Contact with the N Termini in the Central E Domain Enhances the Reactivities of the Distal D Domains of Fibrin to Factor XIIIα*

(Received for publication, May 16, 1995, and in revised form, June 29, 1995)

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The reaction of Factor XIIIα with fibrin is the last enzyme-catalyzed step on the coagulation cascade, leading to the formation of a normal blood clot. The finding that fibrin is preferred by the cross-linking enzyme by about 10-fold over the circulating fibrinogen suggests the operation of a unique substrate-level control for the orderly functioning of the physiological process in the forward direction. An important task is to elucidate the molecular mechanism for the transmission of the signal generated by the thrombin-catalyzed cleavage in the central E domain of fibrin to the distant Factor XIIIα-reactive glutamine residues. By focusing on the substrate sites present in γ chain remnants of D type domains of fibrinogen and by employing the approach of fragment complementation with the regulatory E domain, which represents the thrombin-modified portion of fibrin, we have now succeeded in reconstructing in solution the phenomenon of kinetic enhancement for the reaction with Factor XIIIα.

Two D type preparations (truncated fibrinogen, ~250 kDa and Dγ, ~105 kDa) were obtained by digestion of human fibrinogen with endo Lys-C. Neither product could be cross-linked by Factor XIIIα, but as shown by the incorporation of dansylcadaverine, both were acceptor substrates for the enzyme. The plasmin-derived D (~105-kDa) product, however, could be cross-linked into DD dimers. In all cases, the admixture of E fragments exerted a remarkable boosting effect on the reactions with Factor XIIIα. Even with native fibrinogen as substrate, cross-linking of γ chains was enhanced in the presence of E. Nondenaturing electrophoresis was used to demonstrate the complex forming potential of E fragments with fibrinogen, truncated fibrinogen, Dγ, or D. The GPRP tetrapeptide mimic of the GPRV N-terminal sequence of the α chains in the E fragments, abolished both complex formation and the kinetic boosting effect of E on the reactions of substrates with Factor XIIIα. Thus, the N-terminal α chain sequences seem to act as organizing templates for spatially orienting the D domains, probably during the protofibrillar assembly of the fibrin units, for favorable reaction with Factor XIIIα.

Stabilization of the fibrin network during the last phase of blood coagulation by the Factor XIII system represents a tightly regulated sequence of events. Though clotting times may be normal, failures in regard to the functioning of any aspect of this system could give rise to severe and frequently fatal bleeding (1, 2). The introduction of Nγ-(γ-glutamyl)lysine cross-links by activated Factor XIII (or Factor XIIIα) strengthens the clot (3–5) and renders it more resistant to lytic enzymes (6, 7). Until now, attention was paid mostly to the biochemical regulation of the conversion of circulating Factor XIII (or fibrin stabilizing factor AγBδ) to Factor XIIIα (or Aα*). Activation of this zymogen occurs in two distinct stages, catalyzed by thrombin and promoted by Ca2++: respectively: [AγBδ] → [Aα*Bδ] → Aα* + Bδ (8, 9).

Fibrin, which is the physiological substrate of the cross-linking enzyme, acts as a "feed forward" modulator in the conversion (10–15), ensuring that the transamidating enzyme (Aα*) is produced within the physiologically required time frame for efficient clot stabilization. Factor XIIIα reacts first with the cross-linking sites in the γ chains of fibrin and then with those in the α chains (16–18).

The known preferential reactivity of Factor XIIIα for fibrin over fibrinogen (17, 19) is considered to be yet another regulatory feature for the normal functioning of the clot-stabilizing system. The thrombin-induced alteration in the central N-terminal domains of fibrinogen (or, more precisely, the mere cleavage of fibrinopeptide A from the Aα chains (17)) must generate the signal for activating the distant, Factor XIIIα-reactive sites in the protein. The purpose of the present work is to shed light on this unique substrate level modulation in blood coagulation. By focusing on the γ chain cross-linking sites of the substrate, we succeeded in reconstructing in solution the regulatory mechanism by complementing fragments of the Factor XIIIα-reactive distal D regions of fibrinogen with the central thrombin-modified E domains of fibrin.

MATERIALS AND METHODS

Human Factor XIII was purified from outdated blood bank plasma (10, 20, 21) and was stored for up to 3 months at 4°C at a concentration of 1 mg of protein/ml in 50 mM Tris-HCl, 1 mM EDTA, 1.3 kallikrein-inactivating units/ml of Trasylol (Miles, Inc., West Haven, CT) at pH 7.5. Human α-thrombin (4,570 NIH units/mg), a gift of Dr. J. W. Fenton, III of the New York State Department of Health, Albany, NY, was stored at −20°C as 50 and 1,000 NIH units/ml solutions in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS).1 Hirudin, 650 anti-thrombin units/ml, was purchased from Sigma and was stored at −20°C as 200 units/ml of TBS. Human fibrinogen (a gift from A. B. Kabi, Stockholm, Sweden; lot 84639) was dissolved in TBS, dialyzed against 2 × 2 liters of TBS overnight at 4°C, centrifuged, and stored at −20°C as a 4–6 mg/ml solution. Protein concentration was determined by absorbance at 280 nm using ε280 = 15.1 (22).

A truncated form of fibrinogen was prepared by digestion with endoprotease Lys-C (Promega, Madison, WI) by a procedure similar to that described for bovine fibrinogen (23). Human fibrinogen (50 mg) was dissolved in 5 ml of TBS and dialyzed against 2 liters of the same solution at 4°C. Then 25 µl of 1 M CaCl2 was added to a concentration of 5 mM, and the mixture was warmed to 37°C 30 min prior to adding...

1 The abbreviations used are: TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; GPRP, Gly-Pro-Arg-Pro; GPRV, Gly-Pro-Arg-Val; endo Lys-C, endoprotease Lys-C.
5 μl of 20 units/ml endoprotease Lys-C (final concentration of 0.02 units/ml). Following incubation at 37°C for 2.5 h, 50 μl of 100 mM N-acetyl-L-lysine chloromethyl ketone (Sigma) in TBS was added, to a concentration of 1 mM. The digest was dialyzed against 2 liters of TBS (overnight, 4°C), and the truncated fibrinogen was purified by gel filtration on an Ultrigel AcA 44 column (LKB, Reagents IBF, Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M NH₂HCO₃, pH 8.0, and eluted with a flow rate of 30 ml/h at room temperature. Fractions of 5 ml were collected and analyzed by SDS-PAGE (24); those eluting with the void volume were pooled, lyophilized, dissolved in TBS, and dialyzed at 4°C against the latter overnight. The protein concentration of the product was determined by the bicinchoninic acid assay (Pierce; Reid, D.R., 1971; and reference therein). The fibrinogen as stock solution (0.3 mg/ml) was stored at -20°C. The preparation was free of the intact fibrinogen starting material and comprised one major protein band of about 250 kDa in SDS-PAGE under nonreducing conditions. In reducing SDS-PAGE (40 mM dithiothreitol (Sigma) in the sample buffer, 100°C, 3 min) three components were found with molecular masses of 48, 42, and 28 kDa, respectively. The 48-kDa band was actually a dimer of fibrinogen, with the exception that the digestion time was increased to 4 h at 37°C, and the fractions emerging from the column in the second peak were pooled, dialyzed against 2 × 2 liters of 0.1 M NH₂HCO₃, pH 8.0, overnight at 4°C, lyophilized, and dialyzed against TBS as described for truncated fibrinogen. The preparation (15.8 mg/ml, bicinchoninic acid assay; Pierce (25)) was stored at -20°C. The distal D-like fragment (D') of fibrinogen was also obtained by digestion with endoprotease Lys-C as described for the truncated fibrinogen, with the exception that the digestion time was increased to 4 h at 37°C, and the fractions emerging from the column in the second peak were pooled, dialyzed against 2 × 2 liters of 0.1 M NH₂HCO₃, pH 8.0, overnight at 4°C, lyophilized, and dialyzed against TBS as described for truncated fibrinogen. The preparation (15.8 mg/ml, bicinchoninic acid assay; Pierce (25)) was stored at -20°C.
rates for incorporation of about 2 equivalents of amines reflects mainly on the reactivities of \( \gamma \) chain acceptor sites (16), located in the distal D domains of the protein (45). The \( \alpha \) chain acceptors, themselves capable of reacting with 4–6 equivalents of amines become engaged later (16, 17, 46).

The kinetic advantage for fibrin over fibrinogen can be seen also when, instead of thrombin, a snake venom enzyme, Arvin, is employed for clotting (17). Since Arvin cleaves only fibrinopeptide A from the N termini of the \( \alpha \) chains, this limited proteolytic alteration of fibrinogen alone seems to be responsible for triggering the enhanced reactivity of amine acceptor sites for Factor XIII\(_a\). However, the relevant N-terminal region of the protein, located in its central E domain, is far removed from the Factor XIII\(_a\)-reactive glutamine residues (45–47). This poses the intriguing question as to what the actual mechanism of the long range communication between the two distant loci might be.

In the present work, we focused exclusively on the reactivities of the \( \gamma \) chain acceptor sites in the D domains of fibrinogen. Reactivity to Factor XIII\(_a\), was probed by the incorporation of dansylcadaverine; reaction products were measured quantitatively (42–44) and were also documented on the SDS-PAGE profiles of the proteins photographically under UV illumination (17). Three partially degraded forms of human fibrinogen were used as substrates. Digestion of the parent protein with endo Lys-C yielded a \( \sim 250 \) kDa species, referred to as truncated fibrinogen, and also a \( \sim 105 \) kDa fragment, called \( \Delta' \), while digestion of fibrinogen with plasmin produced the known D fragments. The latter comprise the \( \alpha\Delta\left(105–206\right)B\left(218–327\right)\gamma\left(63/86–406\right) \) segments of the protein (48). On the basis of a variety of tests performed (modification by thrombin, SDS-PAGE, Western blots with antibodies, and lack of cross-linking by Factor XIII\(_a\)) (data not shown), coupled with known amino acid sequences and proteolytic cleavage sites (49–51), it may be suggested with a certain degree of confidence that the constituent chain composition of the endo Lys-C truncated fibrinogen may be represented as \( \alpha\Delta\left(1–206/219/230\right)B\left(54/55/59–461\right)\gamma\left(1–406\right) \), and that the endo Lys-C-derived \( \Delta' \) fragment is comprised of \( \alpha\left(79/82–206/219/230\right)B\left(134–461\right)\gamma\left(63/86–406\right) \).

Experiments with Endo Lys-C-truncated Fibrinogen and with Native Fibrinogen—The endo Lys-C digestion product, having lost the amine incorporation sites from the C-terminal two-thirds portion of its \( \alpha\) chains, retained only the \( \gamma \) chain sites for reaction with Factor XIII\(_a\). However, because of a cleavage at Lys-406, the truncated fibrinogen cannot form protein-to-protein cross-bridges upon treatment with Factor XIII\(_a\). It serves as a pure acceptor substrate for the enzyme, capable of incorporating amines only into the \( \gamma \) chain glutamine sites. Moreover, the N termini of the \( \alpha\) chains of the truncated fibrinogen are still intact and, thus, this region is available for modification by thrombin. A prime question was whether this truncated product would still be susceptible to the thrombin-dependent substrate level regulation toward Factor XIII\(_a\), observed previously for intact fibrinogen (17, 19). The results presented in Fig. 1 clearly answer the question in the affirmative. Although the endo Lys-C truncated fibrinogen has a mass about 30% smaller than the parent protein, it still contains the structural framework necessary for the transmission of information from the thrombin-catalyzed cleavage sites at N termini of the \( \alpha\) chains to the distal Factor XIII\(_a\)-reactive sites in the \( \gamma \) chains. The rate enhancement for the thrombin-modified fibrinogen substrate \( \left(\sim 5\right)\) was well within the range observed for native fibrin.

The next issue was to test whether the Factor XIII\(_a\) reactivity of sites could be enhanced without actually modifying the truncated fibrinogen substrate, itself, by thrombin. In these experiments, a fixed concentration \( \left(4 \mu M\right) \) of truncated fibrinogen was mixed with varying concentrations \( \left(0–16 \mu M\right) \) of the preparation of E fragments, representing the thrombin-modified central domains of fibrin. As seen in Fig. 2, admixture of E produced a dramatic enhancement in the rate of dansylcadaverine incorporation into the Factor XIII\(_a\)-reactive sites of truncated fibrinogen. This finding, coupled with the observation that the aggregation-inhibitory tetrapeptide GPRP \( \left(5 \text{ mM; OZ Chemical Co., Israel}\right) \) abolished the effect, may be taken to indicate that a direct contact with the E domain was required for activating the substrate for Factor XIII\(_a\). Maximal rate enhancement was obtained at an approximate 1:1 molar ratio of truncated fibrinogen to E fragment added. The results predicted that truncated fibrinogen would form a complex with the E fragments. Indeed, the existence of such a complex in solution could be readily demonstrated by nondenaturing electrophoresis (Fig. 3).
Brilliant Blue-stained patterns on the right cated fibrinogen (Fig. 2), the rate of the Factor XIIIa-catalyzed in solution. Moreover, in tune with the observations on truncated fibrinogen could definitely be shown to bind E fragments (Fig. 4) or the Bio-Rad gradient gel system (data not shown), gates (54–59). In reexamining this issue by nondenaturing fragments (a near equivalent of E) growing into large aggregation of complexes of fibrinogen with E and also with N-DSK containing 5 mM CaCl2 for 60 min at 37°C. The control mixtures contained 5 mM GPRP in in TBS. The insets show the SDS-PAGE (12.5% gel, reducing) profiles pertaining to the 60-min time points for the experimental (left, solid circles) and control (open circles) mixtures, with Coomassie Brilliant Blue-stained patterns on the left and UV illumination on the right for each.

FIG. 3. The endo Lys C-truncated fibrinogen can form stable complexes with the E fragments of fibrin in solution. Truncated fibrinogen (tE; 4 mM) was incubated with 0–16 mM of E fragments in TBS containing 5 mM CaCl2 for 60 min at 37°C. The control mixtures contained also the tetrapeptide GPRP (5 mM). Glycerol (68% (v/v) in water) was added to a concentration of 6% (v/v), and the samples were analyzed by nondenaturing electrophoresis (4–20% gradient gel, pH 8.8, Bio-Rad, Laemmli (24) system without SDS and stacking gel). Gels were stained with Coomassie Brilliant Blue. Positions of the complexes between truncated fibrinogen and E are marked as tE:E, whereas the positions of the free E species are indicated as E1, E2, and E3.

production of complexes of fibrinogen with E and also with N-DSK fragments (a near equivalent of E) growing into large aggregates (54–59). In reexamining this issue by nondenaturing electrophoresis, using either the Pharmacia PhastSystem gels (Fig. 4) or the Bio-Rad gradient gel system (data not shown), native fibrinogen could definitely be shown to bind E fragments in solution. Moreover, in tune with the observations on truncated fibrinogen (Fig. 2), the rate of the Factor XIIIa-catalyzed reaction with native fibrinogen could also be significantly increased by admixture of E fragments without the necessity of including thrombin in the medium (Fig. 5). Again, maximal enhancement of γ chain cross-linking for the native fibrinogen was obtained by supplementation with equimolar E, and the GPRP tetrapeptide abolished the augmenting effect of E fragments.

Complementation with E Fragments in Solution Enhances the Reactivities of the D Fragments of Fibrinogen to Factor XIIIa — Further insight into the activation of Factor XIIIa-reactive γ chain sites was sought by studying the behavior of this purified domain of the substrate, obtained by digestion of fibrinogen with either endo Lys-C (yielding D' ) or plasmin (yielding D). Fixed concentrations (8 µM) of the D’ or D fragments were mixed with increasing concentrations of E (0–16 µM), and
tested for reactivities to Factor XIIIa. The experiments presented in Figs. 6 and 7 illustrate our findings. Fig. 6 shows the enhancement obtained for the incorporation of dansylcadaverine into the D' fragment upon complementation with E. Maximal rate enhancement was seen at a mixing ratio of about 2 mol of D/mole of E (i.e. 8-4 μM). By holding this mixing ratio constant for increasing concentrations of the complex and measuring the initial velocity of amine incorporation (data not shown), an approximately 5-7-fold enhancement in the kcat/Km catalytic efficiency index was calculated for the reaction of the [2D'-E] complex with Factor XIIIa in comparison to that of free D'.

Fig. 6. Complementation with E fragments greatly enhances the reactivity of D' fragments to Factor XIIIa. The experimental protocol and procedures were identical to those in Fig. 2, except that endo Lys C-derived D' (8 μM) was used instead of truncated fibrinogen as substrate. The insets show the SDS-PAGE (12.5% gel, reducing) profiles pertaining to the 60-min time points for the experimental (upper) and control mixtures (lower), with Coomassie Brilliant Blue staining on the left and with UV illumination on the right.

Employing the plasmin-derived D fragment of fibrinogen as substrate, the effect of complementation by E could be examined for the formation of γ-γ chain cross-linked D-dimers under the influence of Factor XIIIa. This reaction, too, was greatly promoted by the admixture of E fragments (Fig. 7). As with the reaction involving D' (Fig. 6), the GPRP tetrapeptide abolished the positive modulatory influence of E, indicating that a direct contact with E was necessary to activate the Factor XIIIa-reactive distal glutamine residues, the following must be borne in mind. The GPRP tetrapeptide, a mimic for the GPRV N-terminal sequence of the α chains of fibrin generated by the cleavage of fibrinopeptide A (52), in our experiments abolished both the complex-forming ability and the ki-
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Fig. 8. The endo Lys C-derived D' fragments as well as the plasmin-derived D fragments of fibrinogen can form stable complexes with the E fragments of fibrin in solution. Either the D' (panels A and B) or the D fragments (panels C and D) were incubated at a concentration of 8 μM with 0–16 μM of E fragments in TBS containing 5 mM CaCl₂ for 60 min at 37 °C. Control mixtures contained the tetrapeptide GPRP (5 mM) in addition. Prior to electrophoresis, glycerol (68% (v/v) in water) was added to a concentration of 6% (v/v), and the samples were analyzed by a nondenaturing procedure (4–20% gradient gel, pH 8.8, Bio-Rad, Laemmli (24) system without SDS and stacking gel). Gels were stained with Coomassie Brilliant Blue. The positions of complexes between D' and E were determined by a nondenaturing procedure (4–20% gradient gel, pH 8.8, Bio-Rad, Laemmli (24) system without SDS and stacking gel). Gels were stained with Coomassie Brilliant Blue. The positions of complexes between D' and E or between D and E are marked as D':E and D:E, respectively. The positions of free D', D, E, E', and E₂ are also marked.

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