIII[$\mathbf{A'_2}$]; i.e., preactivating rFXIII[$\mathbf{A_2}$] with thrombin prior to initiation of thrombin catalyzed fibrin formation. In addition, thrombin concentrations were chosen that allowed fibrin formation and polymerization to proceed much faster than γ-chain crosslinking. The influence of fibrin polymerization on the observed rate of crosslinking was assessed by obtaining a full range of initial rate measurements on a large array of rFXIII[$\mathbf{A'_2}$] to fibrinogen concentrations and cross-checked with integrated rate measurements. Thus, by dissecting out confounding reactions and employing highly purified preparations rFXIII[$\mathbf{A'_2}$] and fibrinogen free of endogenous plasma FXIII, it was possible to directly determine the kinetics of γ-chain crosslinking under physiologically relevant conditions.

This approach to fibrin crosslinking allowed us access to several fundamental questions on the mechanism of FXIII:

Scheme 1



e.g., (1) Does rFXIII[$\mathbf{A'_2}$] behave as a Michaelis enzyme when acting on the polymeric, insoluble, gel-phase fibrin substrate? (2) Does the structural nature of the substrate for the γ-chain crosslinking differ from that of α-chain substrates in a way which affects the binding order of the Gln/Lys substrate couple; viz., can acceptor/donor binding occur *both* as a concerted event for γ-chain pairs *and* as independent, sequential events for α-chain products? By comparing the crosslinking kinetics of the γ-chain to those of both the α-chain and a soluble, low molecular weight peptide derived from the γ-chain of fibrinogen, insight was gained into the catalytic order of rFXIII[$\mathbf{A'_2}$] and the structural nature of the fibrin crosslinking substrates. In addition, data from this study, taken together with previous studies, indicate that fibrin contains both crosslinking sites of varying reactivity and affinity toward rFXIII[$\mathbf{A'_2}$] *and* exosites which influence the molecular mobility of rFXIII[$\mathbf{A'_2}$] within the clot matrix. On the basis of our kinetic results, we propose a model for the interaction of rFXIII[$\mathbf{A'_2}$] with fibrin in the broad context of an enzyme diffusing within a complex but highly ordered fibrous structure.

MATERIALS AND METHODS

Fibrinogen. In order to accurately determine the kinetics of the γ-chain crosslinking of fibrin by rFXIII[$\mathbf{A'_2}$], it was necessary to first purify fibrinogen free of plasma proteins known to be substrates for rFXIII[$\mathbf{A'_2}$] (e.g., fibronectin) and also to remove residual plasma factor XIII (i.e., FXIII[$\mathbf{A_2B_2}$], typically $\sim$1 μg/mg) so that the concentrations of added rFXIII[$\mathbf{A_2}$] could be made sufficiently low to allow adequate sampling of the reaction time course. Human fibrinogen (>95% clottable) free of fibronectin and plasmin was obtained from Enzyme Research Laboratories (IN) and further purified to remove all traces of FXIII[$\mathbf{A_2B_2}$] by passing it over a column of anti-human rFXIII[$\mathbf{A_2}$] antibody coupled to CNBr Sepharose (Pharmacia). Aliquots of the resulting fibrinogen solution were quick frozen and stored at -80 °C until used. In all control experiments where thrombin and Ca^{2+} were added to these FXIII-free fibrinogen preparations, neither γ-chain dimerization nor α-chain polymerization was observed after 24 h of incubation.

Thrombin. Bovine α-thrombin (1800 units/mg) was obtained from Enzyme Research Laboratories (IN) and stored at −80 °C in 500 units/mL aliquots in 50% glycerol. Recombinant human thrombin (3000 units/mL), was obtained in house, assayed using a chromogenic assay (Boehringer) and a clotting test, and was also stored at −80 °C in 500 units/mL aliquots in 50% glycerol.

rFXIII[A_2] and rFXIII[A'_2]. Recombinant human rFXIII-[$\mathbf{A_2}$] was expressed in *Saccharomyces cerevisiae*, purified to homogeneity, and characterized as biochemically identical to the native platelet FXIII[$\mathbf{A_2}$] (Bishop et al., 1990b). In experiments where rFXIII[$\mathbf{A_2}$] was preactivated to rFXIII-[$\mathbf{A'_2}$], a stock solution of 80 μg/mL rFXIII[$\mathbf{A_2}$] in TBS buffer (25 mM Tris-HCl, 2.5 mM Ca^{2+}, 100 mM NaCl, pH 7.4) was incubated with 4 units/mL human thrombin for 10 min at 37 °C just prior to the kinetics experiment. All rFXIII-[$\mathbf{A'_2}$] solutions were used within 120 min of thrombin addition. To confirm that complete activation of rFXIII-[$\mathbf{A'_2}$] was achieved by this protocol and that no losses in transglutaminase activity were occurring over the time course of our experiments, solutions of rFXIII[$\mathbf{A'_2}$] were assayed during activation and for 120 min afterward by the dansylcadaverine method of Lorand et al. (1971) as modified by Hornyak et al. (1989).

Fibrin Clots. Since samples of the reaction mixture could not be taken once the mixture had gelled, individual 0.2−0.5 mL samples were prepared in conical Eppendorf centrifuge tubes for each time point. Reactions were performed in TBS with 2.5 mM $CaCl_2$ added at 37 °C. Kinetics experiments were initiated by sequentially mixing solutions of fibrinogen, with thrombin plus either rFXIII[$\mathbf{A_2}$] or rFXIII-[$\mathbf{A'_2}$]. Irrespective of the mixing format, all samples were incubated at 37 °C for measured lengths of time, quenched by quick freezing in a dry-ice methanol bath or directly denatured in a boiling water bath, and stored at −20 °C until analysis. On the basis of the reaction time course extrapolated to $t = 0$, we estimate that the quench time was <30 s using this technique. The time courses followed in these experiments were typically 10−30 min, with sampling at intervals of 1 min or greater.

Fibrin Solubilization. Frozen samples were heat inactivated in a boiling water bath for 5 min and then centrifuged at 14000*g* for 5 min. The resulting supernatants contained no significant protein and were removed to reduce the volume. A solution of 10 M urea, 0.1 M β-mercaptoethanol, 1% sodium dodecyl sulfate, 10 mM EDTA at pH 7 was added to the fibrin pellet to yield a fibrin solution with a final concentration of 1 mg/mL. The fibrin samples were then incubated at 60 °C for 16 h during which time complete dissolution was achieved.

SDS−PAGE. Solubilized fibrin samples (1 mg/mL protein) were loaded onto 5−15% Tris-glycine gels (ISS, Natick, MA) at 4 μg/lane. Protein was visualized by Coomassie blue or amido black staining, and the α, β, γ, and γ-dimer band intensities were quantified by densitometric scanning using a digital CCD camera (BioImage A) and a Sun Sparc computer equipped with imaging software (BioImage, AnnArbor, MI). To account for slight differences in gel loading, α, γ, and γ-dimer band intensities were normalized to the corresponding β band intensities of each lane since the β-chain does not participate in any crosslinking reactions with FXIII[$\mathbf{A'_2}$]. The γ-dimer band stained less intensly than the γ band, but since γ and γ-dimer were the only significant γ-chain containing species initially detected in the reaction mixture, band intensities were further normalized to the combined γ and γ-dimer band intensities. Independent experiments using HPLC rather than SDS−PAGE to analyze the reduced reaction mixtures (data not shown) confirmed that all significant γ-chain species were accounted for and that the normalization procedure was valid. Unless otherwise specified, kinetic parameters were calculated from the consumption of γ-chain expressed as μM fibrinogen (340 000 g/mol).

γ-Chain Peptide. A peptide derived from the known crosslinking sequence of the γ-chain of fibrinogen (TIGEGQQHHLGGAKQAGDV) was synthesized in house for use in kinetic experiments.

Rate Dependence on Thrombin Concentration. The effect of varying thrombin concentration on the velocity of γ-chain crosslinking at constant rFXIII[$\mathbf{A_2}$] or rFXIII[$\mathbf{A'_2}$] was determined using 7.4 μM (2.5 mg/mL) human fibrinogen clotted by the addition of from 0.25 to 5 units/mL human thrombin in the presence of 6.0 nM (1 μg/mL) rFXIII[$\mathbf{A_2}$] or rFXIII[$\mathbf{A'_2}$] at 37 °C, pH 7.4.

Rate Dependence on FXIII Concentration. The effect of varying rFXIII[$\mathbf{A_2}$] and rFXIII[$\mathbf{A'_2}$] concentration on the velocity of γ-chain crosslinking at constant thrombin was determined using 7.4 μM (2.5 mg/mL) human fibrinogen clotted by the addition of 1 unit/mL human thrombin in the presence of varying concentrations of rFXIII[$\mathbf{A_2}$] or rFXIII-[$\mathbf{A'_2}$] at 37 °C, pH 7.4.

Rate Dependence on Fibrinogen Concentration. The effect of fibrinogen concentration on the velocity of γ-chain crosslinking at 1 unit/mL thrombin was determined at several different rFXIII[$\mathbf{A'_2}$] concentrations so that accurate densitometry scans could be obtained. Velocities were normalized to the rFXIII[$\mathbf{A'_2}$] concentration.

Rate Dependence on Peptide Concentration. Reactions using the γ-chain peptide (TIGEGQQHHLGGAKQAGDV) were performed at higher rFXIII[$\mathbf{A'_2}$] concentrations than the fibrin crosslinking reactions; 241 nM (40 μg/mL) rFXIII-[$\mathbf{A'_2}$] and at variable peptide concentration. Reaction rates were determined by analyzing the peptide reaction mixtures using reverse phase HPLC (column, 4.6 × 250 mm Vydac C18; gradient, 0−80% acetonitrile/0.05% TFA in 40 min; flow rate, 1 mL/min) with LC−MS confirmation of peak identities.

RESULTS

Kinetic parameters for the crosslinking of fibrin by rFXIII-[$\mathbf{A'_2}$] were calculated by analyzing the reaction mixture at various times using reduced SDS−PAGE. The simultaneous disappearance of substrate γ-chains and appearance of product γ-dimers were clearly detected by SDS−PAGE (Figure 1A). No significant γ-chain-containing products other than γ-dimers were observed in our initial rate experiments. Typically, reaction times were too short to observe polymerization of α-chains. The reaction time course was expressed as the mole fraction of the γ-chains consumed or the mole fraction of γ-chains incorporated into dimers as a function of time (Figure 1B). Initial rates were determined from the reaction time course plots and expressed as rates in μM fibrin reacted per minute per μM rFXIII-[$\mathbf{A'_2}$]. (There are two γ-chains per fibrin molecule.)

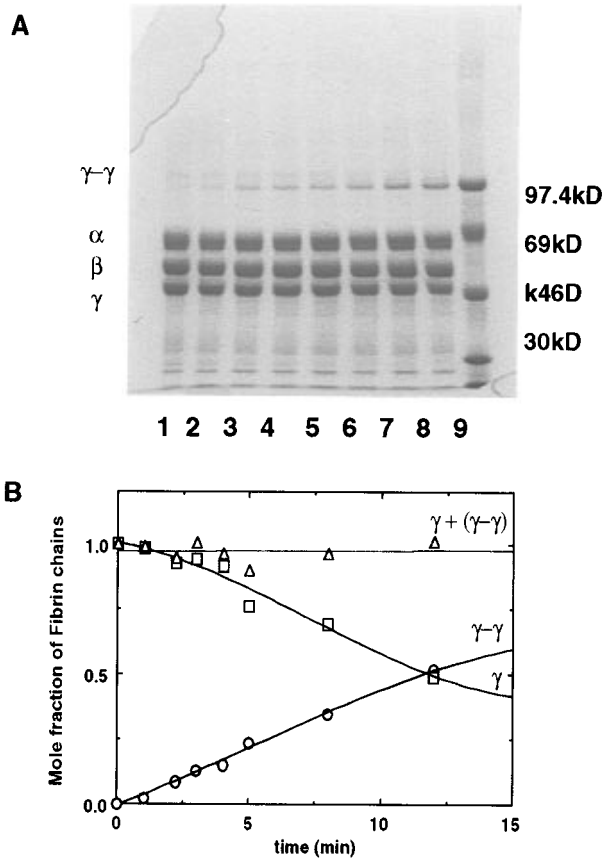


FIGURE 1: (A) Reducing SDS−PAGE analysis of rFXIII[$\mathbf{A'_2}$] catalyzed crosslinking of fibrin. Analysis was determined on timed reactions of 7.4 μM (2.5 mg/mL) human fibrinogen clotted by addition of 1 unit/mL human thrombin in the presence of 1.5 nM (0.25 μg/mL) rFXIII[$\mathbf{A'_2}$] at pH 7.4 and 37 °C. (A) Coomassie blue stained SDS−PAGE gel; lanes 1−8 correspond to reaction times of 0, 1, 2, 3, 4, 5, 8, and 12 min, respectively, and lane 9 contains molecular-weight standards. (B) Reaction time course of crosslinking obtained from the densitometric scanning of the SDS−PAGE gel. Band intensities are normalized (see text) and expressed as mole fraction of each chain relative to unreacted β-chain, γ-chain (γ), γ-dimer (expressed as mole fraction of γ-chains dimerized) (γ-γ), and $\gamma + \gamma$-γ (Δ).

As can be seen in Figure 2A and 2B, thrombin concentration strongly affected the rate of crosslinking by rFXIII. This behavior can be attributed to the action of thrombin in both the formation of the substrate for the crosslinking reaction (fibrin) and the formation of the active crosslinking enzyme (rFXIII[$\mathbf{A'_2}$]). Thrombin concentration affected the rate of γ-chain cross-linking differently with respect to rFXIII[$\mathbf{A_2}$] and rFXIII[$\mathbf{A'_2}$]; when rFXIII[$\mathbf{A_2}$] was used, initial reaction rates appeared to "lag" at low thrombin concentrations (<1 unit/mL), whereas preactivated rFXIII[$\mathbf{A'_2}$] gave no such "lag". In both cases the crosslinking rates appeared to saturate at ~2 units/mL thrombin.

Figure 3A and 3B shows the dependence of γ-chain crosslinking on rFXIII concentration at constant (1 unit/mL) thrombin. Linear behavior with similar specificity constants was observed for both rFXIII[$\mathbf{A_2}$] and rFXIII[$\mathbf{A'_2}$]. Some deviation from linearity was observed at the highest rFXIII concentrations (6 nM; 1 μg/mL).

Figure 4A shows the dependence of crosslinking rate on fibrinogen concentration. Thrombin was held constant at 1 unit/mL, and the concentration of rFXIII[$\mathbf{A'_2}$] was varied with the fibrinogen concentration in order to obtain measurable rates. Figure 4B shows the Lineweaver−Burk plot used to

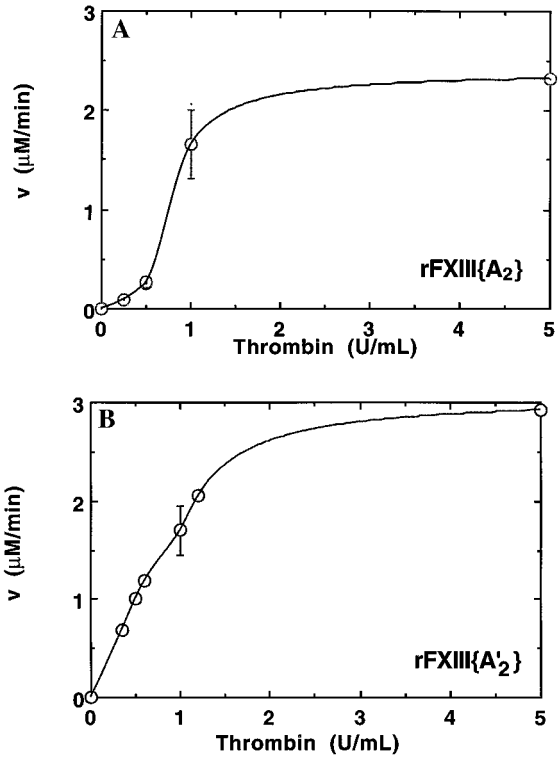


FIGURE 2: Rate dependence of γ-chain crosslinking on thrombin at (A) constant zymogen rFXIII[$\mathbf{A_2}$] and (B) constant preactivated rFXIII[$\mathbf{A'_2}$]. Velocities were determined using 7.4 μM (2.5 mg/mL) human fibrinogen clotted by addition of 0.25, 0.5, 1, and 5 units/mL human thrombin in the presence of 6.0 nM (1 μg/mL) rFXIII[$\mathbf{A_2}$] or preactivated rFXIII[$\mathbf{A'_2}$] at pH 7.4 and 37 °C.

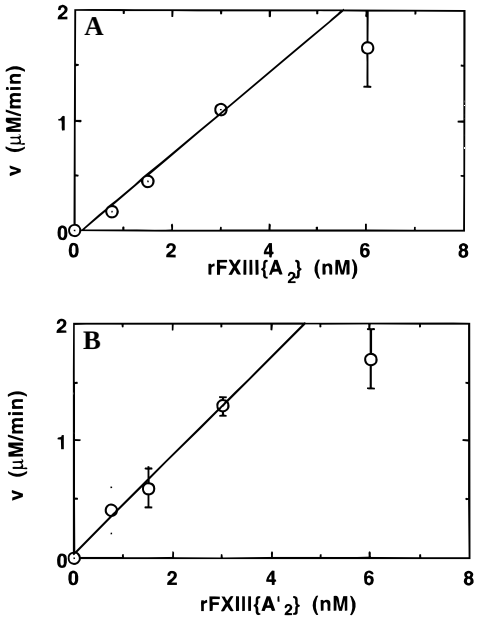


FIGURE 3: Rate dependence of γ-chain crosslinking on rFXIII concentration with (A) zymogen rFXIII[$\mathbf{A_2}$] and (B) preactivated rFXIII[$\mathbf{A'_2}$]. Velocities were determined using 7.4 μM (2.5 mg/mL) human fibrinogen clotted by the addition of 1 unit/mL human thrombin in the presence of varying concentrations of rFXIII[$\mathbf{A_2}$] or preactivated rFXIII[$\mathbf{A'_2}$] at pH 7.4 and 37 °C.

determine the apparent Michaelis constant (K_m = 3.1 μM), the catalytic constant (k_{cat} = 936 min^{-1}), and the specificity constant ($K_{sp} = k_{cat}/K_m$ = 302 min^{-1} μM^{-1}). This specificity constant was independently verified by determining the pseudo-first-order rate constants at low fibrinogen concentration ($\ll K_m$) for both the appearance of γ-chain dimers (311

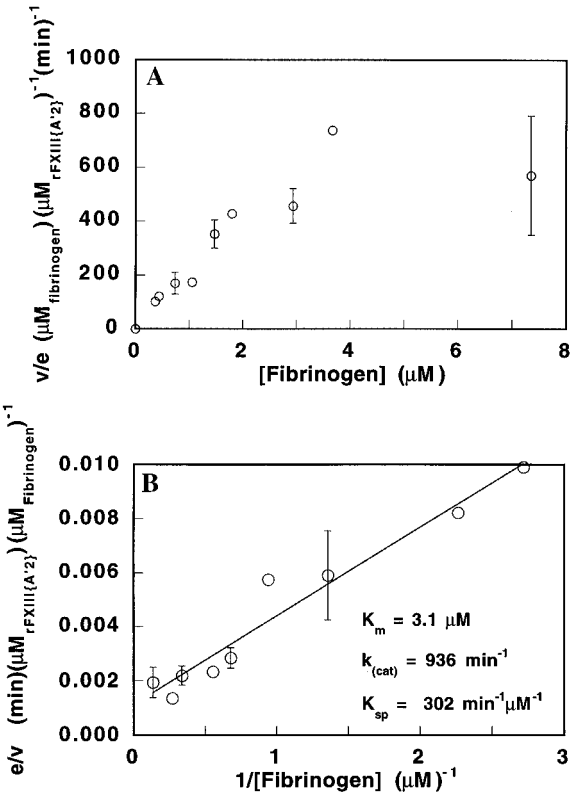


FIGURE 4: Rate dependence of γ-chain crosslinking on fibrinogen concentration at 1 unit/mL thrombin. (A) Plot of velocity per μM rFXIII[$\mathbf{A'_2}$] of γ-chain crosslinking vs fibrinogen concentration. (B) Lineweaver−Burk plot of the same data. (The regression line is weighted to include all data. Error bars are SD; $4 < n < 23$.) K_m and k_{cat} values are calculated per fibrinogen molecule.

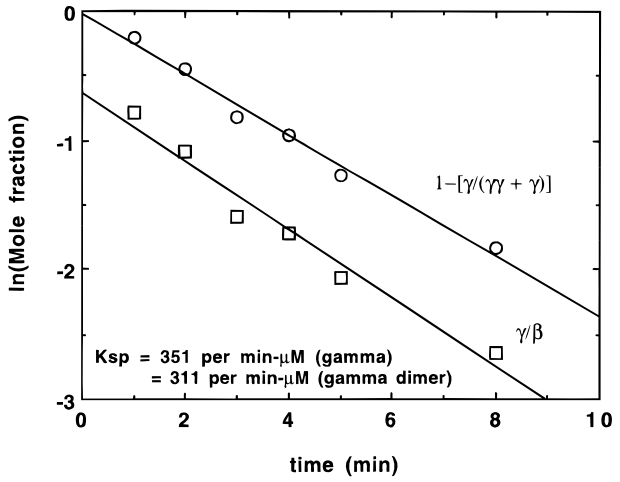


FIGURE 5: Determination of K_{sp} from the pseudo-first-order rate constant of fibrin crosslinking ([fbgn] $\ll K_m$). Reaction conditions: 0.74 μM (0.25 mg/mL) Fbgn, 1 unit/mL thrombin, 0.75 nM (0.125 μg/mL rFXIII[$\mathbf{A'_2}$]). K_{sp} was determined for both γ-chain consumption (□) and γ-dimer production (○).

min^{-1} μM^{-1}) and the disappearance of γ-chain (351 min^{-1} μM^{-1}); see Figure 5.

The rate of crosslinking of the soluble peptide (TIGEGQQHHLGGAKQAGDV) was studied at a constant concentration of rFXIII[$\mathbf{A'_2}$]. In these experiments, thrombin had no effect on the substrate, and the rFXIII[$\mathbf{A'_2}$] was fully activated at the start of the experiment. K_{sp} for this reaction (0.030 min^{-1} μM^{-1}) was determined from the first-order rate dependence on peptide concentration (Figure 6), as Lineweaver−Burk analysis did not yield an interpretable K_m (not shown).

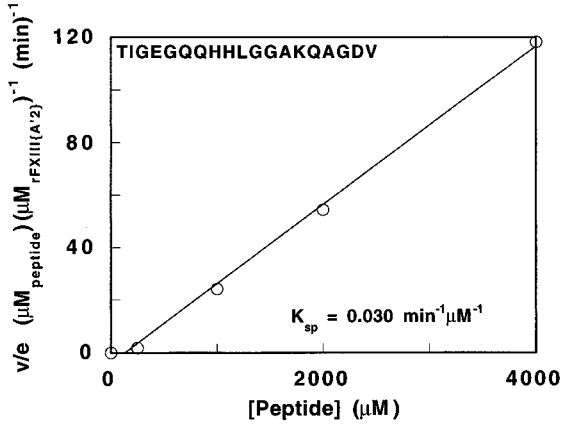


FIGURE 6: Rate dependence of crosslinking on peptide concentration. The peptide (TIGEGQQHHLGGAKQAGDV) is derived from the γ-chain of fibrinogen: 241 nM (40 μg/mL) rFXIII[$\mathbf{A'_2}$] at pH 7.4 and 37 °C. Velocities are normalized to 1 μM rFXIII[$\mathbf{A'_2}$].

DISCUSSION

In this study, analysis of fibrin γ-chain crosslinking by SDS−PAGE and densitometry was used to obtain accurate kinetic data for rFXIII[$\mathbf{A'_2}$] (Figure 1). Initial rate methods were the primary means of determining the enzymatic constants, K_m and k_{cat} for fibrin γ-chain crosslinking and integrated rate methods provided confidence in these values *via* independently determined specificity constants (K_{sp}). Initial rate studies of γ-chain crosslinking also served to minimize potential higher order effects due to cryptic crosslinking, i.e., substrate competition by singly crosslinked γ-chain dimers to form doubly crosslinked γ-chain dimers. Doubly crosslinked γ-chain dimers, in which adjacent γ-chains are crosslinked through both Gln and Lys residues are observed with the soluble peptide (TIGEGQQHHLGGAKQAGDV) derived from the sequence of the γ-chain of fibrinogen.

To perform these studies, it was first necessary to define reaction conditions such that the dependence of crosslinking on thrombin was not rate limiting (see Scheme 1). For example, a high-order dependence of γ-chain crosslinking on thrombin concentration is evident in Figure 2. Comparison of the differential rates of crosslinking when rFXIII[$\mathbf{A_2}$] was employed (panel A) to that when rFXIII[$\mathbf{A'_2}$] was used (panel B) provided information on both the rate of rFXIII-[$\mathbf{A_2}$] activation (i.e., conversion of rFXIII[$\mathbf{A'_2}$]; Scheme 1, rxn 2) and the rate of substrate formation (i.e., conversion of fibrinogen to fibrin; Scheme 1, rxns 1 and 3). The apparent "lag" at low thrombin concentrations seen in reactions starting with rFXIII[$\mathbf{A_2}$] but not with rFXIII[$\mathbf{A'_2}$] indicated that in this range, activation of rFXIII[$\mathbf{A_2}$] was the rate-limiting step. The dependence of crosslinking on thrombin at intermediate concentrations (i.e., between approximately 0.5 and 1 unit/mL) indicated that conversion of fibrinogen to fibrin was rate limiting irrespective of rFXIII-[$\mathbf{A_2}$] or rFXIII[$\mathbf{A'_2}$]. At thrombin concentrations above ~2 units/mL the rate of crosslinking saturated with respect to thrombin concentration; in this regime, rFXIII[$\mathbf{A'_2}$] was rate limiting. Therefore, under these experimental conditions (i.e., 6.0 nM rFXIII and 2.5 mg/mL fibrinogen) thrombin concentrations of >2 units/mL were required for an accurate

Table 1: Comparison of Kinetic Parameters for Factor XIII and Thrombin[a,b]

enzyme	substrate	K_m (μM)	k_{cat} (min^{-1})	K_{sp} (min^{-1} μM^{-1})	ref
rFXIII[$\mathbf{A'_2}$]	γ-chain of fibrin	6.2	1872	300	*g*
				351	
				311	
FXIII[$\mathbf{A_2B_2}$][c]	γ-chain of fibrin[d]	41	21	0.51	*h*
rFXIII[$\mathbf{A'_2}$]	fibrin	1.3[e]			*i*
rFXIII[$\mathbf{A'_2}$]	α-chain of fibrin			0.38	*j*
rFXIII[$\mathbf{A'_2}$]	γ-chain peptide			0.030	*g*
FXIII[$\mathbf{A_2B_2}$]	γ-chain peptide [f]	3500	93	0.027	*h*
α-thrombin	α-chain of fibrin	7.2	5000	700	*k*
				720	
α-thrombin	β-chain of fibrin			250	*k*
α-thrombin	FXIII[$\mathbf{A_2}$]			7.2	*l*

[a] Nomenclature for the various Factor XIII species, substrates and kinetic methodologies are explained in the text. [b] For the sake of comparison with the literature, all values are normalized per half mole of fibrin or per mole of γ-chain peptide; therefore, the apparent values for K_m and k_{cat} in the first row are twice the magnitude given for fibrinogen in Figure 4B. [c] Plasma-derived FXIII (i.e., FXIII[$\mathbf{A_2B_2}$]) was presumed to become activated to FXIII[$\mathbf{A'_2}$] in the course of these experiments. [d] Determined by monodansylcadaverine (MDC) incorporation into the γ-chain of fibrin. [e] Formally determined as the binding constant (K_d) of rFXIII[$\mathbf{A'_2}$] for fibrin gel. [f] Determined by MDC incorporation into a γ-chain peptide. [g] Present study. [h] Gorman & Folk, 1980. [i] Procyk et al., 1993. [j] Bishop et al., 1993. [k] Lewis et al., 1985b. [l] Hornyak et al., 1989.

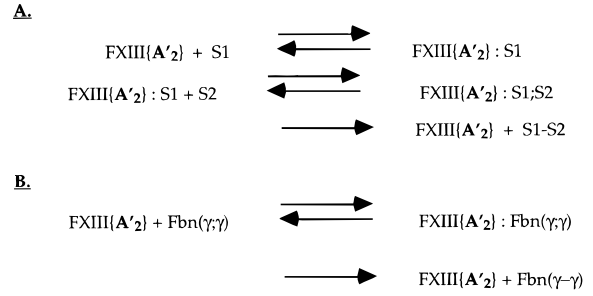
determination the true rate of γ-chain crosslinking. However, in experiments performed with significantly lower concentrations of rFXIII[$\mathbf{A'_2}$] or fibrinogen (e.g., 0.6 nM rFXIII and 0.25 mg/mL Fbgn) crosslinking rates were observed to saturate with respect to thrombin at significantly lower concentrations (e.g., 1 unit/mL, data not shown).

The rate dependence on both rFXIII[$\mathbf{A_2}$] and rFXIII[$\mathbf{A'_2}$] concentrations (Figure 3A,B) exhibited linear behavior at concentrations below 4 nM, consistent with Michaelis kinetics. The deviation from linearity seen at higher rFXIII-[$\mathbf{A_2}$] and rFXIII[$\mathbf{A'_2}$] concentrations was anticipated from results on thrombin dependence (Figure 2), i.e., the rates became thrombin limited.

It should be noted that, in general, for these studies the relative concentrations of reactants was critical. As already pointed out, if the ratio of thrombin to fibrinogen was too low, thrombin was rate limiting. However, if the ratio of thrombin to fibrinogen was too high, inhomogeneous clots resulted due to difficulties in rapid mixing. Equally troubling was the ratio of rFXIII[$\mathbf{A'_2}$] to fibrinogen; too high a ratio gave initial rates which were too fast to be accurately measured ($t_{1/2}$ < 3 min), whereas too low a ratio resulted in slow rates ($t_{1/2}$ > 30 min) where it could not be assumed that rFXIII[$\mathbf{A'_2}$] remained fully active throughout the time course (data not shown). Thus, in order to achieve a full range of accurate initial crosslinking rates, it was necessary to co-vary rFXIII[$\mathbf{A'_2}$] and fibrinogen while keeping thrombin concentrations just above the threshold of being rate limiting. Velocity was therefore normalized to rFXIII[$\mathbf{A'_2}$] concentration in most figures.

The initial rate dependence of γ-chain crosslinking on fibrinogen concentration (Figure 4A) indicates saturation behavior of rFXIII[$\mathbf{A'_2}$] when acting on polymeric fibrin. When replotted as a double reciprocal, Lineweaver−Burk plot (Figure 4B), these data yield an apparent K_m for fibrin(ogen) of 3.1 μM, a k_{cat} of 936 min^{-1} (note that in Table 1 these values are expressed as per γ-chain substrate, i.e., 6.2 μM and 1860 min^{-1}, respectively) and a K_{sp} (k_{cat}/K_m) of 302 min^{-1} μM^{-1}. These values were consistent with the K_{sp} calculated from pseudo-first-order rate constants at low fibrinogen concentration (fibrin $\ll K_m$); viz., 351 and 311 min^{-1} μM^{-1} for γ-chain consumption and γ-dimer formation, respectively (Table 1 and Figure 5). These results indicate that rFXIII[$\mathbf{A'_2}$] crosslinking of the γ-chain of fibrin exhibits apparent Michaelis behavior.

Scheme 2



Interestingly, when rFXIII[$\mathbf{A'_2}$] was reacted with the soluble peptide (TIGEGQQHHLGGAKQAGDV) derived from the sequence of the γ-chain of fibrinogen, Michaelis kinetics were not observed, rather, pseudo-first-order rates were obtained over the entire solubility range of the peptide. The rate constant calculated for this reaction was a factor of 10^4 lower than observed for native fibrin, suggesting that secondary or higher order substrate structures strongly affect the reaction kinetics of rFXIII[$\mathbf{A'_2}$]. Further insight into the specificity of rFXIII[$\mathbf{A'_2}$] for substrate structure can be gained from Table 1 which compares kinetic parameters from this work with those taken from the literature. Note the striking disparity between the kinetic parameters obtained in present study on native fibrin (Table 1) and those measured by Gorman and Folk (1980) on FXIII crosslinking of monodansylcadaverine to the γ-chain of fibrin. The relatively high K_m and low k_{cat} observed by Gorman and Folk reflect weak FXIII−substrate complex binding and correspondingly low rates of catalysis. In contrast, note the impressive similarity in the K_{sp} measured by Gorman and Folk (0.027 min^{-1} μM^{-1}; Table 1) to that of the soluble γ-chain peptide TIGEGQQHHLGGAKQAGDV determined in the present study (K_{sp} = 0.030 min^{-1} μM^{-1}; Table 1) and the crosslinking of α-chains within fibrin gels (K_{sp} = 0.38 min^{-1} μM^{-1}; Bishop et al. (1993)). We attribute the significance of the magnitudes of these kinetic constants as reflecting two likely modes of FXIII[$\mathbf{A'_2}$]−substrate interactions: (1) random (or sequential) binding to soluble, non-interacting substrates imposes an high entropic barrier (i.e., $\Delta G_{binding}$) to the formation of a productive catalytic complex; viz., as per S1 and S2 in Scheme 2 and (2) direct binding to a single interacting substrate effectively lowers the entropic barrier of complex formation; viz., oriented γ-chain pairs within the fibrin strand as per γ;γ in Scheme 2.

This view of γ-chain crosslinking within the fibrin gel is consistent with experiments by Kanaide and Shainoff (1975) showing enhanced crosslinking of fibrinogen at high concentrations where intermolecular interactions and alignment occur even without fibrin formation and recent findings of Samokhin and Lorand (1995) who have examined bulk-phase rFXIII[$\mathbf{A'_2}$] crosslinking of D domains (105 kDa soluble plasmin cleavage fragments of fibrin containing the C-

terminal γ-chain crosslinking site) in the presence of varying concentrations of E-domains (soluble plasmin cleavage fragments of fibrin containing the N-terminal α-chain cleavage sites of the thrombin; viz., GPRV). Significantly, these workers found that rFXIII[$\mathbf{A'_2}$] crosslinking of D domains was greatly enhanced (ca. 8-fold) by the addition of E-domains and that this rate enhancement could be abolished by the addition of the peptide GPRP, which is known inhibit fibrin gel formation ("polymerization"; see Scheme 1, rxn 3). They conclude that the N-terminal α-chain sequences act as organizing templates which orient the D-domains to favor reaction with rFXIII[$\mathbf{A'_2}$].

The nature of the catalytic interaction between FXIII[$\mathbf{A'_2}$] and fibrin is complex. This is both evident from the high number of crosslinks per mole of fibrin monomer which fibrin can attain (Bishop et al., 1993; Mousli & Wakid, 1977) and from direct attempts to quantify and map FXIII[$\mathbf{A'_2}$] binding sites. For example, by perfusing radiolabeled rFXIII-[$\mathbf{A'_2}$] through FXIII free fibrin gels, Procyk et al. (1993) have determined an apparent K_d of 1.3 μM per mole of fibrin for the association of rFXIII[$\mathbf{A'_2}$] to fibrin (Table 1). This is in reasonable agreement with the *apparent* K_m found in the present study, but assumes only a single binding site of rFXIII[$\mathbf{A'_2}$] per mole of fibrin. However, no assumption for the number of FXIII[$\mathbf{A'_2}$] binding sites can yet be justified; for example Procyk et al. (1993) also found that the binding rFXIII[$\mathbf{A'_2}$] to fibrin was unaffected by catalytic inhibition (either removal of co-factor Ca^{2+} or by carboxymethylation of the active site cysteine) and that binding was independent of the state of γ-chain crosslinking of the gel (controls indicated that rFXIII[$\mathbf{A_2}$] zymogen did not bind to fibrin gels). Furthermore their attempts to map the rFXIII[$\mathbf{A'_2}$] binding site(s) demonstrated that binding of rFXIII[$\mathbf{A'_2}$] to fibrin gels could be blocked by both a monoclonal antibody directed to the C-terminal portion of the fibrin α-chain (Aα389-402) and by the fibrin peptide Aα241-476, whereas a monoclonal antibody directed to the C-terminal crosslinking site of the fibrin γ-chain (γ392-406), had no effect on binding. In light of these findings the *apparent* K_m for the fibrin γ-chain determined in the present study may not directly reflect the formation of a classical Michaelis complex. In fact it appears that FXIII[$\mathbf{A'_2}$] binding to fibrin may involve one or more FXIII[$\mathbf{A'_2}$] exosites which are exposed during fibrin activation and which presumably could serve to juxtapose FXIII[$\mathbf{A'_2}$] to the oriented γ-chains within fibrin.

The activity of FXIII[$\mathbf{A'_2}$] in crosslinking the γ-chain of fibrin is very large relative to all other substrates. In order to rationalize this result, we have turned to the structure of the substrate. In the polymerization of fibrin, staggered, double-strand oligomers are formed (protofibrils) which, in turn, condense and grow to create the fibrin fibrils (Hantgan et al., 1983; Weisel & Nagaswami, 1992). In the turbidity investigations of Carr and Hermans (1978) the size and density of fibrin fibers were determined. It was found that the fiber density is 0.28 g/mL and because the protein density in solution is near 1.39 g/mL, the volume of the fibrin fibers is about five times the volume of the protein. With each fibrin monomer having a length of 450 Å, the cross-section is equal to that of the square of 67 Å (Carr & Hermans, 1978) while the protein occupies only an average of 30 Å of that square. Additionally the interstices between the fibrin molecules will be filled with solvent, and the arrangement of that solvent will be in channels between the protofibril filaments. Either very weak exosite binding of FXIII[$\mathbf{A'_2}$] by the fibrin protofibrils or excluded volume (or both) may constrain the enzyme to these channels. Thus, we picture FXIII[$\mathbf{A'_2}$] diffusing in these channels, crosslinking the pre-aligned γ-chains with very high efficiency.

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