Dissociation of the Subunit Structure of Fibrin Stabilizing Factor During Activation of the Zymogen

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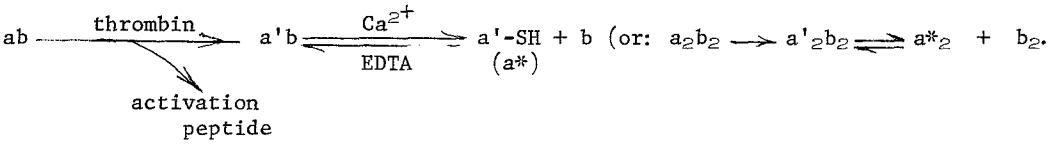
Summary: Calcium ions play an essential role in the zymogenic conversion of the fibrin stabilizing factor (FSF, Factor XIII of plasma) in that they cause a dissociation of the two types of subunits (a' from b) of the thrombin-activated factor (FSF'). Disc gel electrophoresis carried out under non-denaturating conditions and utilizing a newly developed fluorescent activity staining procedure is eminently suited for demonstrating changes in the quaternary structure of this protein. Also, kinetic evidence is presented to show that the b type of subunit, though enzymatically inactive, plays a role in regulating the rate of the calcium dependent activation of the zymogen.

Fibrin stabilizing factor (FSF or Factor XIII) is the zymogenic plasma precursor of the transamidating enzyme, fibrinoligase, which is responsible for the covalent crosslinking of fibrin molecules during clotting. Activation of the zymogen is thus an integral part of the process of normal blood coagulation (1). As isolated from plasma, the zymogenic factor comprises two different types of subunits [a and b, 75,000 and 88,000 daltons, respectively (2)] associating into an oligomeric assembly which has been measured to yield molecular weights both in the 160,000 (3) and 320,000 (2) range, probably with an equilibrium between the two forms: $a_2b_2 \rightleftharpoons 2\ ab$ (4).

Conversion of the zymogen to the enzymatically active transamidase is regulated both by thrombin and by calcium ions (5) and it was shown to proceed in two distinct steps: limited proteolysis by thrombin, followed by a structural change in the protein induced by calcium ions (6). Treatment by thrombin appears to affect only the a subunit of the zymogen (7), cleaving off a peptide fragment (Mw. ca 4,000) from the N-terminal region of the subunit (8), yielding a'. While this limited proteolysis is essential, it is by no means sufficient to bring about total activation of the zymogen. The process is completed by the specific

action of calcium ions (6) causing an unmasking of a cysteine residue in the a' subunit, essential for transamidase activity.

We have now shown that the calcium dependent unmasking of the active center thiol in the a' subunit is coupled to its release from the b subunit. Just as the accessibility of the active center thiol groups to titration with iodoacetamide can be readily reversed by the addition of EDTA (9), a reassociation of the quaternary structure occurs on removal of calcium ions. Hence the complete sequence of zymogen activation may be given as follows:



Dissociation of the zymogen during activation was already suggested by an earlier observation based on disc-gel electrophoresis under non-denaturing conditions (1). In the present work we took advantage of this method which by a transamidase specific staining procedure permitted the unambiguous localization of the active center containing subunits derived from a.

Kinetic evidence will also be presented to show that the b subunit plays a regulatory role in the calcium-dependent activation of the zymogen.

MATERIALS AND METHODS

The plasma zymogen, fibrin stabilizing factor or Factor XIII, was prepared from outdated blood bank citrated human plasma by the DEAE-cellulose chromatographic procedure of Lorand and Gotoh (10) with 1 mM EDTA included in all solvents and using a Sepharose 6 B column (2.5 x 100 cm) with 0.05 M Tris-chloride buffer of pH 7.5 containing 0.1 M sodium chloride as eluant for the final step of purification. A small amount of free b chain which co-chromatographed with the plasma Factor XIII on DEAE-cellulose, was isolated in pure form as a separate and later emerging peak during this step (11). Both Factor XIII and the b chain preparation were stored at 4^0 in 0.05 M Tris-chloride buffer of pH 7.5

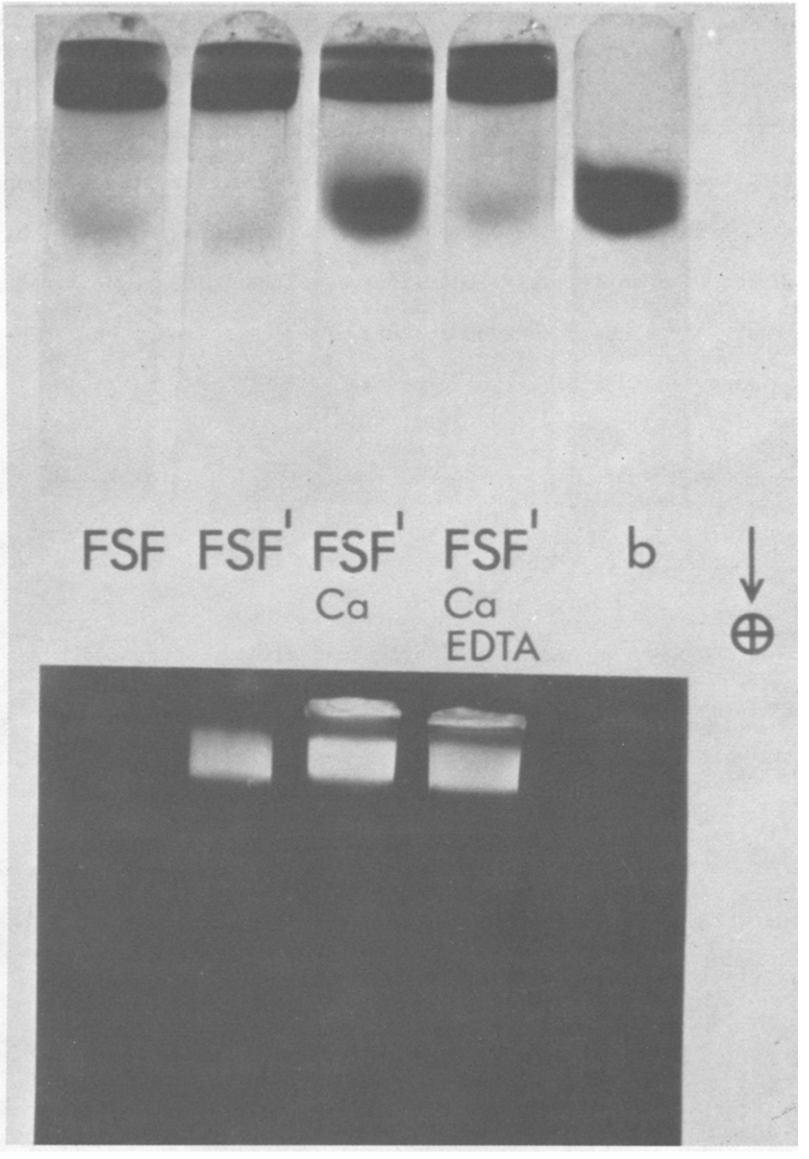


Fig. 1. Disc gel electrophoresis, pH 7.8, of fibrin stabilizing factor (FSF) at various stages of activation. Top: Coomassie blue protein stain. Bottom: Transamidase specific fluorescent activity stain, comprising dansylcadaverine, dimethylcasein and calcium chloride. Approximately 30 μg protein was applied to each gel. The thrombin activated zymogen is denoted as FSF'; b stands for this subunit isolated from the zymogen. For experimental details, see text.

containing 1 mM EDTA. Thrombin was purified from bovine thrombin using a procedure described by Chou (12). Three vials of Parke Davis Thrombin Topical were applied to a cellulose phosphate column (12 x 2.4 cm), equilibrated with 0.05 M potassium

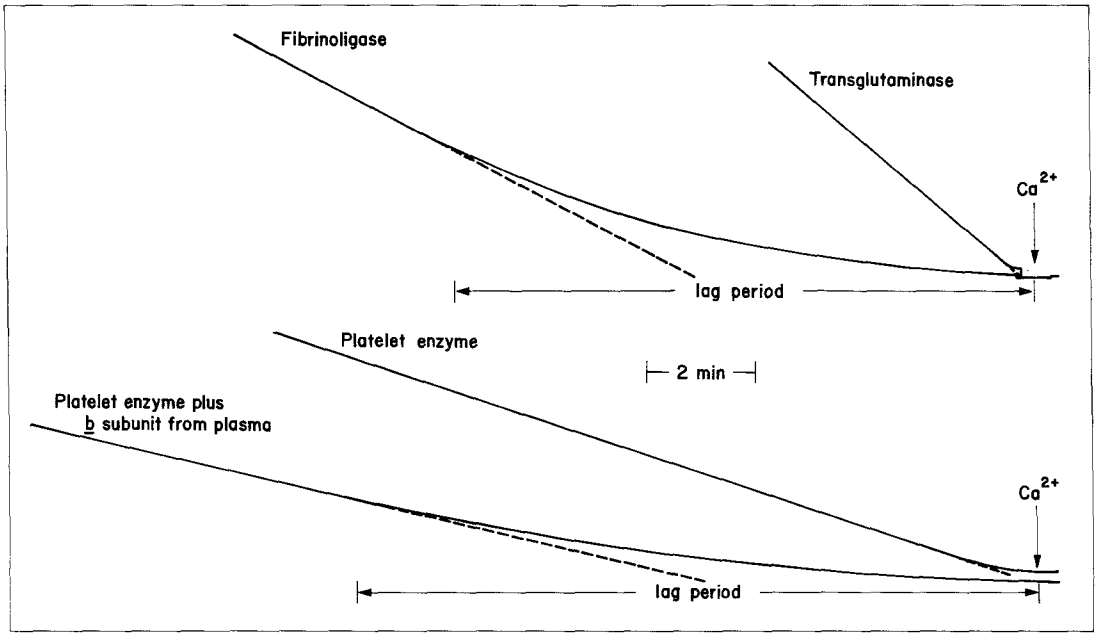


Fig. 2. Progression curves for the formation of the amide product in enzyme-catalyzed reactions between β-phenylpropionylthiocholine iodide and dansyl-cadaverine. For experimental details, see text. Approximately 0.11 mg/ml of platelet Factor XIII and 0.08 mg/ml of b were used.

phosphate, pH 7.0 and eluted with the same. A strong yellow band moved through the column and was removed with 80 ml of the eluant. Next, 250 ml of 0.1 M phosphate buffer were passed through. Thrombin was eluted when, in a third step, a mixture of 0.25 M potassium phosphate and 0.5 M sodium chloride of pH 6.75 were applied. The enzyme was dialyzed against 0.1 M potassium chloride and stored at 4^0.

The limited proteolysis of plasma Factor XIII (approximately one mg per ml) was carried out at 24^0 by the addition of thrombin (3.5 NIH units per ml) for a sufficient time to ensure complete conversion of a subunits to a'. For the experiments described in Fig. 1, this period was 20 min., at which time the action of thrombin was quenched by adding a five-fold excess (17.5 units per ml) of hirudin (Sigma Chemical Co). Tris-acetate (0.05 M) of pH 7.5 was used throughout as buffer. Concentration of the potential enzymatically active centers was measured by titration with [^{14}C]-iodoacetamide in the presence of 0.1 M calcium chloride as described previously (6).

Transglutaminase was prepared from fresh guinea pig livers (13); its functional normality was determined by measuring kinetic burst with trimethylacetyl p-nitrophenyl ester (14). Platelet Factor XIII was isolated by the procedure of Schwartz et al. (7); its activation by thrombin was carried out similarly to that given for the plasma factor.

Protein concentrations were computed by assuming $A^{1\%}_{1\ cm} = 13.8$ at 280 nm (2).

For studying the subunit dissociation of plasma Factor XIII during activation (Fig. 1), the gel electrophoretic procedure of Rodbard and Chrambach (15) was employed with 7% acrylamide and a buffer system of pH 7.8 containing TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), Bis-tris (bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane and cacodylic acid at an ionic strength of 0.01. Electrophoresis at 4° was performed at 1 mA per gel and was terminated when the bromophenol blue tracking dye moved to within a distance of one cm from the bottom of the gels. Parallel samples of gels were stained for protein and carbohydrate contents and were also developed with a transamidase specific activity stain. Coomassie brilliant blue (0.25%) in 50% methanol-10% acetic acid-40% water was used as protein stain and the procedure of Zacharius et al. (16) was employed for carbohydrates. In order to locate enzymatic activity (17) the gels were immersed immediately after electrophoresis in a solution containing 1% N,N-dimethylcasein, 2 mM dansylcadaverine (18), 5 mM calcium chloride and 5 mM dithiothreitol in 0.05 M Tris-acetate of pH 7.5. Following 6 hours of incubation at 24°, the gels were fixed in 10% trichloroacetic acid to remove free dansylcadaverine. Removal of background fluorescence as well as visualization of casein-bound amine was checked by inspection of the gels under UV light (Blak Ray UVL22). - N,N-Dimethylcasein was prepared from Hammersten casein (Schwarz-Mann) by reductive alkylation according to Lin et al. (19).

Kinetics of the reactions catalyzed by the various transamidases (Fig. 2) were measured at 25° in a two-phase system described previously (6). For liver transglutaminase (0.4 μM) and for fibrinoligase (0.5 μM) the 0.1 ml aqueous layer

in which the enzymatic reactions took place, comprised 1.5 mM β-phenylpropionylthiocholine iodide, 1.3 mM dansylcadaverine in 0.05 M Tris-acetate buffer of pH 7.5 and, finally, 10 mM calcium chloride. For the thrombin-activated platelet Factor XIII (0.11 mg/ml) the aqueous phase contained 1.4 mM dansylcadaverine, 0.05 M Tris-acetate of pH 7.5, 6.3 mM β-phenylpropionylthiocholine iodide and 4.2 mM calcium chloride. In all instances, formation of the fluorescent amide coupling product was measured by continuous extraction into 2.0 ml n-heptane as an upper phase in the cuvette and was monitored in the Aminco Bowman Ratio Spectrophotofluorimeter (λ_{exc} = 340 nm; λ_{em} = 460 nm).

RESULTS

Electrophoretic changes in thrombin-activated plasma Factor XIII after exposure to calcium ions. Fig. 1 illustrates the disc gel electrophoretic behavior of fibrin stabilizing factor at various stages of activation. Reading from left to right, gel No. 1 shows the migration of the zymogen (FSF) as isolated from plasma in pure form (10). Gel No. 2 presents the thrombin-activated species (FSF') after all of its a subunits have undergone limited proteolysis to a' [as judged by the procedure given in ref. (7)]. The center gel (No. 3) is the pattern obtained after exposure of FSF' at 25° to 10 mM calcium chloride at μ = 0.15 for 30 min. prior to electrophoresis. This material corresponds (6) to the functionally competent transamidating enzyme, fibrinoligase (FSF*; Factor $XIII_a$). Gel No. 4 relates to this same enzyme but with added incubation with 20 mM EDTA for 15 min. before electrophoresis. Migration of the pure b-chain is shown on the right (gel No. 5) for comparison.

The pictures on top of Fig. 1 were obtained by staining with Coomassie blue. Though not shown, distribution of the carbohydrate specific dye gave qualitatively a quite similar pattern.

The bottom part of Fig. 1 was obtained with the activity staining procedure (17) specific for transamidases. Inasmuch as the staining mixture con-

tained 5 mM calcium chloride, in addition to dansylcadaverine and dimethyl-casein, enzyme activity bands could be demonstrated not only in gel No. 3 but also in gels 2 and 4. In fact, with inclusion of thrombin in the stain, even the native zymogen (gel No. 1) would have generated a fluorescent band in a location corresponding to the dark protein band seen in this gel in the top part of the figure. Neither the pure b subunit preparation (gel No. 5) nor the corresponding fast moving protein band in gel No. 3 ever gave a positive activity stain. Since a is the parent subunit in the zymogen which generates the active center for the transamidase (6), the fluorescent bands, resulting from the covalent incorporation of dansylcadaverine into dimethyl-casein, serve in effect to locate the a-derived subunits on the gels. Once the thrombin-activated zymogen was exposed to calcium (gels 3 and 4), activity stain was also present in a band barely penetrating the gel probably due to formation of aggregates of the a type subunits. However, there is no evidence to support the claims of Takagi and Doolittle (20) as to the origin of these aggregates.

The experiments presented in Fig. 1 clearly show that in the thrombin-activated zymogen (FSF') the a' and b subunits must still be in association with each other, yielding a single electrophoretic entity. Following calcium treatment, however, a reversible dissociation of the quaternary structure of the protein appears to occur. In our preliminary work (1), we referred to the faster moving b band released after addition of calcium as "labile fragment", and we have no explanation as to why at that time we could not demonstrate carbohydrate staining into this band.

The calcium dependent dissociation of the thrombin-activated zymogen must be rather closely coupled to the unmasking of the active center cysteine residue in the a' subunit (6), because only in the preparation corresponding to that shown in gel No. 3 could we demonstrate reaction with [^{14}C]-iodoacetamide. Uptake of the isotope into this species indicated about 40% unmasking of the thiol. None of the other samples could be alkylated.

Role of the b subunit. Inasmuch as it was shown (see gel No. 3 in Fig. 1) that the b subunit is not needed for the enzymatic activity of fibrinoligase, the role played by this subunit had to be examined. Progression curves of transamidation between β-phenylpropionylthiocholine iodide and dansylcadaverine as measured in Fig. 2, display a characteristic lag period prior to a steady state formation of the fluorescent coupling product. Neither transglutaminase from liver, nor thrombin-activated platelet Factor XIII give such an induction period and both of these enzymes contain only a single type of subunit which functionally seems to correspond to that derived from a in fibrin stabilizing factor (2,21). Addition of pure b subunit to transglutaminase caused no significant change in the kinetic behavior of this enzyme but, as seen in Fig. 2, altered that of the apparently more closely related platelet factor. In the latter instance, the presence of the b subunit induced an appreciable lag period and also caused a decrease in the steady-state velocity of the reaction. These effects were observed only if b subunit was added to the platelet enzyme prior to calcium ions. The experiments suggest that platelet enzyme and b subunit can interact in a manner similar to that described for the a' and b subunits of thrombin-activated plasma factor (FSF'). Indeed, hybridization experiments using electrophoretic techniques as employed in Fig. 1, proved this conclusion.

The results shown in Fig. 2 indicate that the b subunit regulates the rate of the zymogenic conversion of fibrin stabilizing factor (Factor XIII) to fibrinoligase (Factor $XIII_a$). No doubt, the biochemical concept of dissociation of heterologous subunits as a means of zymogen activation is of general interest.

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