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Ca^{2+}-related regulatory function of fibrinogen

(regulation of Factor XIII activation/Ca^{2+} requirement of Factor XIII activation/subunit dissociation of Factor XIII/reactions of Factor XIII with iodoacetamide/thrombin-independent activation of Factor XIII)

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ABSTRACT **Fibrinogen displays a regulation of considerable physiological significance by lowering the Ca^{2+} requirement for the conversion of the fibrin-stabilizing factor (Factor XIII) zymogen to the range of concentrations of this ion found in plasma. Fibrinogen modulates both Ca^{2+}-dependent steps in the complex process of zymogen activation, involving the heterologous dissociation of subunits of the thrombin-modified zymogen (Factor XIII′) species ($a'_2 \xrightarrow[b_2]{} a'_2$) and the unmasking of iodoacetamide titratable sites during generation of transamidating activity ($a'_2 \rightarrow a^*_2$). It is interesting that a thrombin-independent pathway of zymogen activation ($a_2b_2 \xrightarrow[b_2]{} a_2 \rightarrow a^0_2$), which we found to operate at Ca^{2+} concentrations above 50 mM, is not affected by the presence of fibrinogen. Regulation by fibrinogen thus appears to be specific for controlling only the physiological pathway of zymogen conversion.**

Fibrinogen has always been characterized as the plasma protein that serves as the precursor for the fibrin network formed during the clotting of blood. Accordingly, only the reaction of fibrinogen with thrombin (1–3) and that of fibrin with fibrinoligase (coagulation Factor $XIII_a$; refs. 4–6) were thought to be of physiological importance. Our recent work reveals that the fibrinogen molecule displays a hitherto unrecognized regulatory property of considerable physiological significance. It will be shown in the present paper that fibrinogen greatly enhances the generation of fibrinoligase activity and lowers the Ca^{2+} requirement for this process. As such, modulation by fibrinogen is somewhat reminiscent of the biochemical functions of troponin C in muscle (7) and of the various Ca^{2+}-dependent regulatory proteins in other tissues (see refs. 7–11).

Fibrinoligase, a transamidase that is responsible for the covalent fusion of fibrin molecules by γ-glutamyl-ε-lysine bridges (5), is the only enzyme of the coagulation cascade (12) that contains a cysteine, rather than a serine, active center residue (13–15). Generation of the enzyme from the fibrin-stabilizing factor zymogen (Factor XIII) requires both thrombin and Ca^{2+} ions (16) and is accomplished in two distinct stages. First, thrombin removes an NH_2-terminal peptide fragment from the *a* subunits of the a_2b_2 zymogen ensemble (17–19). This hydrolytic modification, however, still leaves the subunits heterologously associated as a'_2b_2 (Factor XIII′) and is, by itself, not sufficient for generating transamidase activity. Expression of the latter depends specifically on Ca^{2+} ions, which cause the release of the noncatalytic *b* subunits (14, 20–22) as well as the conversion of a'_2 to a^*_2. The a^*_2 subunits, which represent the functionally competent enzyme, are readily titrated by iodoacetamide (14). As demonstrated in this paper, fibrinogen plays an important regulatory role in the Ca^{2+}-dependent events of subunit dissociation and of unmasking of the active center on the catalytic subunit:

$$a'_2b_2 \xrightarrow{Ca^{2+}} a'_2 + b_2 \qquad a'_2 \xrightarrow{Ca^{2+}} a^*_2$$

MATERIALS AND METHODS

Human fibrinogen (Factor I) was a gift from Kabi, Stockholm, Sweden (batch 1024, lot 32120). The lyophilized powder was dissolved in 50 mM Tris·HCl/0.1 M NaCl/0.5 mM EDTA at pH 7.5 and was dialyzed exhaustively against the same. Protein concentrations were computed from the corrected absorbance at 280 nm, using a value of $E^{1\%}_{1cm} = 15.06$ (23).

The fibrin-stabilizing factor zymogen (Factor XIII) and the pure *b* subunits were isolated from outdated human plasma by published procedures (24). These purified materials were stored at 4° in 50 mM Tris·HCl, pH 7.5/1 mM EDTA. Concentrations of the zymogen and the *b* protein were calculated on the basis of $E^{1\%}_{1cm} = 13.8$ at 280 nm (25).

Human α-thrombin with a specific clotting activity of 2162 National Institutes of Health units per mg of protein was a gift of J. W. Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY.

The functional molarity of Factor XIII was measured by titration with iodoacetamide as described by Curtis *et al.* (14). The molarity of thrombin was determined from pre-steady-state kinetics for the hydrolysis of *p*-nitrophenylguanidobenzoate (26).

Factor XIII zymogen was hydrolytically converted from the a_2b_2 native state to the a'_2b_2 one (Factor XIII′) at 25°, pH 7.5, in solutions of 0.15 ml containing 10 μM zymogen and 0.17 μM thrombin, as well as 50 mM Tris·HCl and 0.5 mM EDTA. After a reaction time of 30 min, sufficient amount of hirudin (Sigma, lot 27B-2330) was added to provide a 2-fold excess of hirudin over the clotting units of thrombin, in terms of the thrombin-neutralizing potency of the hirudin preparation as specified by the supplier. Total conversion of the *a* subunits to *a′* was verified by sodium dodecyl sulfate disc gel polyacrylamide electrophoresis (17). For experiments with a_2b_2 native zymogen rather than the hydrolytically modified a'_2b_2 species, thrombin was omitted, but hirudin was present.

The zymogens (both Factor XIII and XIII′) were alkylated with iodo[*1*-^{14}C]acetamide (58 Ci/mol; Amersham-Searle) at 37°, by the filter paper technique described by Curtis *et al.* (14). Reactions were performed in 0.1-ml solutions containing 0.96 μM zymogens, varying concentrations (0–12 μM) of fibrinogen, 21.5 μM iodoacetamide, 1.5 mM $CaCl_2$, 50 mM Tris·HCl (pH 7.5), and, unless otherwise indicated, NaCl to adjust the ionic

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strength to $\mu = 0.15$. Control mixtures contained all other components, but either none of the Factor XIII′ zymogen or no Ca^{2+}. Aliquots of 10 μl were withdrawn at 30 min and spotted on filter paper discs. After the discs were washed in trichloroacetic acid (10%, twice for 30 min and 5%, three times for 15 min) to remove free isotope, they were rinsed with ethanol/acetone (1:1 vol/vol, 10 min) and in acetone (10 min). The dried discs were placed in vials containing 10 ml of scintillation fluid (0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis[1-(5-phenyloxazoyl)]benzene) and the protein-bound isotope was measured in a Packard Liquid Scintillation Spectrometer, model 3375.

Release of the *b* subunits from the thrombin-modified a'_2b_2 zymogen was analyzed by a procedure reported by Lorand *et al.* (20) and Curtis *et al.* (14), by polyacrylamide disc gel electrophoresis under nondenaturing conditions at pH 7.8 according to Rodbard and Chrambach (27). Samples of 50 μl, corresponding to 16 μg of the zymogen, were placed onto the gels and electrophoresis was performed at 4° and 1 mA per gel until the bromphenol blue tracking dye migrated to within 0.5 cm of the end of the tubes. The gels were stained for 2 hr with 0.5% Coomassie blue dissolved in 10% 2-propanol/10% acetic acid, and were destained with the same solvent mixture. Densitometric scans were obtained at 553 nm with a Beckman DU single beam spectrophotometer, equipped with a Gilford optical density converter and linear transport system. Areas on the protein scans corresponding to the position of *b* subunits were measured in relation to a control gel loaded with 9 μg of the pure *b* protein, which is equivalent to the content of *b* subunits in 16 μg of the a'_2b_2 zymogen electrophoresed on the other gels. Tracings of protein peaks were cut out and weighed.

RESULTS AND DISCUSSION

The present study is concerned primarily with regulatory aspects of the heterologous dissociation of subunits ($a'_2b_2 \rightarrow a'_2 + b_2$) and the unmasking of active centers ($a'_2 \rightarrow a^*_2$ transition) during the Ca^{2+}-dependent conversion of the thrombin-modified fibrin-stabilizing factor zymogen (Factor XIII′) derived from human blood plasma. Release of the *b* subunits from the a'_2b_2 ensemble was monitored by polyacrylamide disc gel electrophoresis under nondenaturing conditions, and the unmasking reaction was observed by titration with iodo[*1*-^{14}C]-acetamide. In the electrophoretic system used, *b* subunits migrated farthest towards the anode (Fig. 1, gel 1) and the a'_2b_2 zymogen moved somewhat less than half as fast (gel 2), but still ahead of the broad fibrinogen band (gel 8). The experiments were carried out at 37° with 1.5 mM Ca^{2+}, so as to approximate events as they are thought to occur in blood plasma. When fibrinogen was omitted, under these conditions there was only a minimal release of *b* subunits (see gel 3) which amounted to less than a 10% dissociation of a'_2b_2. However, it is evident from gels 4, 5, 6, and 7 that when fibrinogen was added at concentrations of 3, 6, 9, and 12 μM, respectively, dissociation was greatly enhanced. This was seen not only by the progressive release of *b* subunits with increasing concentrations of fibrinogen, quantitatively illustrated in Fig. 2, but also by the loss of protein material found at the position of the a'_2b_2 zymogen just below the heavily stained fibrinogen band.

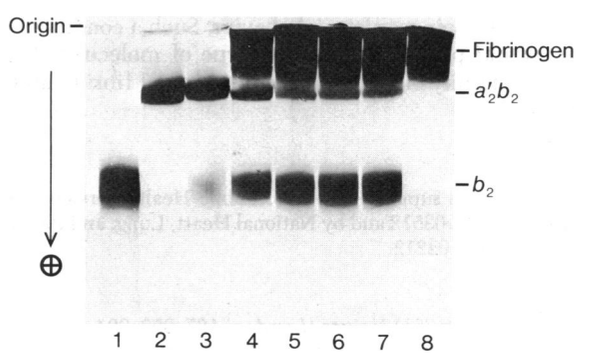

FIG. 1. Fibrinogen-assisted dissociation of the thrombin-modified fibrin-stabilizing factor (Factor XIII′) zymogen (2 μM) during 30 min of incubation at pH 7.5, $\mu = 0.15$, in the presence of 1.5 mM Ca^{2+} ions. Polyacrylamide gel electrophoresis was performed with samples of 50 μl under nondenaturing conditions. Gel 1, pure *b* subunits (2 μM) incubated with Ca^{2+}; gel 2, Factor XIII′ without Ca^{2+}; gel 3, Factor XIII′ with Ca^{2+}; gels 4–7, Factor XIII′ incubated with Ca^{2+} and 3, 6, 9, and 12 μM Kabi fibrinogen, respectively; gel 8, fibrinogen (12 μM) with Ca^{2+}.

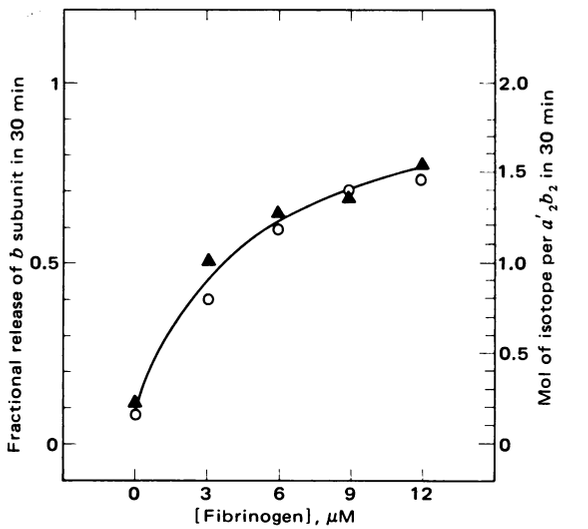

FIG. 2. Comparison between the effects of fibrinogen on the release of *b* subunits from the a'_2b_2 zymogen (Factor XIII′) and on the unmasking of iodoacetamide-titratable sites. Data were obtained from the experiments corresponding to those shown in Fig. 1. Left ordinate (○): fractional release of *b* subunits, determined from the protein patterns of gels 3–7 of Fig. 1, in relation to the standard in gel 1 of Fig. 1. Right ordinate (▲): reaction with iodo[*1*-^{14}C]acetamide.

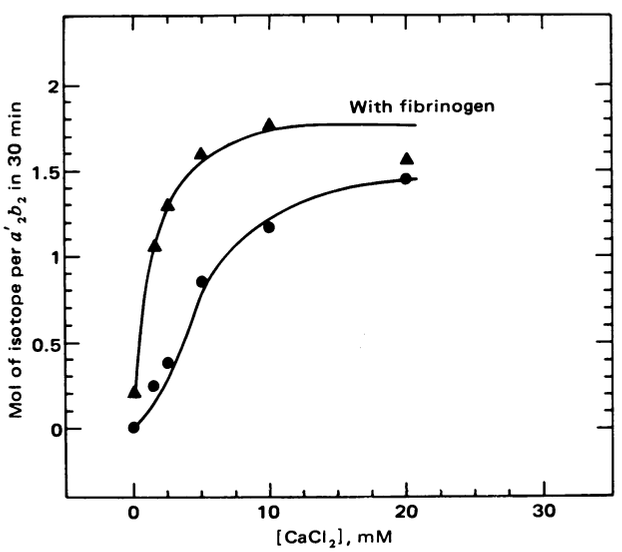

FIG. 3. Ca^{2+} ion requirement for the unmasking of iodoacetamide-titratable sites with 1 μM a'_2b_2 zymogen (Factor XIII′) by itself (●) or in conjunction with 9 μM Kabi fibrinogen (▲). For details of the alkylation reaction with iodo[*1*-^{14}C]acetamide (ordinate) see text.

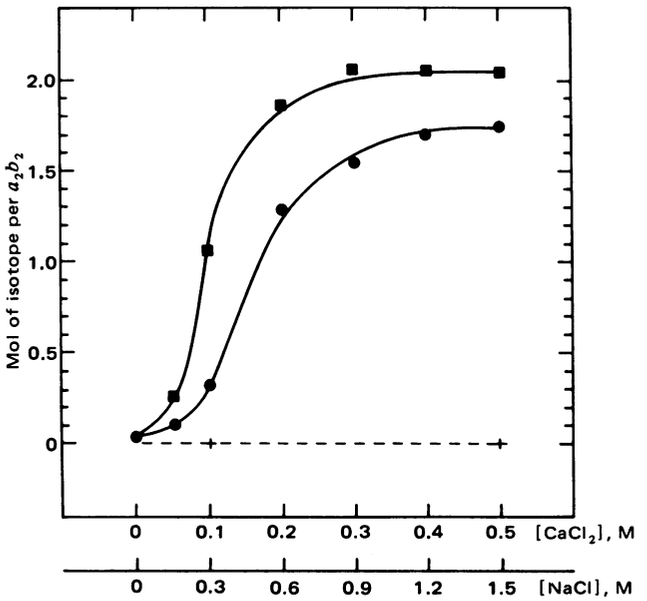

FIG. 4. A Ca^{2+}-specific, but totally thrombin-independent, alternate pathway for unmasking the iodoacetamide-titratable sites directly from the native a_2b_2 Factor XIII zymogen (2 μM). For details of the alkylation reaction with iodo[1-^{14}C]acetamide (ordinate), see text. Addition of Kabi fibrinogen (12 μM) did not alter the titration values shown for either the 10-min or the 60-min period. $CaCl_2$: ●, 10 min; ■, 60 min. Broken line, NaCl for 60 min.

The data in Fig. 2 show that fibrinogen also effectively promoted the unmasking of iodoacetamide-reactive sites. It is important to mention that no protein alkylation whatever was obtained, even with 12 μM fibrinogen, when the reaction mixture did not also contain the thrombin-modified zymogen. Thus, it may be safely concluded that titration with iodoacetamide is restricted to the zymogen itself. In regard to quantitative aspects, the extent of unmasking of iodoacetamide-titratable sites paralleled the fractional release of *b* subunits remarkably well. At a concentration corresponding to that of fibrinogen normally found in human plasma (about 3 mg/ml or 9 μM), the Kabi product caused an approximate 70% dissociation of the subunits and a similar percentage of unmasking of the sites theoretically available for titration with iodoacetamide (i.e., 1.4 equivalents of a total of 2 per mole of a'_2b_2). These figures should be compared with the values obtained for the dissociation and titration of the zymogen (about 10%) without any fibrinogen present. Hence, within the 30-min experimental period, the addition of a physiologically equivalent concentration of Kabi fibrinogen produced a 7-fold enhancement in the activation of pure a'_2b_2 zymogen at 1.5 mM Ca^{2+}.

Fig. 3 provides yet another method for illustrating the regulatory function of fibrinogen. In the experiment presented, the reaction (30 min) between the a'_2b_2 zymogen and radioactive iodoacetamide was examined at various concentrations (0–20 mM) of Ca^{2+}, both in the absence and presence of 9 μM Kabi fibrinogen. The addition of fibrinogen had a very marked effect on the Ca^{2+} ion requirement of the alkylation reaction, reducing it to the range of physiological interest (i.e., 50% titratability at less than 1.5 mM Ca^{2+}). It has been shown by Cooke *et al.* (28) that very high molar concentrations of acetylated and dephosphorylated β-casein (some 30–60 times greater than the fibrinogen used in Fig. 3) could also lower the concentration of Ca^{2+} required for the reaction of a'_2b_2 with 2-chloromercuri-4-nitrophenol or 5,5′-dithiobis(2-nitrobenzoate). Since casein is not a constituent of blood plasma, obviously the effect observed with this protein cannot be regarded as specific.

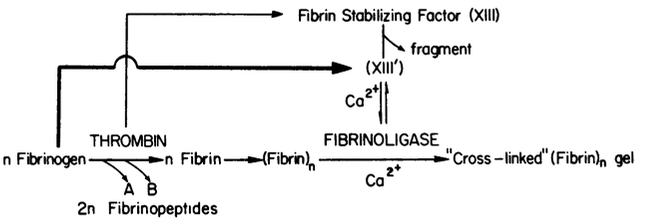

FIG. 5. Outline of physiological controls in the clotting of fibrinogen. The novel aspect of regulation by fibrinogen, operating specifically in regard to the activation of the thrombin-modified fibrin-stabilizing factor zymogen (Factor XIII'), is emphasized by a heavy line.

Modulation by fibrinogen is all the more interesting because the effect of this protein is uniquely restricted to the process of activating the thrombin-modified zymogen (Factor XIII'; a'_2b_2) formed on the physiological pathway. We have recently discovered a direct pathway for activating the native a_2b_2 zymogen that is completely independent of thrombin and that operates at rather high ($\geq$ 50 mM) concentrations of Ca^{2+}. This alternate route also leads to the heterologous dissociation of subunits, unmasking of iodoacetamide-reactive sites (see Fig. 4), and generation of enzyme activity:

$$a_2b_2 \underset{b_2}{\rightleftharpoons} a_2 \rightarrow a^0{}_2$$

but, since the new catalytically active species still carries the NH_2-terminal peptides of the original *a* subunits, it should be distinguished from the $a^*{}_2$ enzyme of the physiological pathway and denoted as $a^0{}_2$. Within the framework of the present discussion concerning modulation, the important point to emphasize is that fibrinogen has no demonstrable effect whatsoever on this thrombin-independent alternate pathway of zymogen conversion. Though not shown, the addition of 12 μM Kabi fibrinogen did not change the Ca^{2+} requirement for the unmasking of titratable sites in the experiment presented in Fig. 4. The hydrolytic conversion of a_2b_2 to a'_2b_2 by thrombin is thus clearly essential for sensitizing the zymogen for regulation by fibrinogen.

In view of these findings, the outline of controls (29) operating in the clotting of fibrinogen should be modified to take into account the specific regulation by fibrinogen. This novel aspect of control is accentuated in Fig. 5 by a heavy line. It is possible that the techniques described in this paper could be used for detecting abnormal fibrinogens that are defective primarily in their regulatory behavior. Such a condition, if it exists, would present yet another type of molecular disease within the family of hemorrhagic disorders of fibrin stabilization (30).

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