Interactions of Factor XIII with Fibrin as Substrate and Cofactor†

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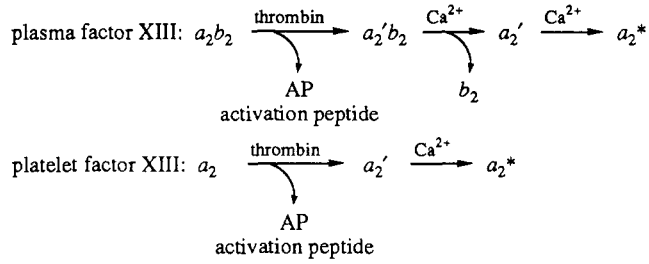
ABSTRACT: Factor XIIIa (a_2') is a homodimeric transglutaminase that is formed via limited α-thrombin-catalyzed proteolysis of the platelet (a_2) or plasma (a_2b_2) factor XIII zymogen in a reaction that results in proteolytic removal of a 37-aminoacyl residue peptide from the N-terminus of the *a* chains and exposure of the active-site thiol group in the resulting *a'* chains of factor XIIIa. In this study, we characterized interactions of factor XIII and factor XIIIa with fibrin, a natural substrate for factor XIIIa and a cofactor for the α-thrombin-catalyzed activation of plasma factor XIII. The carbamylmethyl derivatives of the active-site thiol group of platelet factor XIII (CMa_2) and factor XIIIa (CMa_2') were prepared, and their interactions with fibrin were measured. The enzyme-like derivative (CMa_2') which contained nicked *a'* chains bound more tightly to fibrin (K_d = 2.1 μM) than did CMa_2 (K_d = 14 μM), the platelet zymogen-like derivative with intact *a* chains, but the binding of each was weaker than the binding of plasma factor XIII zymogen (a_2b_2) to fibrin (K_d = 0.20 μM) under the same conditions. Saturation of fibrin with plasma factor XIII zymogen (a_2b_2) did not affect the binding of CMa_2' to fibrin, suggesting that the plasma factor XIII zymogen (a_2b_2) and the active-site-modified form of factor XIIIa (CMa_2') bind to separate, noninteracting sites of fibrin. In contrast to its promoting effect on the activation of plasma factor XIII, fibrin did not promote AP release from platelet factor XIII at concentrations of fibrin high enough to ensure significant binding of platelet factor XIII by fibrin, suggesting that the *b* chains in plasma factor XIII determine not only the affinity of a_2b_2 for fibrin but also the geometric disposition of a_2b_2 in the termolecular thrombin–fibrin–a_2b_2 complex that is involved in fibrin promotion of α-thrombin-catalyzed activation of plasma factor XIII. An analysis of the effects of fibrinogen on the rate of release of *b* chains and concomitant exposure of the active-site thiol group in α-thrombin-cleaved plasma factor XIII ($a_2'b_2$) yielded a value of 2.3 μM for the equilibrium constant for dissociation of the fibrinogen–$a_2'b_2$ complex. The observation that the intact zymogen a_2b_2 did not antagonize the interaction of $a_2'b_2$ with fibrinogen suggested that the proteolytic conversion of a_2b_2 to $a_2'b_2$ results in altered interactions with fibrinogen.

In the terminal stage of blood coagulation, the conversion of factor XIII to factor XIIIa generates a transglutaminase activity which is an important determinant of the strength of the eventual fibrin clot and its resistance to fibrinolysis. Factor XIII is found in the body as plasma factor XIII, an a_2b_2 tetramer, and platelet factor XIII, an a_2 dimer. Plasma factor XIII is cleaved by α-thrombin to release activation peptide (AP)[1] from the N-termini of the *a* chains (Schwartz et al., 1973; Chung et al., 1974). In the presence of calcium ion, the *b* subunits dissociate from the cleaved zymogen to yield catalytically active factor XIIIa (Chung et al., 1974; Lorand et al., 1974). Platelet factor XIII also yields the same active enzyme when cleaved by α-thrombin in the presence of calcium ion. Details of the structures of the factor XIII *a* and *b* subunits have been reviewed (Ichinose et al., 1990). The activation pathways of plasma and platelet factor XIII are summarized in Scheme I according to previous findings (Schwartz et al., 1973; Chung et al., 1974; Lorand et al., 1974).

Studies of platelet factor XIII have yielded insights into the requirements for factor XIII activation. Release of only one activation peptide is required for expression of full factor XIIIa activity in the presence of calcium ion (Hornyak et al., 1989). Factor XIIIa also exhibits half-of-the-sites reactivity of the active-site cysteine with alkylating agents such as iodoacetic acid and iodoacetamide (Chung et al., 1974; Seelig & Folk, 1980; Hornyak et al., 1989). Recently, we have demonstrated that exposure of the active-site thiol groups on both subunits of factor XIIIa occurs and that calcium ion is an allosteric effector for the intersubunit interaction that results in the exposure of both active-site thiol groups (Hornyak & Shafer, 1991). The exposure of both active-site thiol groups of factor XIIIa, even though only one can be alkylated per dimeric molecule, suggests that negative cooperativity may be operative during the factor XIIIa catalyzed cross-linking reaction, either to prevent adventitious cross-linking reactions or to provide a mechanism for the regulation of catalysis (Hornyak & Shafer, 1991).

Scheme I



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[1] Abbreviations: AP, activation peptide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; M_r, molecular weight; PEG, poly(ethylene glycol); PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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Several observations demonstrate specific interactions of factor XIII with fibrinogen and fibrin that may regulate the development of factor XIIIa activity in vivo. Both plasma and platelet factor XIII have been shown to bind directly to fibrinogen (Greenberg & Shuman, 1982). In addition, binding of plasma factor XIII to fibrin I and fibrin II has been demonstrated (Greenberg et al., 1985; Naski et al., 1991). The presence of fibrinogen lowers the calcium ion concentration required for efficient release of the *b* subunits and exposure of the active-site thiol group in $a_2'b_2$ (Credo et al., 1978) and increases the rate of exposure of the active-site thiol group at a given calcium ion concentration (Hornyak & Shafer, 1991). Structurally, this promoting activity of fibrinogen has been localized to the midportion of the fibrinogen Aα chain (Credo et al., 1981). Prior to undergoing cross-linking, fibrin I and fibrin II promote α-thrombin-catalyzed release of AP from plasma factor XIII (Lewis et al., 1985; Greenberg et al., 1987), an activity that may ensure an optimal temporal relationship between the generation of fibrin and factor XIIIa within the normal time frame of in vivo coagulation. Recently, this cofactor activity of fibrin has been shown to be due to formation of a fibrin–factor XIII complex which exhibits a specificity constant for α-thrombin-catalyzed release of AP that is 80-fold higher than that for α-thrombin-catalyzed release of AP from uncomplexed factor XIII (Naski et al., 1991). In contrast to the ability of fibrin to function as a cofactor in the α-thrombin-catalyzed activation of plasma factor XIII, fibrin II appears to exhibit little or no cofactor activity in α-thrombin catalyzed release of AP from platelet factor XIII (Greenberg et al., 1987). This discrepancy in the behavior of fibrin II toward plasma and platelet factor XIII, together with the realization that the a' chains of factor XIIIa must interact with their fibrin substrate, suggests the possibility that different domains of fibrin II may interact with plasma factor XIII (a_2b_2), platelet factor XIII (a_2), and factor XIIIa (a_2'). In the present study, we present evidence that the dual functions of fibrin II as a promoter for factor XIII activation and as a substrate for factor XIIIa catalytic activity involve distinct, noninteracting domains on fibrin II. We also show that the interaction between factor XIIIa and fibrin II does not depend exclusively upon interactions between the active-site thiol residue of factor XIIIa and fibrin II, implying that an exosite, a domain removed from the active site, of factor XIIIa mediates the interaction with its fibrin substrate.

Materials and Methods

Materials. Fibrinogen was purified by repeated precipitation of outdated human plasma (Lewis & Shafer, 1984) and was >95% clottable. The concentration was determined using an $E_{280}^{1\%}$ of 15.1 in 0.3 M NaCl and an M_r of 340000 (Mihalyi, 1968). Human α-thrombin was a gift of Dr. John W. Fenton, II, and possessed >95% activity. Human recombinant platelet factor XIII was a gift from Dr. Paul Bishop, Zymogenetics, Inc. The similarities between recombinant platelet factor XIII and factor XIII purified from human platelets have already been described (Hornyak et al., 1989). Plasma factor XIII tetramer and plasma factor XIII *b* subunits were prepared from precipitation of human plasma followed by chromatography as previously described (Lorand et al., 1981). Concentrations of plasma factor XIII *b* subunits, platelet factor XIII, and plasma factor XIII tetramer were determined using an $E_{280}^{1\%}$ = 13.8 and M_r values of 75000, 150000, and 320000, respectively (Schwartz et al., 1973; Chung et al., 1974). Fibrin II was prepared by treating fibrinogen with α-thrombin as previously described (Lewis et al., 1985). The concentration of fibrin II was determined in 0.02 M acetic acid using an $E_{280}^{1\%}$ = 14.0 and an M_r of 340000. [1-^{14}C]Iodoacetamide (24.1 mCi/mmol) was obtained from New England Nuclear.

Incorporation of [1-^{14}C]Iodoacetamide into Calcium Ion Activated Platelet Factor XIII. Incubates (200 μL) of 1.26 μM platelet factor XIII and 50 mM $CaCl_2$ were prepared, with or without 6.4 mM DTT, in a buffer containing 0.1 M Tris-acetate, 0.15 M NaCl, 1 mM EDTA, and 0.1% PEG, pH 7.5. After preincubation at 37 °C and addition of $CaCl_2$, 20-μL aliquots were pipetted at various times into individual vials containing 2 μL of 420 μM [1-^{14}C]iodoacetamide (final concentration 38 μM). Incorporation of [1-^{14}C]iodoacetamide into the active-site thiol group was allowed to occur for 15 min before aliquots (5 μL) were pipetted onto filter papers and quenched in a 10% TCA bath at 4 °C. The filter papers were then washed with TCA solutions, ethanol/acetone, and acetone as described (Curtis et al., 1973), dried, and counted in 2 μL of Bio-Safe II (Research Products International Corp.) in a Beckman LS 3801 liquid scintillation system.

Preparation of Active-Site-Labeled Derivatives of Cleaved and Uncleaved Platelet Factor XIII. For preparation of cleaved, active-site-labeled platelet factor XIII (CMa_2'), a 1.5-mL or 2.0-mL incubate with a final composition of 1.5 μM platelet factor XIII, 14 mM $CaCl_2$, and 20 nM α-thrombin was prepared in a buffer containing 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5. [It should be noted that 14 mM $CaCl_2$ activates platelet factor XIII only minimally (<10%/h) as mentioned in Hornyak et al. (1989) and that control experiments in which uncleaved plasma factor XIII was incubated with 14 mM $CaCl_2$ showed no activity.] The mixture was preincubated at 37 °C prior to the addition of α-thrombin. Incubation of platelet factor XIII with α-thrombin was performed for 30 min, a sufficient length of time to ensure that >99% of AP cleavage occurred under these conditions (Hornyak et al., 1989) and that an essentially homogeneous population of twice-cleaved factor XIII dimers was obtained. PPACK was then added to inactivate the α-thrombin (final PPACK to α-thrombin ratio = 150:1). After 3 min was allowed for the reaction to be quenched, a small volume of [1-^{14}C]iodoacetamide was added to the incubate to yield 31 μM [1-^{14}C]iodoacetamide. Incorporation of [1-^{14}C]iodoacetamide at the exposed active-site thiol group was allowed to occur for 20 min before the labeled factor XIII was separated from free [1-^{14}C]iodoacetamide and other salts using a column of G-50 Sephadex equilibrated with 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5. The protein-containing fractions were combined in PEG-coated, 1.5-mL polypropylene tubes (Z. Latallo, personal communication) and centrifuged for 3 min in a tabletop centrifuge. This step was necessary because it was found that factor XIII, once activated in the presence of calcium ion, had limited solubility under these conditions and, in addition, was quite surface active. Centrifugation was necessary to pellet any remaining precipitate after gel filtration, and the use of PEG-coated tubes was important to prevent adsorptive loss of labeled platelet factor XIII. The resulting supernatant solution was used in the fibrin II binding experiments after measurement of the A_{280} to determine protein concentration.

Uncleaved, active-site-labeled platelet factor XIII (CMa_2) was prepared in a similar manner, except that a 1.0-mL incubate was prepared with a final composition of 1.5 μM platelet factor XIII and 50 mM $CaCl_2$ in 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5. The factor XIII/Tris solution was preincubated at 37 °C before the addition of $CaCl_2$ to activate factor XIII. Activation with $CaCl_2$ (Credo et al., 1978) occurred for 10 min before addition of [1-^{14}C]iodo-

acetamide to alkylate exposed active-site thiol groups. Alkylation, separation of labeled factor XIII, and analysis were performed as described for cleaved, labeled factor XIII.

Binding of Active-Site-Labeled Platelet Factor XIII to Fibrin II. Binding experiments were performed with 250-μL incubates in PEG-coated, 0.5-mL polypropylene tubes. Samples of centrifuged, labeled factor XIII solution (CMa_2' or CMa_2) were preincubated at 37 °C in a buffer of 0.1 M Tris-HCl/0.15 M NaCl, pH 7.5. Small volumes of $CaCl_2$ solutions were added (if indicated) to achieve the desired final $CaCl_2$ concentration. Then, fibrin II from a concentrated solution of fibrin II in 0.02 M acetic acid was added to achieve a final concentration of 1.0 μM (2.0 μM fibrin II protomer). Because of the high concentration of the fibrin II solution in acetic acid, addition of less than 10 μL was necessary to attain a final concentration of 1.0 μM. It was necessary to add the fibrin II solution gradually into the preincubate with slow vortexing to disperse the fibrin in solution before polymerization occurred. Labeled factor XIII was incubated with fibrin for 30 min. (Control experiments demonstrated that binding equilibrium was reached within 15 min.) A control containing no fibrin II was also prepared and incubated under the same conditions. After incubation, the samples were spun in a tabletop centrifuge for 3 min to dislodge the clot. Samples (50 μL) of the supernatant solution from each experiment and control were counted for radioactivity in 2 mL of Bio-Safe II scintillation fluid using a Beckman LS 3801 liquid scintillation system. A_{280} measurements of the supernatant solutions from the fibrin clotted under these conditions and centrifuged showed that clottability was >99%. To determine accurately the concentration of labeled factor XIII and to eliminate errors due to precipitation during the course of incubation, aliquots of the centrifuged, labeled factor XIII solution were counted as well from the controls without fibrin. The measurements of fibrin binding were corrected for loss of factor XIII observed in the controls without fibrin. The fibrin-independent loss of factor XIII was less than 15% for concentrations of CMa_2 and $CMa_2' > 100$ nM, and it was less than 25% at concentrations of CMa_2' as low as 13 nM.

For measurement of the binding of unlabeled a_2 to fibrin II, 0.4 μM platelet factor XIII was incubated with 2.0 μM fibrin II (4.0 μM fibrin II promoter) for 30 min. The incubate was centrifuged for 6 min to dislodge the clot. Supernatant solutions (50 μL) were assayed for unbound factor XIII using the dansylcadaverine incorporation assay (Curtis & Lorand, 1976; Hornyak et al., 1989) to determine the concentration of unbound factor XIII relative to a control incubated without fibrin.

Experiments designed to observe the effect of added *b* chains upon the binding of CMa_2 to fibrin II were performed as described above except that a solution of concentrated *b* chains, previously dialyzed against 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5, was included in the binding incubate prior to the addition of fibrin.

Simultaneous Binding of Cleaved, Labeled Platelet Factor XIII (CMa_2') and Plasma Factor XIII (a_2b_2) to Fibrin. Labeled factor XIII (CMa_2') was prepared as described above. Binding experiments and controls were also performed as described except that aliquots of a solution of 39.9 μM plasma factor XIII (a_2b_2), previously dialyzed against 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5, were added to the binding incubates. Additional experiments were performed to measure binding of plasma factor XIII to fibrin in the absence of CMa_2'. Portions of the supernatant solutions containing a_2b_2 were assayed for factor XIII activity, after activation with α-thrombin (Naski et al., 1991), using the dansylcadaverine incorporation assay (Curtis & Lorand, 1976; Hornyak et al., 1989), and the results were used to quantify plasma factor XIII binding to fibrin as well as to assess any inhibitory effect of a_2b_2 upon the binding of CMa_2' to fibrin.

Determination of Activity of Calcium-Activated, Uncleaved Platelet Factor XIII. An incubate of platelet factor XIII, $CaCl_2$, and 0.1 M Tris–acetate/0.15 M NaCl/1 mM EDTA/0.1% PEG, pH 7.5 as buffer was prepared with final concentrations of 0.40, 0.10, or 0.025 μM platelet factor XIII and 0.1 M $CaCl_2$. Platelet factor XIII and buffer were mixed first and equilibrated at 37 °C before the addition of $CaCl_2$ (from a solution of 2.0 M $CaCl_2$ prepared in 50 mM Tris-HCl, pH 7.5) at time = 0. At successive time points, 100-μL aliquots of the 0.10 and 0.025 μM platelet factor XIII solutions and 50-μL aliquots of the 0.40 μM platelet factor XIII solution were removed from this incubate and assayed for factor XIIIa activity using the dansylcadaverine incorporation assay (Curtis & Lorand, 1976; Hornyak et al., 1989). To compare the activity of calcium-activated platelet factor XIII with the activity of α-thrombin-cleaved platelet factor XIII, solutions of platelet factor XIII at the above concentrations in 0.1 M Tris–acetate/0.15 M NaCl/1 mM EDTA/0.1% PEG, pH 7.5, containing 14 mM $CaCl_2$ to stabilize factor XIIIa activity (Hornyak & Shafer, 1989) were activated by the addition of α-thrombin to a concentration of 220 nM and incubation at 37 °C for 5 min before the assay of factor XIIIa activity.

Kinetics of Fibrinogen-Promoted Exposure of the Active-Site Thiol Group in Cleaved Plasma Factor XIII ($a_2'b_2$). Plasma factor XIII was cleaved in the absence of $CaCl_2$ and fibrinogen by incubating 15.6 μL of 9.63 μM plasma factor XIII, 21.0 μL of 306 nM α-thrombin, and 18.0 μL of 840 μM [1-^{14}C]iodoacetamide in a buffer of 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5. The volume of buffer for the incubation was chosen so that the sum of the volumes of plasma factor XIII, buffer, and fibrinogen solution (to be added later) was 300 μL. After α-thrombin was allowed to cleave the factor XIII for 30 min, 33 μL of 30 μM PPACK was added to quench the α-thrombin. The indicated amount of fibrinogen (previously dialyzed against 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5) was added. The cleaved factor XIII ($a_2'b_2$) was then activated by the addition of 13.9 μL of 0.04 M $CaCl_2$ in 0.1 M Tris-HCl/0.15 M NaCl/0.1% PEG, pH 7.5. The final concentrations of the reactants in the $CaCl_2$-containing incubate were 0.39 μM plasma factor XIII, 39.1 μM [1-^{14}C]iodoacetamide, and 1.5 mM $CaCl_2$. Aliquots (15 μL) were pipetted from the solution onto filter papers at various times up to 60 min and quenched in a 10% TCA bath. Rates were determined from a semilogarithmic plot of fraction incorporation, normalized to a final value of 1, versus time. At higher concentrations of fibrinogen (>3 μM), values for the final fraction incorporation significantly greater than 0.5 were observed with increasing concentrations of fibrinogen extending to a final value of 0.75 with 11 μM fibrinogen. This deviation is attributed to a factor XIII contamination (~1%) in the fibrinogen. The computer fit to the experimental rate data (obtaining the parameter K_d and the standard deviation) was performed using the BMDX85 program from the Health Science Computing Facility at the University of California, Los Angeles.

Kinetics of AP Release. These were measured as described previously (Janus et al., 1983) in experiments wherein 1.0 μM a_2 was incubated with 4.0 nM α-thrombin with and without 8.0 μM fibrin II for various times. A volume of 0.2 M Tris-HCl/0.30 M NaCl/0.2% PEG, pH 7.5, equal to the

Table I: Dissociation Constants for CMa_2' and CMa_2 Binding to Fibrin II

	K_d[a] (μM)	
$[Ca^{2+}]$ (mM)	CMa_2'	CMa_2
0	2.1 (±0.4)[b]	14 (±4)[c]
1.5	1.5	
14	1.0	
50	1.1	5

[a] K_d was calculated using the relationship $K_d = [CMa_2']_{free}([\alpha\beta\gamma] \times [CMa_2']_{bound})/[CMa_2']_{bound}$ or $K_d = [CMa_2]([\alpha\beta\gamma][CMa_2]_{bound})/[CMa_2]_{bound}$. Use of this equation to calculate K_d assumes 1:1 binding of labeled factor XIII to fibrin II protomer ($\alpha\beta\gamma$) and equivalence of the factor XIII binding sites in fibrin. [b] Average of 10 observations with the standard deviation of the mean in parentheses. [c] Average of six observations with the standard deviation of the mean in parentheses.

volume of fibrin II in 0.02 M acetic acid was used in each incubate to increase its buffering capacity. The same volumes of 0.02 M acetic acid and 0.2 M Tris-HCl/0.30 M NaCl/0.2% PEG, pH 7.5, were included in the samples not containing fibrin II.

Results

Binding of Thrombin-Processed Carbamylmethyl Platelet Factor XIII (CMa_2') and Uncleaved Carbamylmethyl Platelet Factor XII (CMa_2) to Fibrin II. Measurement of the binding of CMa_2' to fibrin II yielded a K_d of 2.1 (±0.4) μM (Table I). The presence of 50 mM $CaCl_2$ only modestly increased the affinity of CMa_2' for fibrin (Table I). To assess the effect of the AP domain upon the interaction of platelet factor XIII and fibrin, uncleaved labeled platelet factor XIII (CMa_2) was generated by thrombin-independent activation of platelet factor XIII zymogen with 50 mM $CaCl_2$ followed by alkylation of the exposed active-site thiol group with [1-^{14}C]iodoacetamide. A K_d of 14 (±4) μM was obtained for the dissociation of CMa_2 from fibrin II in the absence of $CaCl_2$. It should be noted that platelet factor XIII nonenzymatically activated by 50 mM $CaCl_2$ was observed to incorporate only approximately 0.4 [1-^{14}C]iodoacetamide molecule per platelet factor XIII *a* subunit (Figure 1), even after extensive incubation with iodoacetamide. Additional experiments (Figure 2) revealed that platelet factor XIII activated by high concentrations of calcium ion possessed a maximal activity about 80% that of platelet factor XIII activated by α-thrombin. Hence, the ratio of the fraction of thiol alkylated to the fraction of maximal activity was 0.5, suggesting that platelet factor XIII activated by 50 mM $CaCl_2$ exhibits half-of-the-sites reactivity, like platelet factor XIII activated after cleavage by α-thrombin followed by exposure to a low concentration (1.5 mM) of calcium ion (Chung et al., 1974; Hornyak et al., 1989). Higher concentrations of $CaCl_2$ (0.1 and 0.2 M) did not increase the fraction of *a* subunits alkylated with iodoacetamide.[2] A comparison of the equilibrium constants for the dissociation of CMa_2 and CMa_2' from fibrin that are depicted in Table I reveals that removal of AP results in a 5–7-fold increase in the affinity of carbamylmethylated platelet factor XIII for fibrin II.

Affinity of Unlabeled Intact Platelet (a_2) and Plasma (a_2b_2) Factor XIII Zymogens for Fibrin II. Separate studies of the binding of a_2b_2 to fibrin II yielded a value of 0.20 μM for K_d when the concentrations of fibrin II and plasma factor XIII were expressed in protomeric units ($\alpha\beta\gamma$ and *ab*, respectively),

[2] When the concentration of CMa_2 was determined from measurements of radioactivity the incorporation of 0.4 carbamylmethyl groups per *a* subunit as determined from independent measurements was used to relate radioactivity to the amount of CMa_2.

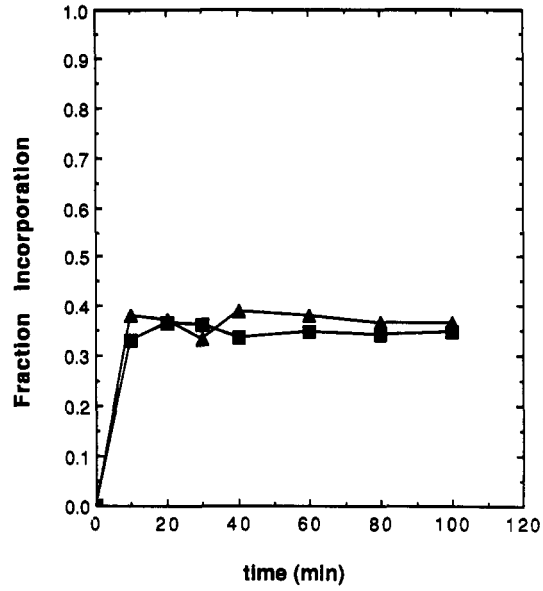

FIGURE 1: Half-of-the-sites reactivity of calcium ion activated platelet factor XIII. Factor XIII was activated by 50 mM $CaCl_2$ in the presence (▲) and absence (■) of 6.4 mM DTT. Incorporation of [1-^{14}C]iodoacetamide is described in the Materials and Methods section. Fraction incorporation represents the fraction of total active-site thiol groups alkylated with [1-^{14}C]iodoacetamide. Plotted on the *x*-axis is the length of time that platelet factor XIII was incubated with $CaCl_2$ before [1-^{14}C]iodoacetamide was added.

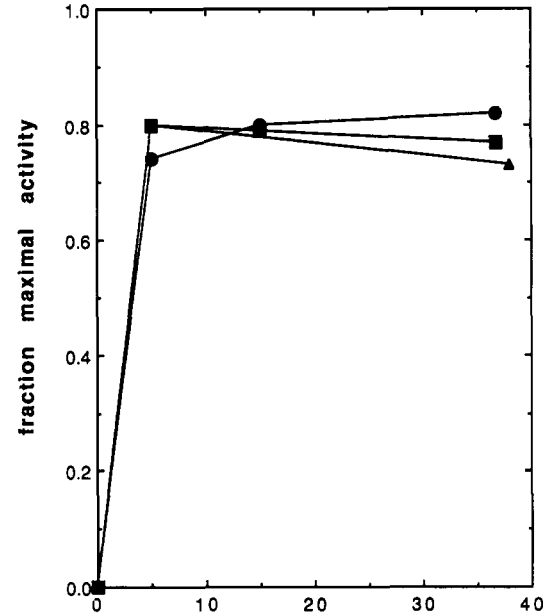

FIGURE 2: Activity of uncleaved platelet factor XIII activated by calcium ion. Solutions containing 0.40 μM (■), 0.10 μM (●), and 0.025 μM (▲) platelet factor XIII were activated by 0.1 M calcium ion as described in the Materials and Methods section. Aliquots were assayed at the indicated times for factor XIIIa activity, and the activity was compared to the maximal factor XIIIa activity obtained by proteolysis of platelet factor XIII by α-thrombin in the presence of 14 μM $CaCl_2$ as described.

and the stoichiometry for the fibrin II–factor XIII complex of 1.6 fibrin protomeric units binding per plasma factor XIII protomeric unit determined by Naski et al. (1991) was assumed. Studies of the binding of a_2 to fibrin II yielded a value of 24 (±8) μM for the K_d for dissociation of the complex between fibrin II and a_2, compared to the value of 14 (±4) μM for K_d observed for dissociation of the complex between

fibrin II and CMa_2. Thus, carbamylmethylation of the active-site cysteine has little or no effect on the affinity of the intact platelet zymogen for fibrin. Finally, addition of isolated b chains (up to a concentration of 10.0 μM) did not increase the affinity of CMa_2 for fibrin II, suggesting that alkylation of the active-site thiol group by [1-^{14}C]iodoacetamide prevents formation of a CMa_2b_2 complex with binding properties similar to those of a_2b_2.

It has been previously reported (Greenberg et al., 1987) that fibrin II does not promote the activation of platelet factor XIII. This result contrasts with the striking effect of fibrin II upon plasma factor XIII activation as measured by enhancement of the rate of α-thrombin-catalyzed AP release from plasma factor XIII (Lewis et al., 1985). It is possible that the difference in the fibrin affinity of platelet factor XIII (K_d = 14 μM) and plasma factor XIII (K_d = 0.2 μM) may explain why no promotion of platelet factor XIII activation by fibrin was observed. We found, however, that, at a concentration of fibrin II (16.0 μM fibrin II protomer) sufficient to bind close to half of the platelet factor XIII in the incubate of platelet factor XIII, fibrin, and α-thrombin, promotion of AP release was not observed relative to a control containing platelet factor XIII and α-thrombin at the same concentrations but no fibrin II.

Competition of a_2b_2 and CMa_2' for Binding to Fibrin II. The interaction between CMa_2' and fibrin II was unaffected by the addition of plasma factor XIII zymogen (a_2b_2) up to concentrations of 5.0 μM (Figure 3). Since the equilibrium constant for dissociation of a_2b_2 from fibrin II under these experimental conditions is 0.20 μM,[3] binding sites for a_2b_2 in fibrin II were >97% saturated at 5.0 μM a_2b_2.

Interaction of $a_2'b_2$ and Fibrinogen. Previous studies by Lorand and his co-workers (Credo et al., 1978, 1981) have elegantly demonstrated that calcium ion and fibrinogen facilitate α-thrombin-catalyzed activation of plasma factor XIII by promoting dissociation of the b chains from $a_2'b_2$ and concomitant exposure of the active-site thiol group. Recent studies from this laboratory (Hornyak & Shafer, 1991) indicate that the binding of two calcium ions to the $a_2'b_2$ tetramer that is produced upon α-thrombin-catalyzed release of AP from a_2b_2 increases the rate of dissociation of the b chains from $a_2'b_2$ and concomitant exposure of the active-site thiol group. The studies of the enhancement of this process by fibrinogen that are depicted in Figure 4 are consistent with Scheme II, Scheme II

$$a_2'b_2 + \text{Fgn} \overset{K_D}{\rightleftharpoons} \text{Fgn}\text{-}a_2'b_2$$

$$a_2'b_2 + 2Ca^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} a_2'b_2\text{-}(Ca^{2+})_2 \xrightarrow{k_2}$$

$$a_2'\text{-}(Ca^{2+})_2 + (b_2 \text{ or } 2b)$$

$$\text{Fgn}\text{-}a_2'b_2 + 2Ca^{2+} \underset{k_{-1,\text{Fgn}}}{\overset{k_{1,\text{Fgn}}}{\rightleftharpoons}} \text{Fgn}\text{-}a_2'b_2\text{-}(Ca^{2+})_2 \xrightarrow{k_{2,\text{Fgn}}}$$

$$\text{Fgn}\text{-}a_2'\text{-}(Ca^{2+})_2 + (b_2 \text{ or } 2b)$$

[3] Data from the experiments with 0.5 μM a_2b_2 and 1.0 μM a_2b_2 yielded a K_d of 0.20 (±0.01) μM for the equilibrium constant for dissociation of a_2b_2 from fibrin, assuming n = 1.6 for the number of fibrin protomers ($\alpha\beta\gamma$) bound by one factor XIII protomer (ab). The fraction of total factor XIII that was free in experiments at higher factor XIII concentrations was too great to yield an accurate value for K_d at these concentrations. Saturation of fibrin II was determined directly, using the difference between the measured values of free and total factor XIII to determine the concentration of bound factor XIII for the experiments wherein the concentrations of a_2b_2 were either 0.5 or 1.0 μM. In experiments where the concentration of factor XIII was 3.0 or 5.0 μM, the fraction of bound factor XIII was calculated using the above stoichiometry and the dissociation constant of 0.20 μM for the fibrin–factor XIII complex.

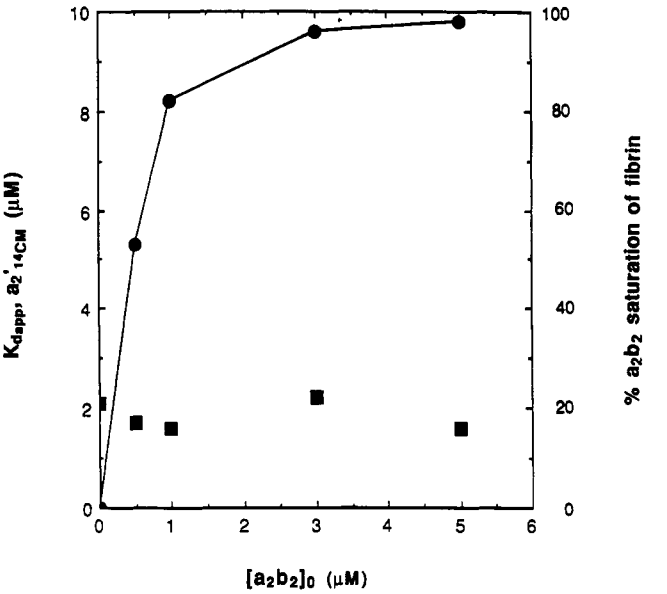

FIGURE 3: Competition of CMa_2' and a_2b_2 for fibrin. The apparent K_d observed for CMa_2' binding to fibrin (■) and the percent saturation of fibrin by plasma factor XIII zymogen (●) are noted on the left and right axes, respectively, as a function of the total plasma factor XIII zymogen concentration ($[a_2b_2]_0$). Saturation of fibrin by factor XIII was determined as described in the text.

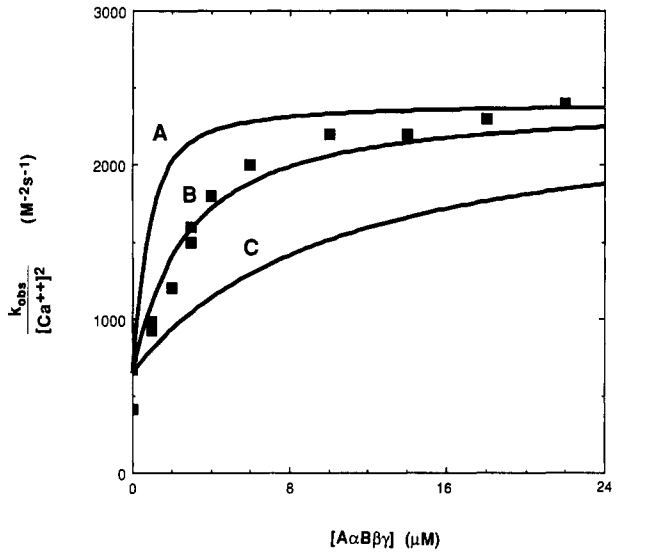

FIGURE 4: Fibrinogen promotion of the exposure of the active-site thiol group from $a_2'b_2$. The y-axis label $k_{obs}/[Ca^{2+}]^2$ reflects the apparent second-order rate constant for active-site thiol group exposure, measured as described in the Materials and Methods section. The data points (■) represent $k_{obs}/[Ca^{2+}]^2$ determined at varying concentrations of fibrinogen protomer ($[A\alpha B\beta\gamma]$). The solid lines are plots of eq 5 as a function of $[Fgn]_0$ with k' = 650 M^{-2} s^{-1} (determined from eq 1 and the rate constant observed with no fibrinogen present), k'_{Fgn} = 2400 M^{-2} s^{-1} (determined from eq 1 and rate constant observed in the presence of 22 μM fibrinogen protomer), $[a_2'b_2]$ = 0.39 μM, and K_d set at 0.4 μM (A), 2.30 μM (B), and 10.0 μM (C), respectively.

wherein fibrinogen forms a complex with $a_2'b_2$ (Fgn–$a_2'b_2$) that is more susceptible to calcium ion mediated dissociation of the b chains from $a_2'b_2$ than is $a_2'b_2$ uncomplexed with fibrinogen. When the steady state concentrations of $a_2'b_2$–$(Ca^{2+})_2$ and Fgn–$a_2'b_2$–$(Ca^{2+})_2$ are low, the dependence of the observed rate constant (k_{obs}) for exposure and alkylation of the active-site thiol group should be given by

$$\frac{k_{obs}}{[Ca^{2+}]^2} = \frac{[a_2'b_2]}{[a_2'b_2]_0}k' + \frac{[a_2'b_2\text{–Fgn}]}{[a_2'b_2]_0}k'_{Fgn} \qquad (1)$$

where

$$k' = \frac{k_1 k_2}{k_{-1} + k_2} \quad (2)$$

and

$$k'_{Fgn} = \frac{k_{1,Fgn} k_{2,Fgn}}{k_{-1,Fgn} + k_{2,Fgn}} \quad (3)$$

The 100-fold molar excess of [1-^{14}C]iodoacetamide over plasma factor XIII together with the minimal extra alkylation observed with addition of high concentrations of fibrinogen (see Materials and Methods) ensured that the addition of fibrinogen was not interfering with the alkylation of the active-site thiol group on factor XIIIa. Also

$$K_d = \frac{[a_2'b_2][Fgn]}{[Fgn\text{–}a_2'b_2]} \quad (4a)$$

$$[a_2'b_2] = [a_2'b_2]_0 - [Fgn\text{–}a_2'b_2] \quad (4b)$$

$$[Fgn] = [Fgn]_0 - [Fgn\text{–}a_2'b_2] \quad (4c)$$

where the subscript zero denotes total initial concentration. Substituting eqs 4b and 4c in eq 4a, solving eq 4a for Fgn–$a_2'b_2$, and substituting the result in eq 1 yields eq 5, an expression of the dependence of the observed rate constant for exposure and alkylation of the active-site thiol group as a function of the K_d and the initial concentrations of fibrinogen and $a_2'b_2$. The data (Figure 4) depict the observed increase

$$\frac{k_{obs}}{[Ca^{2+}]^2} = \left\{[a_2'b_2]_0 - [Fgn]_0 - K_d + \sqrt{([a_2'b_2]_0 + [Fgn]_0 + K_d)^2 - 4[a_2'b_2]_0[Fgn]_0}\right\} \times k'/2[a_2'b_2]_0 + \left\{[a_2'b_2]_0 + [Fgn]_0 + K_d - \sqrt{([a_2'b_2]_0 + [Fgn]_0 + K_d)^2 - 4[a_2'b_2]_0[Fgn]_0}\right\} \times k'_{Fgn}/2[a_2'b_2]_0 \quad (5)$$

in the rate constant for exposure of the active-site thiol group of $a_2'b_2$, with increasing fibrinogen concentration, to a limiting value of 2400 M^{-2} s^{-1} for $k_{obs}/[Ca^{2+}]^2$. A nonlinear, least-squares fit to eq 5 of the dependence of $k_{obs}/[Ca^{2+}]^2$ on the initial concentration of fibrinogen ($[Fgn]_0$) and cleaved plasma factor XIII ($[a_2'b_2]_0$) yielded a value of 2.30 (±0.30) μM for the equilibrium constant (K_d) for dissociation of fibrinogen from $a_2'b_2$ (with the fibrinogen concentration expressed in terms of the fibrinogen protomeric unit AαB$\beta\gamma$). In an attempt to determine whether a_2b_2 and $a_2'b_2$ bind similarly to fibrinogen, the effect of 5.0 μM a_2b_2 on the fibrinogen (1.5 μM) mediated enhancement in the rate of exposure of the active-site thiol group in $a_2'b_2$ (0.39 μM) was measured. The rate of exposure of the active-site thiol group in the presence of 5.0 μM a_2b_2 was 1420 M^{-2} s^{-1}, compared to 1600 M^{-2} s^{-1} in the absence of a_2b_2. The failure of a_2b_2 to effect substantial inhibition of the enhancement by fibrinogen of *b* chain dissociation from $a_2'b_2$ and subsequent generation of factor XIIIa activity suggests that the proteolytic cleavage which converts a_2b_2 to $a_2'b_2$ markedly alters the interaction with fibrinogen and that $a_2'b_2$ must bind to fibrinogen much more tightly or at a different site than does a_2b_2.

Discussion

The binding of carbamylmethylated platelet factor XIII (CMa_2') to fibrin II (K_d = 2.1 μM) observed in the present study demonstrates that the interaction of factor XIIIa with its fibrin substrate is not exclusively dependent upon an interaction between the active site of factor XIIIa and the cross-linking sites on fibrin, but instead it involves a domain on factor XIIIa distinct from the active site (an exosite). Release of activation peptide appears to be a significant determinant of the exosite interaction, since even in the presence of 50 mM $CaCl_2$, where uncleaved platelet factor XIII zymogen assumes an enzymically active conformation, the fibrin affinity of uncleaved CMa_2 does not increase to that observed for CMa_2'. Interestingly, factor XIII that has been activated by 20 mM $CaCl_2$ (rather than by α-thrombin cleavage) cross-links the γ-chains of fibrin I as does α-thrombin-activated factor XIII. In contrast to α-thrombin-activated factor XIII, however, the $CaCl_2$-activated factor XIII does not catalyze formation of cross-links between α-chains of fibrin or between fibrin and fibronectin (Okada et al., 1985). The unique catalytic properties of factor XIIIa activated solely by high concentrations of calcium ion suggest that the activation peptide domain, when present, may alter interactions between the exosite of factor XIII and fibrin. In this regard, it is noteworthy that Janus (1984) reported a K_m for the incorporation of [^{14}C]putrescine into *N*,*N*′-dimethylcasein of 8.5 (±1.9) × 10^{-5} M for factor XIIIa activated by exposure to 60 mM $CaCl_2$, whereas the K_m for the α-thrombin-activated enzyme is 3.4 (±0.4) × 10^{-5} M. This observation suggests that the domain encompassing the activation peptide may influence the interaction of the factor XIIIa exosite with protein substrates other than fibrin.

Although platelet factor XIII combines readily with free *b* chains to form a fibrin binding a_2b_2 complex (Chung et al., 1974) whose behavior and response to α-thrombin and calcium ion are indistinguishable from that of native plasma factor XIII (Hornyak & Shafer, 1991), carbamylmethyl uncleaved platelet factor XIII (CMa_2) was incapable of forming a fibrin binding CMa_2b_2 complex upon incubation with excess *b* chains. This observation suggests that steric perturbations in the region of Cys-*a*314 are incompatible with proper association of *b* chains with *a* chains in plasma factor XIII. Recently, we have found that, in the absence of calcium ion, the active-site thiol group of a_2' is exposed (as measured by reactivity toward iodoacetamide), whereas the presence of the *b* chains in the $a_2'b_2$ complex prevents exposure of the active-site thiol group (Hornyak & Shafer, 1991). Lack of exposure of the active-site thiol group in $a_2'b_2$ would be consistent with an inability to form a CMa_2b_2 complex, if a region of the *b* chain critical for tetramer assembly were in close contact with Cys-*a*314 and thereby impeded access of iodoacetamide to the active site of *a*′ in $a_2'b_2$.

The finding that saturation of fibrin with a_2b_2 had no effect upon the binding of CMa_2' to fibrin indicated that CMa_2' and a_2b_2 bind to distinct, noninteracting sites in fibrin. The existence of separate fibrin binding sites for the plasma zymogen and for a derivative of the active enzyme suggests that the domain of fibrin that binds plasma factor XIII zymogen during the fibrin-promoted α-thrombin-catalyzed activation of plasma factor XIII (Naski et al., 1991) is distinct from the domain of fibrin that binds the exosite of factor XIIIa and thereby anchors factor XIIIa to its fibrin substrate. The equilibrium constant for dissociation of plasma factor XIII zymogen and fibrin reported in this study (200 nM) is greater than that reported by Naski et al. (65 nM). The higher ionic strength used in the present studies may be responsible for this difference. A similar variation in affinity with ionic strength has been reported for the interaction of factor XIII and fibrinogen

(Greenberg & Shuman, 1982). The existence of separate binding sites in fibrin for the plasma factor XIII zymogen and the factor XIIIa exosite suggests that upon activation of fibrin-bound factor XIII, reorientation of the fibrin–factor XIII complex occurs to facilitate factor XIIIa-catalyzed cross-linking of the γ-chains and α-chains of adjoining fibrin units.

It has been reported previously (Greenberg et al., 1987) that fibrin II does not promote platelet factor XIII activation by α-thrombin. These experiments, however, were performed at a concentration of fibrin II protomer (1.24 μM) well below the K_d for the platelet factor XIII–fibrin II interaction of 14 μM determined in this study. The absence of a promoting effect upon platelet factor XIII activation, even at concentrations of fibrin II comparable to the K_d (ensuring that about half of the platelet factor XIII is bound to fibrin), suggests that the thermodynamics of factor XIII binding to fibrin II alone do not determine the potential for promotion of factor XIII activation but that the mode of binding, influenced by the presence of *b* subunits, is undoubtedly important. The *b* chains of plasma factor XIII may orient the protein in the ternary complex of α-thrombin, fibrin, and factor XIII to ensure efficient in vivo cleavage of the scissile bond in the factor XIII *a* chain.

The dissociation constant (K_d = 2.3 μM) determined for the interaction of $a_2'b_2$ and fibrinogen in this study is much greater than that determined by Greenberg and Shuman (1982) for the interaction of plasma factor XIII zymogen (a_2b_2) and fibrinogen (K_d = 10–20 nM), suggesting that activation peptide cleavage significantly weakens the interaction of factor XIII with fibrinogen. To determine whether the weakened binding of $a_2'b_2$ reflects binding of $a_2'b_2$ to a site distinct from that involved in the binding of a_2b_2, we measured the effect of 5.0 μM a_2b_2 on the rate of exposure of the active-site thiol group in 0.39 μM $a_2'b_2$ in the presence of 1.5 μM fibrinogen. Even if the affinity of plasma factor XIII zymogen for fibrinogen under our conditions were no greater than the affinity of a_2b_2 for fibrin II found in this study (K_d = 200 nM instead of the K_d of 10–20 nM reported by Greenberg and Shuman), at least 85% of the fibrinogen in the incubate would have been complexed with a_2b_2. If a_2b_2 and $a_2'b_2$ were competing for the same site on fibrinogen, less than 20% of the added fibrinogen would be available to interact with $a_2'b_2$. Under these conditions eq 5 predicts that the rate constant for exposure of the active-site thiol group $a_2'b_2$ should drop from 1600 M^{-2} s^{-1} (in the absence of a_2b_2) to 900 M^{-2} s^{-1}. The failure to observe a significant rate difference upon addition of a_2b_2 suggests that a_2b_2 and $a_2'b_2$ interact with different domains of fibrinogen.

Together with previous studies, the findings in this paper demonstrate further the roles of the AP domain and of the *b* subunits in mediating interactions of factor XIII with other proteins in the terminal part of the blood coagulation cascade. AP cleavage, in addition to facilitating the binding of calcium ion to factor XIII to cause release of the *b* subunits (Schwartz et al., 1973; Chung et al., 1974) and inducing the interaction between the *a* subunits necessary for catalytic competence (Hornyak & Shafer, 1991), appears to increase the affinity of the factor XIIIa exosite for the fibrin clot. Additionally, loss of the AP domain from plasma factor XIII (a_2b_2) produces an intermediate ($a_2'b_2$) which binds fibrinogen more weakly and at a different site than does a_2b_2. The *b* subunits of plasma factor XIII not only contribute to the affinity of the plasma factor XIII zymogen a_2b_2 for fibrin, as demonstrated in this study and by Naski et al. (1991), but also orient the *a* chains of fibrin for efficient cleavage of AP by α-thrombin.

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