Expression of Primary Polymerization Sites in the D Domain of Human Fibrinogen Depends on Intact Conformation*

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Fragments D₁ and DD, plasmin degradation products of human fibrinogen and cross-linked fibrin, respectively, originate from the COOH-terminal domain of the parent molecule. Since a specific binding site for fibrin resides in the COOH-terminal region of the γ chain, the primary structure of the two fragments was compared and their affinity for fibrin monomer measured. Fragments D₁ and DD contained the same segments of the three fibrinogen chains, corresponding to the sequences 1α̲ 105–206, β̲ 134–461, and γ̲ 63–411. Fragment DD had a double set of the same chain remnants. Fragments D₁ and DD inhibited polymerization of fibrin monomer in a dose-dependent manner; 50% inhibition occurred at a molar ratio of fragment to monomer of 1:1 and 0.5:1, respectively. To prevent fibrin monomer polymerization and render it suitable for binding studies in the liquid phase, fibrinogen was decorated with Fab fragments isolated from rabbit antibodies to human fragment D₁. Fibrinogen molecules decorated with 6 molecules of this Fab fragment did not clot after incubation with thrombin, and the decorated fibrin monomer could be used to measure binding of fragments D₁ and DD in a homogeneous liquid phase. The data analyzed according to the Scatchard equation and a double-reciprocal plot gave a dissociation constant of 12 nM for fragment D₁ and 38 nM for fragment DD. There were two binding sites/fibrin monomer molecule for each fragment. After denaturation in 5 M guanidine HCl, the inhibitory function on fibrin polymerization was irreversibly destroyed. Denatured fragments also lost binding affinity for immobilized fibrin monomer. The preservation of the native tertiary structure in both fragments was essential for the expression of polymerization sites in the structural D domain.

Two major structural and functional regions are distinguished in the fibrinogen molecule. The E domain resides in the center encompassing the NH₂ termini of the Aα, Bβ, and γ polypeptide chains (1, 2). The D domain involves the COOH termini of the β and γ chains (1); it is recovered in fragment D₁ (M̲, 103,000) cleaved by plasmin from either fibrinogen or non-cross-linked fibrin (3, 4). Degradation of cross-linked fibrin with plasmin results in the formation of fragment DD (5–8). Although its amino acid sequence has not been completely determined, it was inferred from the polypeptide chain composition analyzed by polyacrylamide gel electrophoresis that fragment DD contains two D moieties linked covalently by (γ-glutamyl)lysine isopeptide bonds between the COOH-terminal regions of the two γ chain remnants (5). Fragments D₁ and DD inhibit fibrin monomer polymerization (9, 10). This phenomenon results from the presence of a fibrin polymerization site “a” localized in the COOH terminus of the γ chain (11–13). The conclusion was derived mainly from the interaction of fragments D₁ and D₂ with the polymer fibrin clot formation. The two fragments have very similar a and β chain remnants, but their γ chains differ, containing at the COOH termini Val₄₁₁ and Lys₃₀₂, respectively (14). Since fragment D₁ neither binds to immobilized fibrin monomer (11, 15) nor inhibits fibrin monomer polymerization (9) it is apparent that a 109-residue segment of the γ chain COOH terminus has an important contribution in the expression of fibrin polymerization site “a.” The complementary site “A” appears in the E domain after removal of fibrinopeptides A by thrombin or batroxobin (12, 13). The binding of sites A with a seems to propagate the assembly of fibrin clots.

The formation of fibrin oligomers in aqueous solution at neutral pH (16, 17) complicates direct binding studies with fibrin monomer because of the phase transition. In this work we addressed the question of the affinity of fragments D₁ and DD for fibrin monomer. The fragments were obtained from human fibrinogen and cross-linked fibrin, respectively; their polypeptide chains isolated, and the NH₂- and COOH-terminal amino acid sequences determined. The characterized fragments D₁ and DD were then tested for interference with the polymerization process and for binding to either immobilized or soluble fibrin monomer. Fibrin monomer was prevented from polymerizing by decoration with Fab fragments isolated from antibodies against fibrinogen fragment D₁. In this system, polymerization sites A were available on the decorated fibrin monomer and the complementary sites a on fragments D₁ and DD. To correlate the inhibitory and binding functions with protein conformation, the fragments were studied before and after denaturation with guanidine HCl.

MATERIALS AND METHODS

All reagents were of analytical grade obtained from commercial sources. Human plasmin (10.2 CTA (Committee on Thrombolytic Agents) units/ml, 12.7 CTA units/mg of protein) was kindly provided by Dr. David Aronson, Bureau of Biologics, Food and Drug Administration, Rockville, MD.

Preparative Procedures

Digestion of Fibrinogen—Fibrinogen (human, grade L, Kabo) digestion with plasmin was done after the addition of streptokinase (Varidase, Lederle Laboratories) at 10,000 units/g of fibrinogen in the
presence of 25 mM calcium chloride for 4 h at 37 °C. The digest contained homogeneous fragment D1 (18) and fragment E, which after thrombin treatment had the same electrophoretic mobility as fragment E0 (2). Fragment D1—This was obtained from a plasmic digest of human fibrinogen (100 mg) by gel filtration on a column (2.5 x 130 cm) of Sephadex G-25 (Pharmacia) equilibrated in 0.025 M sodium acetate buffer containing 1.5 M NaCl, pH 5.3, at 37 °C for 30 min. Under these conditions fragment D1 precipitated. The precipitate was recovered by centrifugation at 3000 x g for 10 min, dissolved in 0.025 M Tris phosphate buffer containing 0.14 M guanidine HCl, pH 8.4, and dialyzed against 3 changes (each 900 volumes) of the same buffer.

Digestion of Cross-linked Fibrin—In the presence of 25 mM calcium chloride for 4 h at 37 °C. The digest was homogeneous in SDS-PAGE and contained remnants of α (M, 12,000), β (M, 42,000), and γ (M, 39,000) chains (Fig. 1, lane 1).

Cross-linked Fibrin—This was prepared from human fibrinogen enriched with factor XIII, clotted, and freeze-dried as described previously (20).

Fragments DD-This was purified from cross-linked fibrin digested by isolation of the D-D complex using gel filtration on Sepharose 4B (Pharmacia) as described before (23). The D-D complex, which contained predominantly fragment DD, was isolated in 25 mM guanidine HCl buffer containing 1.5 M NaCl, pH 5.3, at 37 °C for 30 min. Under these conditions fragment DD precipitated. The precipitate was recovered by centrifugation at 3000 x g for 10 min, dissolved in 0.025 M Tris phosphate buffer containing 0.14 M guanidine HCl, pH 8.4, and dialyzed against 3 changes (each 900 volumes) of the same buffer.

Alternatively, nondenatured fragment DD was isolated from an exhaustive cross-linked fibrin digest using gel filtration on Sepharose 4B as described before (20).

Fibrinogen Decorated with Fab Fragments—Two ml of fibrinogen (0.5 mg/ml) were mixed with anti-D, Fab at a molar ratio of 1.5:1 and incubated at 4 °C for 3 h. After that, the Fab-decorated fibrinogen was digested at 37 °C for 2 h with thrombin using 0.4 unit/mg of protein. The reaction was stopped by the addition of 0.01 M EDTA, pH 5.5, and digested at 37 °C for 2 h. The reaction was stopped by the addition of 0.01 M iodoacetamide in 0.2 M Tris-HCl buffer, pH 7.5, to a final concentration of 0.1% SDS either under nonreducing conditions or after reduction of the samples with 2-mercaptoethanol (26).

Polypeptide Chains of Fragments D1 and DD—These were obtained by reduction of the protein (20 mg/ml) in 0.2 M Tris-HCl buffer containing 0.01 M iodoacetamide in 0.2 M Tris-HCl buffer, pH 7.5, prior to mixing with various amounts of radiodinated fragments D1 or DD and reincubation at room temperature for 30 min. The concentration of the decorated fibrin monomer was 0.25 μM and that of 125I-D1 or 125I-DD varied from 0.025 to 5 μM. In the binding studies, aliquots of decorated fibrin monomers were mixed with increasing amounts of 125I-labeled fragment D1 or DD and incubated with Ultrogel AcA 34 (LKB) at room temperature for 2 h. The concentration of the radioactive fragments outside the gel in the presence and absence of the decorated fibrin monomers was determined. The amounts of bound and free peptides were calculated according to the method of Hirose and Kano (29) and the data were plotted according to the Scatchard equation (29) and a double reciprocal plot (30).

Fibrin Monomers—These were prepared according to method of Belitsky and colleagues (31). Polymerization of fibrin monomers generated from fibrinogen by thrombin or reaggregation of purified fibrin monomers was monitored spectrophotometrically at 350 nm as described previously (10).

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; dansyl, 5-dimethylaminonaphtalene-1-sulfonyl; EGTA, [ethylenebis(oxyethylenenitro)tetraacetic acid.
1 ml of fibrin monomer-Sepharose CL-4B at room temperature for 30 min. Nonspecifically bound protein was washed out with 0.05 M Tris phosphate buffer containing 1.0 M NaCl and 0.005 M EDTA, pH 7.8. The complex of D1 or DD with immobilized fibrin monomer was dissociated at pH 5.3 using 0.05 M Tris/H3PO4 containing 1.0 M NaCl and 0.005 M EDTA, pH 5.3. The binding of native and denatured fragments D1 or DD was studied under the same conditions. In some experiments, a plasmic digest of fibrinogen was used in addition to purified fragment D1.

RESULTS

Structural Characteristics of Fragment D1, DD, and Their Polypeptide Chains—Reduced and carboxymethylated fragments D1 and DD showed the presence of three different chains corresponding to the α, β, and γ remnants, respectively (Fig. 1, lanes 1 and 5), consistent with published data (4, 5). The purified chains were homogeneous (Fig. 1, lanes 2–4 and 6–8); the electrophoretic mobility of the α and β remnants in both fragments was the same; the mobility of fragment DD γ chain remnant (Fig. 1, lane 7) was slow due to its dimeric structure. The amino acid composition of the purified polypeptide chain remnants was very similar for fragments D1 and DD when results for the latter were expressed as residues/0.5 mol of the fragment. The amino acid composition of the chain remnants was also in good agreement with that calculated from the sequences of human fibrinogen chains (33–36).

NH2- and COOH-terminal amino acid sequences were determined to establish the exact length of each chain remnant, with the results summarized in Table I. The primary structure of fragment DD was identical to that of fragment D1 except that the former contained a double set of the same segments of the α, β, and γ chains.

Interference of Fragments D1 and DD with Fibrin Polymerization—Both fragments D1 and DD used in these studies had a strong inhibitory effect on the rate of clot formation. The effect of these fragments was analyzed over a wide range of concentration (Fig. 2). Fragments D1 and DD efficiently inhibited both thrombin-induced fibrinogen polymerization (Fig. 2A) and polymerization of fibrin monomers (Fig. 2B). A 50% decrease of the maximal reaction rate was observed at a molar ratio of the fragment to fibrin monomer of 0.5:1 and 1:1 for DD and D1, respectively. The inhibition appeared to be critically dependent upon the native conformation of both derivatives. Fragments D1 and DD which had been exposed to 5 M guanidine HCl prior to equilibration in the polymerization buffer lost their ability to inhibit both thrombin-induced fibrinogen clotting (Fig. 2A) and fibrin monomer polymerization (Fig. 2B). Denatured fragments had the same primary structure as the native ones, and, as judged by SDS-PAGE of nonreduced samples, there was no degradation or aggregation of polypeptide chains in either fragment associated with denaturation.

Binding of Fragments D1 and DD to Immobilized Fibrin Monomer—To confirm the importance of the native conformation of the fragments in determining affinity for fibrin, the binding of native and denatured fragments D1 and DD to immobilized fibrin monomer was compared. Untreated fragment D1 bound to the immobilized fibrin monomer as evidenced by the retention of protein applied in buffer 1 and its elution with buffer 2 (Fig. 3, bottom). The peak contained fragment D1, as shown by SDS-PAGE of nonreduced samples (inset to Fig. 3). The treatment of fragment D1 with 5 M guanidine HCl, followed by dialysis, resulted in a loss of affinity for fibrin. Only 9% of the denatured fragment bound to fibrin monomer as compared to the untreated counterpart. Denaturation of fragment DD under the same conditions resulted in a total loss of affinity for fibrin monomer (Fig. 3, top).

Binding Affinity of Primary Polymerization Sites—Spontaneous polymerization of fibrin monomers (16, 17) does not allow measurement of binding constants by equilibrium techniques. Therefore, we attempted to block selectively polymerization sites which are on the D domains of the fibrinogen molecules. For this purpose, Fab fragments were obtained

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**Table I**

| Chain remnant | Fragment D1 | Fragment DD* |
|---------------|-------------|--------------|
|               | NH2 terminus | COOH terminus | NH2 terminus | COOH terminus |
| α             | Asp-105     | Lys-206      | Asp-105     | Lys-206       |
| β             | Asp-134     | Gln-461      | Asp-134     | Gln-461       |
| γ             | Ala-63      | Val-411      | Ala-63      | Val-411       |

* Fragment DD contained 2 identical sets of the α, β, and γ chain remnants.

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**Fig. 2.** Dependence of polymerization on fragment D1 (□) and DD (▲) concentration. Panel A represents thrombin-induced polymerization of fibrinogen, and panel B shows fibrin monomer polymerization. Experiments were done in the presence of intact fragments D1 and DD (open symbols) and denatured by treatment with 5 M guanidine HCl with subsequent dialysis (closed symbols).
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Fig. 3. Binding of fragments DD (top) and D₁ (bottom) to immobilized fibrin monomers. SDS-PAGE patterns on the right show protein composition of peaks eluted from the affinity column by Buffer 2. Buffer 1, 0.05 M Tris phosphate buffer containing 1.0 M NaCl and 0.005 M EDTA, pH 7.8. Buffer 2, 0.05 M Tris phosphate buffer containing 1.0 M NaCl and 0.005 M EDTA, pH 5.3. Intact (open symbols) and denatured (closed symbols) fragments were studied.

Fig. 4. The kinetics of thrombin-induced polymerization of fibrinogen. Fibrinogen (Fg), 0.3 mg/ml, was mixed with thrombin (Thr), 3 units/ml, and the absorbance increase recorded in a spectrophotometer at 350 nm. When fibrinogen was preincubated at a molar ratio of 1:6 with Fab fragments isolated from anti-D₁ antiserum and then treated with thrombin, clot formation did not occur.

from affinity-purified anti-D₁ antibodies. These antibody fragments recognized only the epitopes located on the surface of the fibrinogen molecule. The Fab fragments were potent inhibitors of fibrin monomer polymerization as assessed by absorption measurements at 350 nm. In the presence of a 6-fold molar excess of anti-D₁ Fab fragments, fibrinogen became incoagulable by thrombin (Fig. 4).

The incoagulable derivative of fibrin monomer was purified by gel filtration on a column of Ultrogel AcA 22. The elution profile showed the presence of high Mₛ species and only a small amount of contamination by noncomplexed proteins. The composition and apparent molecular weight of the decorated fibrin monomers was tested by SDS-PAGE of nonreduced samples (Fig. 5). The decorated fibrin monomers had polymerization site a of the D domain blocked with Fab fragments, but the functional polymerization site A in the E domain was apparently available. This property allowed us to measure the binding affinity of fragments D₁ and DD in a homogeneous liquid phase system. The binding studies were performed using the gel equilibrium technique (28). The Fab-decorated fibrin monomer was prepared immediately before the binding experiments.

A Scatchard plot of the binding data gave values of Kₐ for fragment D₁ and DD of the same order of magnitude, that is 12 and 38 nM, respectively. The values were calculated from the solid lines in Fig. 6 derived from experiments done at low concentrations of fragments. The limiting value of γ, corresponding to the molar ratio of bound fragment to fibrin monomer was found to be equal to 2, indicating that there

Fig. 5. Purification of fibrin monomer decorated with Fab fragments by gel filtration on Ultrogel AcA 22. Fibrin monomer (FM) decorated with Fab (1.5 mg) was applied to the Ultrogel AcA 22 column (1 x 90 cm). The column was equilibrated and eluted with 0.15 M sodium chloride buffered with 0.05 M Tris-Cl, pH 7.3. The major peak (fibrin monomer-Fab) contained fibrin monomer decorated with Fab fragments as verified by SDS-PAGE in the inset. In addition, two small peaks containing noncomplexed fibrinogen (Fg) and Fab were observed. The elution profile of fibrinogen alone (C) from the same column serves as a control.

Fig. 6. Scatchard analysis of binding data using the ¹²⁵I-D₁ (panel A, □) or ¹²⁵I-DD (panel B, △) and Fab-decorated fibrin monomers. The binding was measured using the Ultrogel AcA 34 equilibrium method. The ratio of the number of fragment D₁ or DD molecules bound per molecule of the decorated fibrin monomer is plotted on the abscissa. The ratio of bound to free peptide is given on the ordinate. Increase of fragment concentration above 1.5 μM (corresponding to the molar ratio of 2 of fragment bound per fibrin monomer (FM)) is accompanied by aggregation. The dashed lines show the increase of aggregates formed in fragment D₁ (panel A) and DD (panel B) solutions as demonstrated by glutaraldehyde cross-linking.

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were two binding sites for each fragment on the decorated fibrin monomers. The course of the experimental points in the Scatchard plot obtained from experiments done at a high concentration of fragments showed the characteristic nonlinearity predicted by the simulation studies of Calvert and co-workers (37). These points are not connected with a line, although the experimental conditions were the same, and calculation gave $K_d$ values of 1.6 and 0.8 $\mu M$, respectively, for fragments $D_1$ and $DD$.

As postulated before (37), deviations from linearity in a plot of the binding data represent either cooperativity between binding sites or the presence of heterogeneous interactions. The latter may result from nonspecific aggregation of fragments $D_1$ or $DD$, particularly at high protein concentrations. In order to explore the origin of the interaction, glutaraldehyde cross-linking of the interacting proteins was performed. This technique has been found to be useful in the analysis of quaternary structure of protein molecules (38). For this purpose, increasing concentrations of fragments $D_1$ or $DD$, corresponding to those used in binding studies, were treated with 50 mM glutaraldehyde at room temperature for 10 min. Aliquots preincubated with 1 M glycine were analyzed by SDS-PAGE for the presence of oligomeric forms. The stained gels were scanned and the percentage of cross-linked fragments calculated.

It was found (Fig. 6, broken lines) that there was a linear increase in the amount of aggregated species of fragments $D_1$ and $DD$ starting from a concentration of approximately 1.5 $\mu M$. Above this concentration, dimeric, trimeric, and tetrameric derivatives of fragments $D_1$ and $DD$ were observed in SDS-PAGE of nonreduced glutaraldehyde-cross-linked samples. This fact demonstrated that fragments $D_1$ and $DD$, at concentrations higher than 1.5 $\mu M$, tend to form aggregates and that this reaction may account for the nonlinearity observed during binding studies.

**DISCUSSION**

Fragment $D_1$, a terminal product of plasmin digestion of fibrinogen in the presence of Ca$^{++}$, binds to immobilized fibrin monomer and inhibits fibrin monomer polymerization. It loses its anticoagulant activity when further digested to fragment $D_2$ after chelation of Ca$^{++}$ by EDTA or EGTA. The production of fragment $D_2$ from $D_1$ is associated with the release of a peptide $\gamma$Ala-357-Val-411 (39) that seems to be involved in fibrin polymerization site $a$ which is complementary to binding site $A$ exposed by thrombin in the E domain. The present results demonstrate for the first time that expression of the polymerization site depends upon the integrity of the D domain conformation. Dramatic changes in the inhibitory activity of fragments $D_1$ and $DD$ were observed during polymerization and binding studies when fragments denatured with guanidine HCl were used. As judged by gel analysis, treatment of the fragments with this denaturant did not result in cleavage in the COOH-terminal region of the $\gamma$ chain. Spectropolarimetric studies showed that fragment D lost 95% of $\alpha$-helical structure without any fragmentation after incubation in 5 M guanidine HCl (40). Apparently, fragment $D_2$ after denaturation contains the entire $\gamma$ chain peptide segment corresponding to the sequence $\gamma$Ala-63-Val-411. Therefore, a loss of the inhibitory activity in denatured fragments $D_1$ and $DD$ must have been caused by conformational changes occurring in and around the polymerization site.

Two hypotheses can be proposed to explain the importance of the native D domain conformation in the expression of polymerization site $a$. First, the polymerization site may be formed simply by a linear sequence of amino acid residues in a segment of the $\gamma$ chain. This segment would occupy a highly accessible location on the surface of the fibrinogen molecule. Second, the polymerization site may have a complex structure, requiring for complete expression the presence of several not necessarily contiguous peptide segments in close proximity. Both the simple and complex models can explain the effect of 5 M guanidine HCl on the inhibitory activity of fragments $D_1$ and $DD$. Unfolding of the fragments after denaturation may result in steric hindrance of binding at the polymerization site resulting from the altered arrangement of the immediate sequence around the site. On the other hand, dissociation of separate peptide segments forming the polymerization site may cause the loss of inhibitory activity.

Direct binding studies using Ultrogel AcA 34 to separate free from bound ligand showed that there were two binding sites for fragments $D_1$ and DD on a fibrin monomer decorated in the D domain with Fab fragments. The finding of two binding sites for fragment DD molecules on the decorated fibrin monomer was unexpected. Since fragment DD contains two identical subunits, it could be expected to express double polymerization sites “$aa$” and form an equimolar complex with the decorated fibrin monomer. Since the cross-linking reaction occurs after completion of fibrin polymerization, the formation of two $\epsilon$-(glutamyl)lysine bonds may distort the distance between two a sites on the $\gamma$ chains. Thus, there could be an imperfect fit with two A sites in the native E domain. In addition, fragment DD, when released from cross-linked fibrin by plasmin, might undergo a conformational change which could change either shielding of one polymerization site or its dislocation. In either case, binding of fragment DD to the decorated fibrin monomer could only be accomplished through one polymerization site $a$. Thus, fragment DD would become functionally monovalent.

Plasmin digestion of fragment $D_1$, in the presence of EDTA results in its degradation to fragment $D_3$ (41) and several peptides. Using affinity chromatography on immobilized fibrin monomer, we isolated peptide $\gamma$374-411 that inhibited fibrin polymerization and bound to a thrombin-treated NH$_2$-terminal disulfide knot with a $K_d$ of 1.4 $\mu M$ (42). Horwitz et al. (43) confirmed this observation and obtained with staphylococcal protease peptide $\gamma$374-396 that inhibited polymerization in stoichiometric proportions. The findings prompted conclusions about the localization of polymerization site $a$ in that segment of the $\gamma$ chain. However, two recent publications are at variance. Southan and colleagues (44) did not find any inhibitory activity either in peptides $\gamma$303-356, $\gamma$357-373, $\gamma$374-405, or in their mixtures, leaning to our concept of complex tertiary-structured polymerization sites (45). Vardaci and Scheraga (39) purified by high pressure liquid chromatography noninhibitory peptide $\gamma$374-411 and proposed that the segment $\gamma$356/357-411 is important for maintenance of a polymerization site in this region. Regardless of the identification of peptide segments participating in polymerization site $a$, there is little doubt about its localization in the $\gamma$ chain between Ala-357 and the COOH terminus. However, contributions of other parts of the $\gamma$ chain to the expression of binding affinity should be taken under serious consideration. Abnormal fibrinogen Milano I with the amino acid replacement $\gamma$Asp-330 → Val (46) and fibrinogen Haifa, $\gamma$Arg-275 → His (47) are characterized by defective fibrin clot formation and provide supportive evidence for long-range cooperativity of charged residues in an extended polymerization site.

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