α-Chain Domain of Fibrinogen Controls Generation of Fibrinoligase (Coagulation Factor XIIIa). Calcium Ion Regulatory Aspects†

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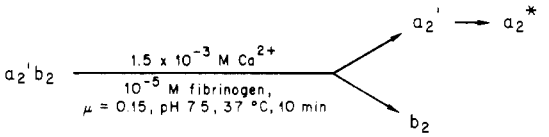
ABSTRACT: Fibrinogen ($\sim 10^{-5}$ M) labilizes heterologous interactions within the thrombin-modified factor XIII zymogen (i.e., XIII′ = $a_2'b_2$) so that, in the time frame (ca. 10 min) of normal clotting in plasma (37 °C, μ = 0.15, pH 7.5), 1.5 mM Ca^{2+} is sufficient to cause the release of the noncatalytic b subunits and also the unmasking of 1 equiv of iodo[1-^{14}C]acetamide-titratable group per catalytic a subunit. Under similar conditions, but in the absence of fibrinogen, approximately 10 mM Ca^{2+} would be needed to achieve the same effect. Thus, by promoting the conversion of XIII′ to XIIIa (i.e., $a_2'b_2 \rightarrow a_2^* + b_2$), fibrinogen functions as a physiologically important Ca^{2+}-modulator protein. Total plasmin digests of fibrinogen display the regulatory phenomenon nearly as well as the parent protein. In an attempt to identify the structural domain on the fibrinogen which is responsible for this novel function of the molecule, it was found that two overlapping fragments derived from the midsections of the α chains, either by CNBr cleavage (residues 243–476) or by plasmin digestion (residues 242–424), are active.

Until quite recently, fibrinogen was thought to contain two types of functional domains relevant for clotting in blood plasma. First to be recognized were the thrombin-sensitive N-terminal regions of the Aα and Bβ chains of the molecule which comprise fibrinopeptides A and B (Lorand, 1951, 1952; Bettelheim & Bailey, 1952; Blomback & Vestermark, 1959). As long as the fibrinopeptides remain attached to the parent protein, clot formation (i.e., aggregation) at the ionic milieu of plasma is prevented (Lorand, 1954). Thus the thrombin-catalyzed removal of these peptides during the $(\alpha\beta\gamma)_2A_2B_2 \rightarrow (\alpha\beta\gamma)_2 + 2A + 2B$ conversion seems to be the prerequisite for unmasking the fibrin-to-fibrin contact sites (i.e., aggregation sites). The second type of functional domains is involved in the covalent fusion of fibrin molecules by γ-glutamyl-ε-lysine cross-bridges. This polymerization reaction is catalyzed by the last enzyme, factor XIIIa or fibrinoligase, of the coagulation cascade [for a recent review, see Lorand et al. (1980a)]. Arising from its precursor (factor XIII), the transamidase reacts first with the γ and then with the α chains of fibrin (Lorand & Chenoweth, 1969; Lorand et al., 1972a), and cross-linking sites are located in the C-terminal portions of the γ chains as well as in the midsections of α chains (Chen & Doolittle, 1971; Fretto et al., 1978).

Recent work from this laboratory (Credo et al., 1978) revealed the existence of yet a third type of functional domain on the fibrinogen molecule. In a continuing effort to reconstruct in vitro the systems which regulate clot formation in normal plasma [see Lorand (1975)], we found that fibrinogen controls the rate of generating factor XIIIa activity. Specifically, by interacting with the thrombin-modified but still inactive form of the zymogen (XIII′ = $a_2'b_2$), fibrinogen reduces the Ca^{2+} concentration needed for the dissociation of this heterologous ensemble and for the unmasking of the active-center cysteines in the catalytic subunits (XIIIa = a_2^*) to that of the free Ca^{2+} (1.5 mM) in plasma. In the presence of physiological concentrations of fibrinogen ($\sim 10^{-5}$ M), production of XIIIa is accelerated so that it is completed within the time frame of normal coagulation of about 10 min.



Thus, fibrinogen acts as a true Ca-modulatory protein with regard to XIIIa generation.

The main topic of the present paper is to delineate in a structural sense the functional domain on the fibrinogen molecule which is responsible for this novel type of regulation. It will be shown that the total plasmin digest of the fibrinogen is nearly as effective in promoting the conversion of XIII′ to XIIIa as the parent molecule. Furthermore, two overlapping fragments derived from the midsections of the Aα chains either by CNBr cleavage (Blomback et al., 1974; called HI2DSK) or by plasmin digestion (Nussenzweig et al., 1961; Harfenist & Canfield, 1975; Fretto et al., 1978; called fragment A, H, or P121) are likewise effective.

Materials and Methods

Fibrin stabilizing factor (FXIII) zymogen and its b subunit were purified from outdated human plasma by previously published procedures (Lorand & Gotoh, 1970; Curtis & Lorand, 1976). The zymogen and b subunit were stored at 4 and −10 °C, respectively, in solutions of 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA. Plasma fibronectin (cold insoluble globulin, or CIg) was obtained as a byproduct of the preparation of the factor XIII zymogen (Molnar et al., 1979). Human α-thrombin was a gift of Dr. J. W. Fenton, II, Division of Laboratories and Research, New York State Department of Health, Albany (Fenton et al., 1977). Plasminogen was prepared from human plasma by using a lysine-coupled Sepharose-4B affinity column according to the procedure of Brockway & Castellino (1972). Urokinase (13 000 CTA units per mg of protein, lot 2120-215) was obtained from Abbott Laboratories (Barlow, 1976). Trasylol (aprotinin, 10 000 kallikrein inactivator units per mL) was obtained from Mobay Chemical Co. Hirudin (lot 378-2330), 7000 anti-thrombin clotting units per mL, as defined by the supplier, was pur-

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chased from Sigma Chemical Co.

Iodo[1-^{14}C]acetamide (57 Ci/mol) was obtained from Amersham-Searle, Radiochemical Centre.

Human fibrinogen was purchased from IMCO (batches F-149 II, F-144, and F-146) and was also obtained as a gift from A. B. Kabi (lots 32120 and 32128). Highly purified fibrinogen I-4 and I-8 were kindly provided by Dr. D. K. Galanakis and Dr. M. Mosesson, Downstate Medical Center, State University of New York. All were dialyzed exhaustively against 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and 0.5 mM EDTA. The plasmin-mediated fibrinogen degradation products X (stage 1), X (stage 2), D, E, and fragment A were kindly provided by Dr. A. Z. Budzynski, Health Sciences Center, Temple University, Philadelphia. [For nomenclature of these fragments, see Latallo (1973).] The carboxymethylated Aα chain of human fibrinogen and the CNBr derivative, Hi-2 DSK, were kindly provided by Dr. B. Hessel and Dr. B. Blomback, Karolinska Institute, Stockholm. Calmodulin was kindly provided by Dr. D. Storm, University of Washington, Seattle. Concentrations of these proteins were determined by using an extinction coefficient of $E^{1\%}_{1cm}$ (280 nm) = 15.1 for both IMCO and Kabi fibrinogens (Mihalyi, 1968), 15.5 for fibrinogen I-4 and I-8 (D. Galanakis, private communication), 20 for fragment D (Olexa & Budzynski, 1979), 12 for fragment E (Olexa & Budzynski, 1979), 12.8 for plasma fibronectin (CIg) (Mossesson & Umfleet, 1970), and 1.8 for calmodulin (D. Storm, private communication).

Typically, activation of the FXIII zymogen with α-thrombin was performed at 25 °C in 0.145-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-5} M zymogen and 1.8×10^{-7} M α-thrombin, the latter expressed as *p*-nitrophenyl guanidobenzoate titratable active sites (Chase & Shaw, 1969). After incubation for 30 min, a 2-fold excess of hirudin (35 units/mL, based upon the inhibition of thrombin clotting units) was added.

The dissociation of the a′ and b subunits of the thrombin-modified zymogen (FXIII′) was achieved at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 9.6×10^{-7} M FXIII′, 5.2 units/mL hirudin, 8.9×10^{-6} M fibrinogen (Kabi, lot 32120), 2.16×10^{-5} M iodoacetamide, 1.5 mM $CaCl_2$, and NaCl to maintain an ionic strength of $\mu = 0.15$. After a 30-min incubation, each sample was mixed with 7 μL of 50% glycerol containing 0.1% bromophenol blue tracking dye. Aliquots of 50 μL, containing approximately 16 μg of FXIII′ protein equivalents, were then subjected to electrophoresis under nondenaturing conditions (Rodbard & Chrambach, 1971). As a reference for dissociation, a sample containing 8 μg of purified b subunit (corresponding to the amount of b subunit expected for complete dissociation of FXIII′) was treated in a similar manner. In addition, control samples of the factor XIII zymogen, not exposed to thrombin, but nevertheless containing a comparable amount of hirudin, were also treated at 37 °C for 30 min and subjected to analysis.

The unmasking of potential active centers, as judged by titration with iodo[1-^{14}C]acetamide, was carried out by the method of Curtis et al. (1974). Alkylations were performed at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing as required for each experiment 3.4 units/mL hirudin, 2.1×10^{-5} M or 4.4×10^{-5} M iodo[1-^{14}C]acetamide, 0–30 mM $CaCl_2$, 0–1.8×10^{-5} M fibrinogen (or fibrinogen fragment), 0–10^{-5} M plasma fibronectin, and in all cases NaCl to maintain a constant ionic strength of $\mu = 0.15$. At the designated times (0–4 h), aliquots of 10 μL were removed and spotted onto filter paper disks which were immediately washed in trichloroacetic acid and processed as described by Lorand et al. (1972b). Protein-bound isotope is reported as moles of [^{14}C]carbamidomethyl groups incorporated per mole of thrombin-modified zymogen of $a_2'b_2$ structure.

Conversion of plasminogen to plasmin was achieved in 10 min at 37 °C in solutions of 0.05 M Tris-HCl, pH 7.5, containing 0.05 M lysine, 6.9×10^{-5} M plasminogen, 29 CTA units of urokinase, and 0.05 M Tris-HCl.

Fibrinogen was digested in 0.26-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 4.55×10^{-5} M fibrinogen I-4 and 5.4×10^{-7} M plasmin. After a 12-h incubation at 37 °C, an additional aliquot of plasmin (2 μL of 6.8×10^{-5} M, dissolved in the same Tris/lysine buffer as above) was added to ensure complete digestion. After a further 12 h at 37 °C, an 8-fold molar excess of Trasylol (9×10^{-6} M) over plasmin was added to the reaction mixtures. Control samples of fibrinogen, which were not digested, were treated in a similar manner with buffer in lieu of plasmin.

Plasmin-digested IMCO fibrinogen and its control were prepared by first mixing 0.25 mL of IMCO fibrinogen (lot F-149 II, 4.68×10^{-5} M, dissolved in 0.05 M Tris-HCl, 0.1 M NaCl, and 0.5 mM EDTA, pH 7.5) with either Trasylol (6×10^{-5} M) or plasmin (1.97×10^{-5} M). After a 12-h incubation at 37 °C, a second dose (7 μL) of either Trasylol or plasmin was added. After a total period of 24 h, the samples of fibrinogen (0.27 mL) were then mixed with 5 μL of Trasylol (2.1×10^{-3} M, dissolved in 0.05 M Tris-HCl, pH 7.5).

Plasmin-digested IMCO fibrin was prepared by mixing 0.25 mL of IMCO fibrinogen (4.68×10^{-5} M, dissolved in the same Tris/NaCl/EDTA buffer as above) with a 5-μL solution containing 1.3 NIH clotting units of α-thrombin. The resulting clots were then wound out onto a glass rod, separated from the clot liquor, and washed (5 × 3 mL with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.5 mM EDTA). After the final washing, the clot mixtures were adjusted to their original volumes by the addition of Tris/NaCl/EDTA buffer at pH 7.5. Proteolytic digestion was initiated by the addition of 7 μL of 1.97×10^{-5} M plasmin (dissolved in 0.05 M Tris-HCl, pH 7.5). After an incubation of 12 h at 37 °C, an additional dose (7 μL) of plasmin was added to the clots.

Fibrin clots, which were not washed, so as not to remove fibrinopeptides, were also treated with plasmin in the above manner. After 24 h in the presence of plasmin, the clots (0.27 mL) were mixed with 15 μL of 0.05 M Tris-HCl buffer, pH 7.5, containing 3.65 units of hirudin and 10^{-5} M Trasylol.

Cross-linking of IMCO fibrin and its digestion by plasmin was performed by first incubating the FXIII zymogen (3.5×10^{-7} M) for 10 min at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.2×10^{-7} M esterase sites of α-thrombin (11 NIH clotting units/mL) and 0.19 M NaCl. The thrombin-modified zymogen was then mixed with 0.06 mL of 30 mM $CaCl_2$ (dissolved in Tris buffer). After a 10-min incubation at 37 °C, cross-linking was initiated by the addition of 0.135 mL of fibrinogen solution (2.8×10^{-5} M IMCO, lot F-149 II, dissolved in 0.05 M Tris-HCl, 0.1 M NaCl, and 0.5 mM EDTA, pH 7.5). The complete cross-linking solution (0.3 mL) contained final concentrations of 1.24×10^{-5} M fibrinogen, 1.23×10^{-7} M FXIII′, 4.4×10^{-8} M esterase sites of α-thrombin (3.88 NIH clotting units per mL), 6.15 mM $CaCl_2$, 0.1 mM EDTA, 0.113 mM NaCl, and 0.05 M Tris-HCl, pH 7.5, $\mu = 0.175$. Control samples containing fibrinogen, rather than clotted fibrin, were treated in a similar manner, with the omission of both FXIII′ and $CaCl_2$. When required for digestion, the fibrin clots or fibrinogen were mixed with 5 μL of plasmin solution (1.55×10^{-5} M, dissolved in 0.05 M Tris-HCl, pH 7.5, with 0.01 M lysine). After a 12-h

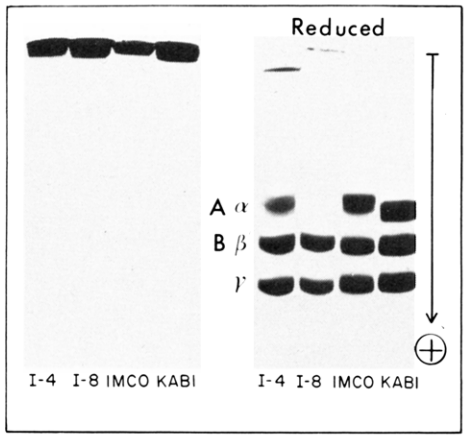


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis (7.8%) of the different preparations of human fibrinogen employed in this work. Track 1, fibrinogen I-4; 2, I-8; 3, IMCO; 4, Kabi. Tracks 5–8, in the same order, represent the patterns of the reduced proteins.

incubation at 37 °C, a second aliquot of plasmin (5 μL) was added to ensure digestion. The proteolytic activity of plasmin was then terminated by the addition of a 2-fold molar excess of Trasylol over plasmin (i.e., 10^{-6} M Trasylol).

The various samples containing fibrinogen, fibrin, cross-linked fibrin, and their plasmin digests were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (Weber & Osborn, 1969). For quantitation of protein-bound isotope in the acrylamide matrix, after electrophoresis the gels were fixed and then sliced into 2.5-mm segments for treatment with H_2O_2 (Tishler & Epstein, 1968).

Blocking of the FXIIIa-reactive glutamine-acceptor sites of fibrinogen with hydroxylamine was performed at 25 °C by the method of Lorand & Ong (1966) in 7-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 8.86×10^{-6} M IMCO fibrinogen, 1 unit/mL hirudin, 1.5×10^{-7} M thrombin-modified FXIII, 5 mM $CaCl_2$, 22.5 mM hydroxylamine, 0.3 M NaCl, and 20 mM cysteine. After a reaction time of 24 h, a 0.1-mL solution of 10 mM iodoacetamide (dissolved in 0.05 M Tris-HCl, pH 7.5) was added. Fifteen minutes later, EDTA was added to a final concentration of 5 mM to terminate the activity of FXIIIa. The final reaction mixture was then dialyzed exhaustively at 25 °C against 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and 0.5 mM EDTA. Estimation of the incorporation of hydroxylamine into fibrinogen was performed by the procedure of Bergman & Segal (1956) as employed by Seifter et al. (1960) and by Lorand & Ong (1966).

Concentrations of fragment A and Hi-2 DSK were determined by amino acid analysis (Hirs et al., 1954; Spackman et al., 1958). Duplicate samples of both proteins were hydrolyzed in vacuo at 108 °C for 24, 48, and 72 h. The average yields of glycine, glutamic acid, and aspartic acid (extrapolated to zero hydrolysis times) for the duplicate analyses were used to calculate the concentration of fragment A and Hi-2 DSK, based upon the reported amino acid compositions (Gardlund et al., 1972; Doolittle et al., 1977; Fretto et al., 1978).

Results

Specificity of Fibrinogen in Modulating the Reaction of the Thrombin-Modified Factor XIII Zymogen (i.e., Factor XIII′) with Iodoacetamide. Alkylation of purified human Factor XIII′ (9.6×10^{-7} and 1.26×10^{-6} M) with iodo[1-^{14}C]acetamide (2.2×10^{-5} and 4.4×10^{-5} M) was examined at 37 °C, $\mu = 0.15$, pH 7.5, with the filter paper assay described by Curtis et al. (1974) for measuring protein bound radioactivity. Reaction times (0–60 min) and fibrinogen (0–1.5×10^{-5} M)

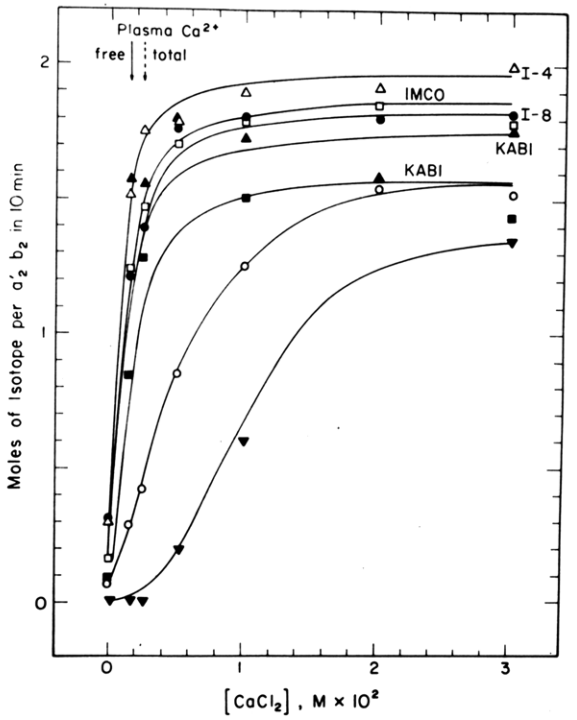


FIGURE 2: Calcium requirement for the alkylation of thrombin-modified factor XIII with iodoacetamide, in the absence and presence of fibrinogen. Alkylation was performed for 10 min at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.2×10^{-6} M factor XIII′, 3.4 units/mL hirudin, 4.4×10^{-5} M iodo[1-^{14}C]acetamide, 0–30 mM $CaCl_2$, and NaCl to maintain $\mu = 0.15$ (▼, ○). Reaction mixtures also contained either I-4 (△, 9.2×10^{-6} M), I-8 (●, 1.1×10^{-5} M), IMCO (□, 8.8×10^{-6} M, lot F-149 II), or Kabi fibrinogen (▲, ■, 8.8×10^{-6} M, lot 32120). The ordinate denotes protein-bound isotope. Vertical arrows indicate the concentration of free and total Ca^{2+} in human plasma (i.e., 1.5 and 2.5 mM, respectively).

and $CaCl_2$ concentrations (0–3×10^{-2} M) were varied. Four different types of fibrinogen preparations (I-4, I-8, IMCO, and Kabi) were used and their sodium dodecyl sulfate electrophoretic profiles, with and without reduction with dithiothreitol, are shown in Figure 1. While all preparations gave clottabilities of about 97% with thrombin, which is the practical limit (Lorand, 1951, 1952), major differences regarding the Aα chains were evident. The Aα chain of the Kabi preparation was significantly smaller than that of either I-4 or IMCO. On the basis of literature reports (Mosesson et al., 1974), we can assume that the Aα chain in fibrinogen I-8 was cleaved into large fragments which comigrated with the β and γ chains.

By setting of the time of alkylation with iodo[1-^{14}C]acetamide (4.4×10^{-5} M) to 10 min, at similar concentrations of fibrinogen (9.2×10^{-6} M for I-4, 1.1×10^{-5} M for I-8, 8.8×10^{-6} M for IMCO, and 8.8×10^{-6} M for Kabi), the Ca^{2+} dependence of the reaction is shown in Figure 2. Total unmasking of active-center cysteines would give rise to the incorporation of 2 mol of the isotope for each mol of the $a_2'b_2$ ensemble (i.e., one thiol per a′ unit of catalytic potential), and it is seen that essentially full blocking can be achieved with 10 min of alkylation.

The Ca^{2+} requirement, which we may define as the Ca^{2+} concentration necessary for 50% labeling and which would signify an average degree of alkylation of 0.5 equiv of a cysteine residue per a′ subunit, varies somewhat from one factor XIII′ preparation to the other, but all our measurements to date fall between the two sets of control values shown in Figure 2 (○ and ▼). In the absence of fibrinogen, as measured for the 10-min reaction, 6–12 mM Ca^{2+} is needed. In the presence of any of the four fibrinogens, however, this value

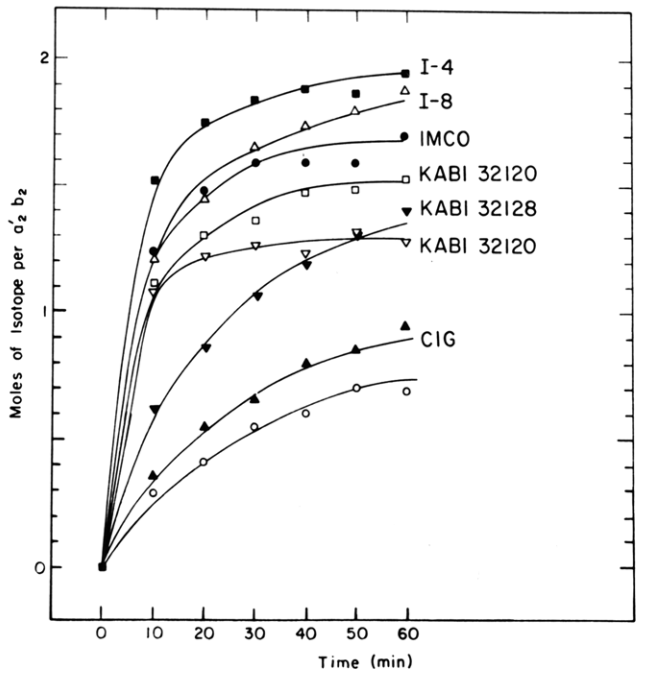


FIGURE 3: Time course for the alkylation of thrombin-modified factor XIII with iodoacetamide in the absence and presence of various preparations of human fibrinogen and plasma fibronectin (CIG). Reactions were performed at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-6} M factor XIII′, 4.4×10^{-5} M iodo[1-^{14}C]acetamide, 1.5 mM $CaCl_2$, 3 units/mL hirudin, and NaCl to maintain $\mu = 0.15$ (○). The following also contained either (■) 9.2×10^{-6} M I-4, (△) 1.1×10^{-5} M I-8, (●) 9.2×10^{-6} M IMCO (lot F 149 II), (□, ▽) 9.2×10^{-6} M Kabi fibrinogen (lot 32120), (▼) 8.8×10^{-6} M Kabi fibrinogen (lot 31218) or (▲) 7.5×10^{-7} M plasma fibronectin (CIG). At the designated times (abscissa), aliquots were taken to measure protein-bound isotope (ordinate).

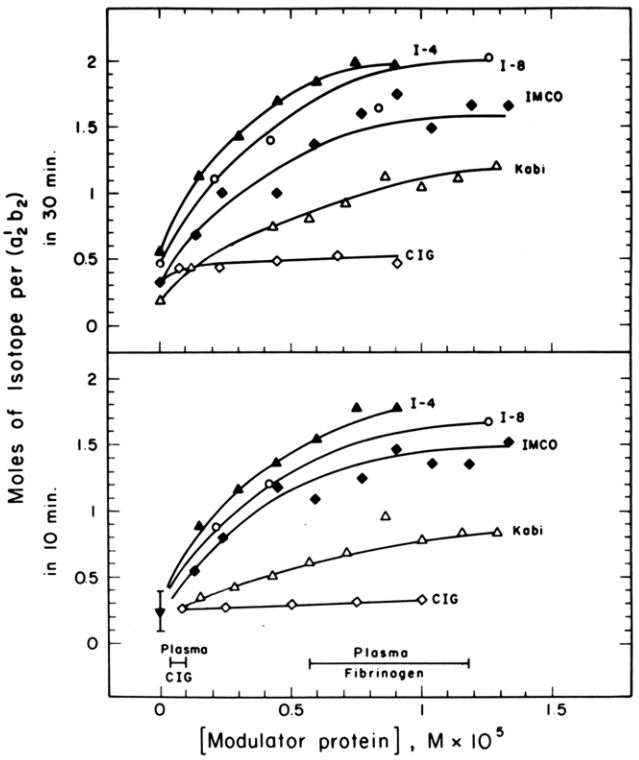


FIGURE 4: Dependence of the alkylation of thrombin-modified factor XIII with iodoacetamide on the concentration of protein modulators. Reaction was carried out for 10 (bottom panel) and 30 min (top panel) at 37 °C in 0.1-mL solutions of 0.5 M Tris-HCl, pH 7.5, containing 9.6×10^{-7} M factor XIII′, 2.16×10^{-5} M iodo[1-^{14}C]acetamide, 2.28 units/mL hirudin, 1.5 mM $CaCl_2$, NaCl to maintain $\mu = 0.15$, and 0–1.5×10^{-5} M of protein modulators (abscissa) as follows: (▲) fibrinogen I-4, (○) I-8, (♦) IMCO (Lot No. F-146), (△) Kabi (1024, lot 32120), and (◊) plasma fibronectin (i.e., cold-insoluble globulin or CIG). The ordinate denotes protein-bound isotope. Horizontal bars show the physiological range of concentrations for fibrinogen and CIG in human plasma.

was reduced to less than 2 mM Ca^{2+}. In order to indicate the variability inherent in such studies, we included two sets of measurements (▲ and ■) using the same batch of Kabi fibrinogen, but tested on two different preparations of factor XIII′.

Because it was thus shown that the presence of fibrinogen reduces significantly the Ca^{2+} requirement for the reaction of factor XIII′ with iodoacetamide, the concentration of Ca^{2+} for all future experiments ($\mu = 0.15$, pH 7.5, 37 °C) was set to 1.5 mM, which is equivalent to the free concentration of Ca^{2+} in plasma and serum (Linder & Blomstrand, 1958).

Figure 3 shows progression curves for the alkylation of factor XIII′ (at 1.5 mM Ca^{2+} and 37 °C). Reactivity with iodo[1-^{14}C]acetamide was greatly enhanced by the presence of fibrinogen but, again, significant differences were found with regard to the effectiveness of the various fibrinogen preparations in assisting this alkylation reaction. CIG (cold-insoluble globulin or plasma fibronectin), at a concentration equivalent to that in plasma, had very little effect.

At 1.5 mM Ca^{2+}, nearly complete unmasking of the iodoacetamide-titratable sites could be obtained at physiological concentrations of fibrinogen within reaction periods of 10–30 min (Figure 4). By contrast, CIG had no effect even at a concentration ten times higher than found in plasma, and the fact that fibrinogen is a rather specific modulator of factor XIII′ conversion is also borne out by the finding that calmodulin, up to 1.5×10^{-5} M concentration, was also ineffective.

We have previously developed a nondenaturing electrophoretic technique to monitor the release of the noncatalytic b subunits during the activation of the zymogen and showed that the heterologous dissociation of the a′b protomeric ensemble parallels the unmasking of the titratable thiols in the

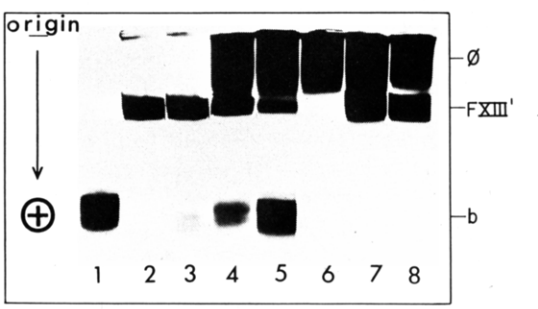


FIGURE 5: Fibrinogen-assisted dissociation of thrombin-modified factor XIII as seen by polyacrylamide gel electrophoresis under nondenaturating conditions at pH 7.8. Factor XIII′ (9.6×10^{-7} M) was incubated for 30 min at 37 °C in a 0.1-mL solution of 0.05 M Tris-HCl, pH 7.5, containing 2.16×10^{-5} M iodoacetamide, 4 units/mL hirudin, NaCl to maintain $\mu = 0.15$, and, as required, one or both 1.5 mM $CaCl_2$ and 8.9×10^{-6} M fibrinogen (Kabi lot 32120). The factor XIII zymogen itself (9.6×10^{-7} M) and purified b subunit (9.6×10^{-7} M) served as markers. Aliquots of 50 μL were taken for electrophoresis. Track 1, b subunit; 2, factor XIII; 3, factor XIII′ and $CaCl_2$; 4, factor XIII′ and fibrinogen; 5, factor XIII′ with $CaCl_2$ and fibrinogen; 6, fibrinogen and $CaCl_2$; 7, factor XIII and fibrinogen; 8, factor XIII with $CaCl_2$ and fibrinogen. Positions of fibrinogen (ϕ), free b subunit, and the catalytic subunit containing factor XIII′ species are indicated.

a′ subunits in factor XIII′ (Credo et al., 1978). As shown in Figure 5, the release of free b subunits is promoted by the combined presence of fibrinogen and Ca^{2+} (compare track 3 with no fibrinogen to track 4 with no Ca^{2+} and to track 5 with both Ca^{2+} and fibrinogen). It is interesting to note that a

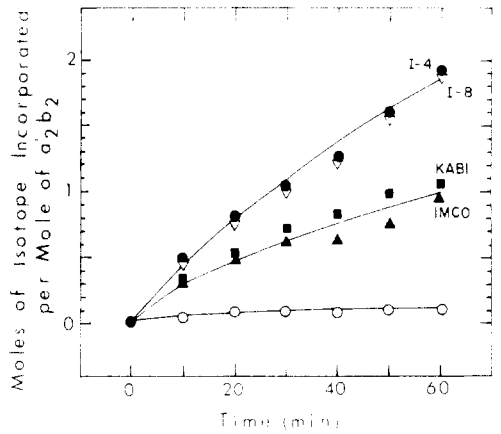


FIGURE 6: Alkylation of thrombin-modified factor XIII with iodoacetamide in the presence of fibrinogen, but without $CaCl_2$. The reaction was performed at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-6} M factor XIII′, 3 units/mL hirudin, NaCl to maintain $\mu = 0.15$ (○), and also one of the following additions as required: 8.9×10^{-6} M IMCO fibrinogen (▲, lot F-144), 8.9×10^{-6} M Kabi (■, lot 32120), 1.1×10^{-5} M I-4 (●), or 1.2×10^{-5} M I-8 (▽). The ordinate denotes protein-bound isotope.

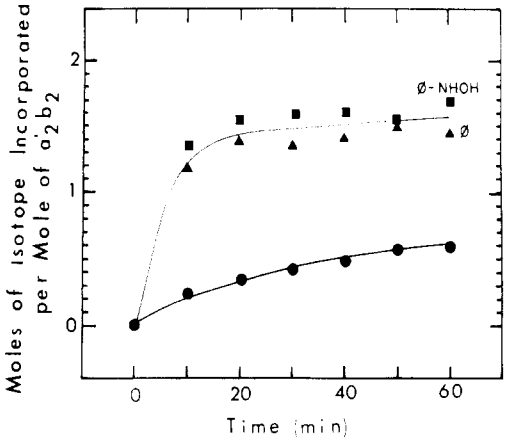


FIGURE 7: Effectiveness of hydroxylamine-blocked IMCO fibrinogen in promoting the alkylation of thrombin-modified factor XIII by iodoacetamide. The reaction was carried out at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.5 mM $CaCl_2$, 9.6×10^{-7} M factor XIII′, 3 units/mL hirudin, 2.16×10^{-5} M iodo[1-^{14}C]acetamide, NaCl to maintain $\mu = 0.15$ (●), and, as indicated, 8.9×10^{-6} M of either native fibrinogen (▲) or fibrinogen in which the factor XIIIa reactive γ-glutamine residues have been previously blocked with hydroxylamine (Lorand & Ong, 1966). Protein-bound isotope is shown on the ordinate.

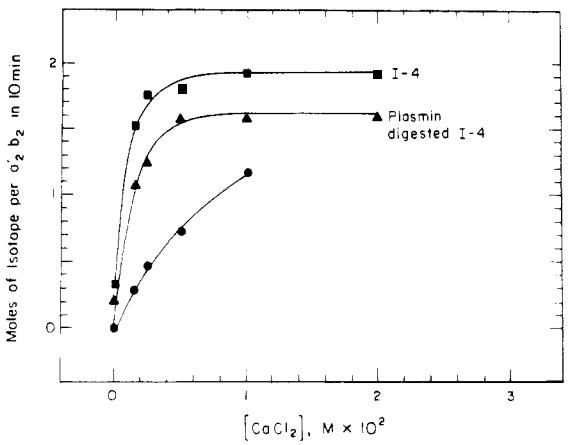


FIGURE 8: Calcium requirement for the alkylation of thrombin-modified factor XIII in the presence of fibrinogen I-4 and of its plasmin digest. Reactions were carried out at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-6} M factor XIII′, 3 units/mL hirudin, 2.2×10^{-5} M iodo[1-^{14}C]acetamide, 0–20 mM $CaCl_2$ (abscissa), NaCl to maintain $\mu = 0.15$ (●), and, as required, 9.2×10^{-6} M fibrinogen I-4 (■) or the same concentration of its plasmin digest (▲). Protein-bound isotope is shown on the ordinate.

significant degree of dissociation of b subunits from factor XIII′ was found to occur in the presence of fibrinogen even when precautions were taken to remove Ca^{2+} from the proteins by exhaustive dialysis (track 4 in Figure 5). Thus, it would seem that fibrinogen alone can assist the conversion of factor XIII′. This effect was confirmed by titration with iodo[1-^{14}C]acetamide for all preparations of fibrinogen (Figure 6). Though reactivity toward alkylation is more sluggish than with Ca^{2+} also present, it is nevertheless obvious that essentially full unmasking of titratable groups can be achieved simply by allowing factor XIII′ to interact with fibrinogen.

In order to examine the question whether the modulatory behavior of fibrinogen, in assisting the conversion of factor XIII′ to factor XIIIa, was in any way related to the specific interaction of the latter enzymatic species with glutamine functionalities in the γ and α chains of the protein substrate (Lorand et al., 1968a), we prepared a hydroxylamine-derivatized form of fibrinogen according to Lorand & Ong (1966) by blocking these glutamine residues in the protein molecule. The derivatization involved prior treatment of fibrinogen with factor XIIIa, $CaCl_2$, and hydroxylamine and resulted in the incorporation of approximately 7 mol of hydroxamate/mol of fibrinogen, which signifies complete coverage of reactive sites. Upon reisolation, the fibrinogen derivative proved to be refractory to cross-linking when incubated with thrombin, Ca^{2+}, and factor XIII, which is a good indication that the glutamines in question were, indeed, modified. The blocked fibrinogen, however, as shown in Figure 7, was just as effective in promoting the alkylation of factor XIII′ with iodo[1-^{14}C]acetamide as the native fibrinogen control.

Identification of a Functional Domain in the Aα Chain of Fibrinogen Which Promotes the Conversion of the Thrombin-Modified Factor XIII Zymogen (i.e., of Factor XIII′). The effectiveness of the I-8 preparation (see Figures 1–4) suggested that fibrinogen may undergo rather extensive cleavages and yet retain its ability to assist in the conversion of the factor XIII′ zymogen. This suggested the possibility that there might be a distinct domain in the fibrinogen molecule with the specialized function of promoting this conversion. Various avenues were explored to identify such a functional sequence within the structure of fibrinogen, including the analysis of total limit digests of human fibrinogen, fibrin, and cross-linked fibrin with human plasmin and the testing of some previously characterized and isolated plasmin fragments (A, D, E, and X) and also of the so-called HI2DSK fragment, derived from the Aα chain of the molecule by cleavage with CNBr.

The reactivity of factor XIII′ with iodo[1-^{14}C]acetamide was examined in the presence of all of these products by assays in which either the concentration of Ca^{2+} was fixed (at 1.5 mM) and the time of the alkylation reaction was varied or the time was set to 10 min for different concentrations of Ca^{2+}. As seen in Figures 8 and 9, the total plasmin digest of I-4 fibrinogen proved to be almost as effective as the native protein by either experimental criterion. Essentially identical results were obtained with plasmin digests of fibrin or of cross-linked fibrin.

Results of the testing of known, purified plasmin fragments of fibrinogen are presented in Figure 10, and it is obvious that only fragment A, and perhaps X of the stage 1 plasmin digest, can be considered to display significant activity. A Kabi preparation was used as reference because this type of fibrinogen, albeit a different batch, served as the parent protein

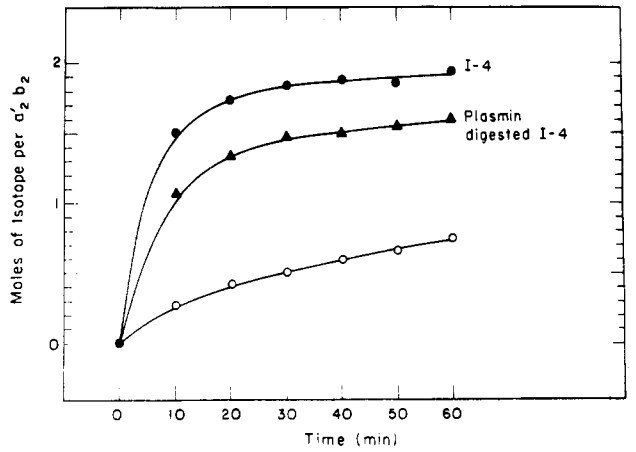


FIGURE 9: Time course for the alkylation of thrombin-modified factor XIII in the presence of fibrinogen I-4 and of its plasmin digest. Reactions were performed with 1.5 mM $CaCl_2$ under the conditions given in the legend to Figure 8. (○) Factor XIII′, control; (●) factor XIII′, with fibrinogen I-4; (▲) factor XIII′, with plasmin-digested fibrinogen I-4.

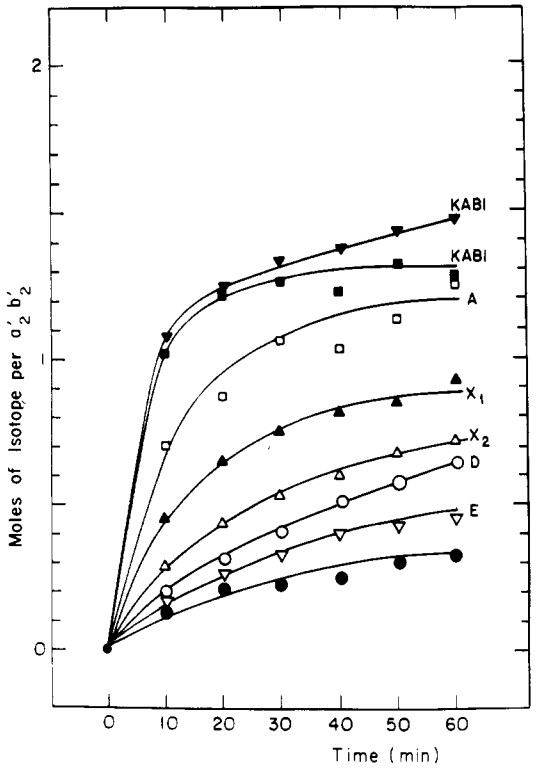


FIGURE 10: Time course for the reaction of thrombin-modified factor XIII with iodoacetamide in the presence of fibrinogen and that of known, isolated plasmin degradation products. Alkylations were performed at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-6} M factor XIII′, 4.4×10^{-5} M iodo[1-^{14}C]-acetamide, 1.5 mM $CaCl_2$, NaCl to maintain $\mu = 0.15$ (●), and either 9.2×10^{-6} M Kabi fibrinogen (▼, ■), 1.1×10^{-5} M fragment A (□), 9.2×10^{-6} M fragment X of a stage 1 plasmin digest (▲), 9.2×10^{-6} M fragment X of a stage 2 plasmin digest (△), 1.8×10^{-5} M fragment D (○), or 1×10^{-5} M fragment E (▽). Protein-bound isotope is shown on the oridinate.

for isolating the plasmin fragments examined.

The CNBr cleavage product of the Aα chain of fibrinogen, HI2DSK, was derived from an IMCO preparation and was therefore analyzed in relation to this fibrinogen, though again a different lot. HI2DSK is significantly larger (29 000) than plasmin fragment A (18 000) and contains the entire sequence of the latter in its structure. It was examined at concentrations which were close to equivalent and 2-fold in excess of the reference fibrinogen (Figure 11). Since there are two Aα

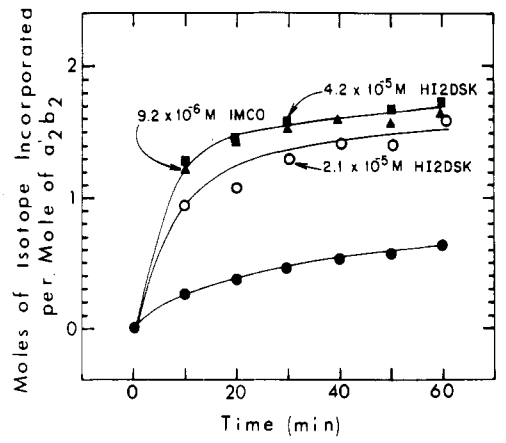


FIGURE 11: Time course for the alkylation of thrombin-modified factor XIII with iodoacetamide in the presence of the CNBr fragment from the Aα chain of fibrinogen, HI2DSK. Reactions were performed at 37 °C in 0.1-mL solutions of 0.05 M Tris-HCl, pH 7.5, containing 1.26×10^{-6} M factor XIII′, 3 units/mL hirudin, 1.5 mM $CaCl_2$, 4.4×10^{-5} M iodo[1-^{14}C]acetamide, NaCl to maintain $\mu = 0.15$ (●), and either 9.2×10^{-6} M IMCO fibrinogen (▲) or 4.2×10^{-5} M (■) and 2.1×10^{-5} M or HI2DSK (○). Ordinate shows protein-bound isotope.

chains in the fibrinogen molecule ($M_r \sim 340\,000$) and since each gives rise to a HI2DSK fragment, 2.1×10^{-5} M HI2DSK (○) is slightly more than the equivalent of 9.2×10^{-6} M IMCO material (▲), and 4.2×10^{-5} M HI2DSK represents slightly more than 2-fold excess (●). As seen from the graphs, the effectiveness of HI2DSK in promoting the reaction of factor XIII′ with iodoacetamide approximates that of the IMCO fibrinogen.

Discussion

Two experimental pathways are known for activating the fibrin-stabilizing-factor (factor XIII) zymogen: a thrombin-independent and a thrombin-dependent one. In terms of the a_2b_2 structure of the plasma zymogen (i.e., factor XIII), generation of the enzyme by the thrombin-independent route (I) can be expressed as

$$a_2b_2 \rightarrow a_2^0 + b_2 \qquad (1)$$

where the a^0 designation stands for the catalytic species. Existence of this pathway can be demonstrated (Credo et al., 1978) simply by exposing factor XIII to relatively high concentration of Ca^{2+} (about 100 mM at 37 °C, $\mu = 0.5$, pH 7.5). We have recently shown that the Ca^{2+} requirement for the conversion can be lowered significantly (to about 50 mM Ca^{2+}) in the presence of chaotropic anions, such as Br^-, I^-, or SCN^- (Curtis et al., 1981).

The other pathway (eq 2) requires both thrombin (or a similar proteolytic enzyme) as well as Ca^{2+} (Lorand & Konishi, 1964; Lorand et al., 1968b), with these two acting in a sequential manner (Janus et al., 1974).

$$a_2b_2 \xrightarrow{\text{thrombin}} a_2'b_2 \xrightarrow{Co^{2+}} a_2^* + b_2 \qquad (2)$$

(activation peptide)

The hydrolytically cleaved $a_2'b_2$ (or factor XIII′) structure is a modified form of the zymogen without catalytic activity. It would appear that the hydrolytic step, just as the addition of chaotropic solutes, reduces the interaction between the heterologous subunits to the point where significantly less Ca^{2+} (6–10 mM Ca^{2+} at pH 7.5, $\mu = 0.15$, 37 °C) can bring about the necessary dissociation of a_2' from b_2 as well as the unmasking of the iodoacetamide-titratable active sites for generating the a_2^* enzyme (i.e., factor XIIIa). However, in terms

of the 1.5 mM free Ca^{2+} in plasma, 6–10 mM Ca^{2+} is still too high. Thus, from the point of view of reconstructing the physiological events, the next important question was to ask whether a system could be defined in which the factor XIII′ to factor XIIIa conversion could proceed within the constraints of normal blood coagulation (i.e., 37 °C, μ = 0.15, pH 7.5, 10–30 min).

In 1978, we succeeded in showing that the fibrinogen molecule exerted a special regulatory effect by reducing the Ca^{2+} requirement to about 1.5 mM to allow the complete activation of factor XIII′ in the physiological time frame (Credo et al., 1978). The present paper is an extension of this work. Using mostly the method of titration with iodo[1-^{14}C]acetamide, we have shown that fibrinogen preparations of very similar clottabilities differ significantly in their properties of promoting the conversion of the factor XIII′ zymogen.[1] This novel function of the fibrinogen molecule thus seems to be unrelated to clotting (i.e., reactivity toward thrombin, release of fibrinopeptides, aggregation of fibrin monomers) as such. With certain fibrinogen preparations (e.g., I-4; see Figure 4), nearly total unmasking of iodoacetamide reactives sites (i.e., 2 equiv per $a_2'b_2$) could be obtained in about 10 min, with 1.5 mM Ca^{2+} and less than 10^{-5} M of fibrinogen (ϕ), which corresponds to its concentration in plasma. Therefore, the physiological event can be summarized as

$$a_2'b_2 \xrightarrow[Ca^{2+}\ (1.5 \times 10^{-3}\ M)]{\phi\ (10^{-5}\ M)} a_2^* + b_2 \qquad (3)$$

Actually, as seen in Figures 5 and 6, the interaction of $a_2'b_2$ with fibrinogen alone promotes the dissociation of subunits and the unmasking of cysteines, and it may be assumed that formation of the complex with fibrinogen is the important element for weakening the heterologous interaction between the a_2' and b_2 subunits. This could be illustrated in the following manner:

$$\phi + a_2'b_2 \rightleftharpoons \phi\cdot a'b_2 \rightleftharpoons \phi\cdot a_2' + b_2 \qquad (4)$$

$$\phi + a_2^*$$

and physiological concentrations of Ca^{2+} (1.5 mM) can apparently shift the overall equilibrium in eq 4 to the right.

Considering that fibrinogen itself is a substrate for the a_2^* enzyme, one could propose that the formation of a Michaelis complex ($\phi\cdot a_2^*$) and an acyl-enzyme intermediate (ϕ–a_2^*) might contribute to the observed phenomena:

$$\phi + a_2^* \rightleftharpoons \phi\cdot a_2^* \rightarrow \phi\text{–}a_2^* \qquad (5)$$

Several lines of evidence, however, would seem to argue against this interpretation. Though adding the steps shown in (5) might help explain the finding that fibrinogen promotes the dissociation of b_2 from $a_2'b_2$, occupying the active center of a_2^* by fibrinogen as in the acyl-enzyme intermediate, if anything, would run counter to the experimental results of enhancing the reactivity of iodoacetamide-titratable sites. The active-center thiols in a_2^* can be set free only either by deacylation with water (eq 6) or by reaction with another fibrinogen molecule (ϕ') as nucleophile (eq 7). Reaction 6 is

$$\phi\text{–}a_2^* + H_2O \rightarrow \phi + a_2^* \qquad (6)$$

$$\phi\text{–}a_2^* + \phi' \rightarrow \phi\text{–}\phi' + a_2^* \qquad (7)$$

thought to be quite slow, and there is no reason to suggest that it would contribute significantly to a turnover of a_2^*. We believe that the same may be said also with regard to reaction 7, particularly since in our experiments the concentration of fibrinogen is only about 5×10^{-6} M.

The complexation of a_2^* with fibrinogen as substrate to give an acyl-enzyme intermediate, as indicated in (5), is unlikely to contribute for other reasons, too. Cold-insoluble globulin (plasma fibronectin) is a known substrate of the a_2^* enzyme (Mosher, 1975), yet, as shown in Figure 4, it is unable to promote the conversion of the $a_2'b_2$ zymogen. Furthermore, after modifying all the a_2^*-reactive glutamines in fibrinogen by incorporation of hydroxylamine (Figure 7), the protein was as good in assisting the conversion of the $a_2'b_2$ zymogen as its native counterpart. Thus, interaction of the active-center region of a_2^* with its specific γ-glutamine targets in the fibrinogen molecule, as such, does not seem to be important. The same conclusion may be drawn from the observation that the plasmin digest of cross-linked fibrin, in which the sensitive γ-glutamines are linked with ϵ-lysines, is just as effective as fibrin or fibrinogen.

The results with plasmin fragment A (see Figure 10) and with CNBr cleavage product HI2DSK (Figure 11) indicate that the critical interaction domain for contact with $a_2'b_2$ corresponds to a stretch of residues in the midsection of the Aα chain of the fibrinogen molecule (i.e., 243–476 for HI2DSK and 242–424 for fragment A). The A or H fragment, which is probably identical with P1 21 (Fretto et al., 1978), contains two glutamine substrate sites for a_2^*. However, in view of what was discussed above, the reaction of these glutamines as such is probably of no regulatory significance in the conversion of the factor XIII′ zymogen.

Future work is needed before a precise stoichiometry for the interactions of fibrinogen, HI2DSK, and A fragments with the thrombin-modified zymogen can be given. Since one fibrinogen molecule contains 2 equiv of either of these fragments and since it is possible that, at the 10^{-6} M concentration used in our experiments, factor XIII′ dissociates into two protomeric units

$$a_2'b_2 \rightleftharpoons 2(a'b) \qquad (8)$$

a formulation such as

$$\phi + 2(a'b) \longrightarrow \phi\begin{matrix}\diagup\ a'b\\ \diagdown\ a'b\end{matrix} \qquad (9)$$

would be more realistic.

Fibrinogen is thought to bind 3 equiv of Ca^{2+} (Marguerie et al., 1977; Marguerie, 1977), and two of the binding sites are in the γ chains of the molecule (Lindsey et al., 1978; Lawrie & Kemp, 1979; Nieuwenhuizen et al., 1979). It would be of interest if the third (actually, since fibrinogen has a symmetrical structure, perhaps even the fourth) would be located in the HI2DSK or, rather, the fragment A or P1 21 regions which we found to be the active domain in assisting in the conversion of Factor XIII′. Binding of Ca^{2+} to these Aα sites in the molecule could produce a more favorable configuration for interaction with the $a_2'b_2$ zymogen:

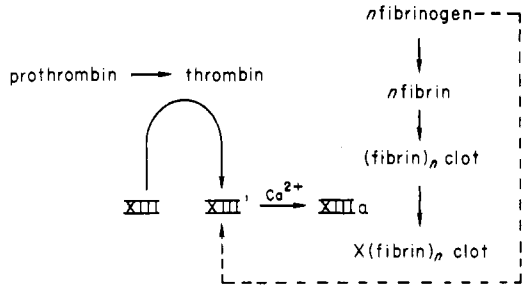
$$\phi + Ca^{2+} \rightleftharpoons \phi\cdot Ca^{2+} \rightleftharpoons \bar{\phi}\cdot Ca^{2+}$$

$$\bar{\phi}\cdot Ca^{2+} + a_2'b_2 \rightleftharpoons \bar{\phi}\cdot Ca^{2+};\ a_2'b_2$$

$$\bar{\phi}\cdot Ca^{2+};\ a_2'b_2 \rightleftharpoons \bar{\phi}\cdot Ca^{2+};\ a_2' + b_2 \rightleftharpoons \bar{\phi}\cdot Ca^{2+} + a_2^* \qquad (10)$$

[1] We do not know the reason for the observed differences. Even if one could show that the preparations with the relatively poor activities had both HI2DSK (or A, H, P121) portions in the Aα chains intact, one could still envisage that these critical regions might assume some abnormal configuration in a protein which suffered extensive (plasmin?) cleavages in the same chain not too far from this region. If these alterations lead to a "masking" of the site, one would clearly have a less reactive fibrinogen species. It cannot be assumed a priori that the various domains of fibrinogen behave independently of each other in every respect when they are still incorporated in a single structure.

Scheme I



The situation might be analogous to that when calmodulin, in the presence of Ca^{2+}, interacts with one of its protein targets [see Cheung (1980)]. From a kinetic point of view, the similarity is so striking that we actually tested calmodulin to examine whether it could enhance the conversion of factor XIII', but the results were negative, nor could we detect obvious sequence similarities between the primary structures of calmodulin and those of the HI2DSK and A or P1 21 fragments from fibrinogen.

Whatever the ultimate interpretation of the molecular mechanism for the described, rather unique regulation by fibrinogen may be, there can be little doubt that we are dealing here with a control of great biological significance in blood clotting. Factor XIIIa is the last enzyme generated on the coagulation cascade, and it would seem to be logical that its appearance be synchronized with clot formation (Scheme I). As illustrated by the broken line in Scheme I, interaction of fibrinogen with the thrombin-modified form of the XIII' zymogen species would ensure that the transamidase (i.e., XIIIa) is available as needed to act on the fibrin network to produce a cross-linked (X) clot. It is quite possible that the relevant structural domain in the fibrinogen molecule (as represented by human fragments HI2DSK, A, H, or P1 21) may contain amino acid sequences which are highly conserved throughout the vertebrate phylum, where fibrin stabilization by factor XIIIa is a universal feature in clotting.

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Susceptibility of Type V Collagen to Neutral Proteases: Evidence That the Major Molecular Species Is a Thrombin-Sensitive Heteropolymer, $[\alpha 1(V)]_2\alpha 2(V)$†

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ABSTRACT: The susceptibility of human type V collagen to several neutral proteases was examined. Thrombin cleaved both the $\alpha 1(V)$ and $\alpha 2(V)$ chains of this protein at 34 °C, producing two pairs of fragments with apparent molecular weights of 95 000 and 10 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Two-dimensional ^{125}I-labeled peptide mapping of the larger fragments demonstrated that the upper band [which comigrated with $\alpha 1(I)$] was derived from both the $\alpha 1(V)$ and $\alpha 2(V)$ chains, while the other component [which comigrated with $\alpha 2(I)$] was a product of $\alpha 1(V)$ alone. Cleavage of type V collagen, containing $\alpha 3(V)$ chains, with thrombin produced an analogous pattern with three high molecular weight bands. Chymotrypsin and trypsin cleaved type V collagen at 37 °C but not at lower temperatures. Digestion of type V collagen with elastase at 37 °C resulted in selective proteolysis of $\alpha 2(V)$, leaving $\alpha 1(V)$ essentially intact. Pepsin treatment of type V collagen from which $\alpha 2(V)$ had been removed by elastase treatment resulted in nearly complete degradation of $\alpha 1(V)$. These data support the hypothesis that a major fraction of native type V collagen is a heteropolymer with the chain composition $[\alpha 1(V)]_2\alpha 2(V)$. Cleavage of type V collagen by thrombin may have physiologic significance in that breakdown of pericellular matrix may be an important step in the response of a tissue to injury.

Collagens associated with basement membranes or with cell surfaces (types IV and V)[1] are not substrates for human skin collagenase under conditions which result in cleavage of interstitial collagens (types I, II, and III) (Woolley et al., 1978; Crouch & Bornstein, 1979; Sage et al., 1979; Sage & Bornstein, 1979; Liotta et al., 1979). These observations have prompted several investigations of the reactivities of other neutral proteases toward types IV and V collagens and consideration of the possible significance of such processes in inflammation, wound repair, and metastatic invasion. The concept that specific collagenases exist for certain collagen types has been reinforced by the finding of a granulocyte collagenase which exhibited preferential activity toward type I as compared to type III collagen (Horwitz et al., 1977) and by the isolation of a collagenase from a metastatic murine tumor which cleaved only type IV collagen (Liotta et al., 1979).

The collagen triple helix is unusually resistant to proteolytic attack. However, recent reports from several laboratories have shown that enzymes other than "classical" collagenases are capable of degrading native, triple-helical collagen molecules. Trypsin and neutrophil elastase cleaved type III collagen at or near the collagenase-sensitive site (Miller et al., 1976; Gadek et al., 1980). In addition, a neutral protease extracted from human leukocytes degraded native type IV collagen into several products of M_r <70 000 (Uitto et al., 1980), and an elastase purified from polymorphonuclear leukocyte granules digested type IV collagen at 37 °C into several fragments (Mainardi et al., 1980). Native type IV procollagen and collagen, but not types I, III, and V, were degraded by a neutral serine protease present in the secretory granules of rat mast cells (Sage et al., 1979; Crouch et al., 1980; Woodbury & Neurath, 1980). It should be noted, however, that the helix in type IV collagen is interrupted in several places by sequences that cannot form the triple helix (Shuppan et al., 1980) and that proteolysis may occur preferentially in these locations.

The collagen protein family is comprised of at least nine structurally distinct genetic chain types, three of which are found in type V collagen [for a review, see Bornstein & Sage (1980)]. This collagen type has been purified from a number of tissues which are enriched in basement membranes by solubilization with pepsin (Burgeson et al., 1976; Chung et al., 1976) and from cells in culture with which it appears to be preferentially associated (Sage et al., 1981b; Pöschl & von der Mark, 1980). However, although immunoferritin studies at the ultrastructural level have shown that types IV and V collagens are codistributed in kidney basement membranes (Roll et al., 1980), a distinction has been made between the localization of type IV collagen, which exists as an integral structural component of morphologically defined basement membranes, and that of type V collagen, which may be a ubiquitous pericellular collagen found in the extracellular matrix or exocytoskeleton (Gay et al., 1980).

Type V collagen consists of two or three α chains, depending upon the tissue of origin; the molecular organization of the

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[1] Collagen containing B and A chains and previously described as type AB has been referred to as type V collagen, and the chains as $\alpha 1(V)$ and $\alpha 2(V)$, respectively (Bornstein & Sage, 1980). Type V collagen from some tissues contains an additional chain, $\alpha 3(V)$, which was previously referred to as the C chain (Sage & Bornstein, 1979).

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