Binding Phenomena of Isolated Unique Plasmic Degradation Products of Human Cross-linked Fibrin*

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Proteolysis of human cross-linked fibrin by plasmin results in the formation of a DD-E complex, and Fragments DD and E as the major degradation products. Three species of Fragment E, which differ both in molecular weights (E1, M = 60,000; E2, M = 55,000; E3, M = 50,000) and in charge, have been isolated from a digest of cross-linked fibrin. Each Fragment E species reacts with monospecific anti-E antiserum. Fragments E1 and E2 bind with Fragment DD to form a DD-E complex but Fragment E3 is inactive. This binding is specific since these Fragments E do not bind to fibrinogen or to degradation products of fibrinogen or of noncross-linked fibrin. Fragments E1 and E2 incubated with plasmin are degraded to Fragment E3, suggesting that the three species represent sequential degradation products. Plasmic-treated Fragments E1 and E2 no longer bind with Fragment DD; therefore, it appears that the peptides cleaved from Fragment E3 by plasmin contain or modify the sites responsible for complex formation. On the other hand, Fragment DD binds not only to Fragments E1 and E2, but also to fibrinogen, Fragments X (Stage 1), X (Stage 2), Y, and NH2-terminal disulfide knot, but only after thrombin treatment, suggesting that Fragment DD binds to complementary sites on the NH2-terminal region of fibrinogen which are exposed after thrombin treatment.

Plasmic digestion of fibrinogen produces Fragments D and E as the main terminal, high molecular weight end products (1) while the digestion of cross-linked fibrin results in the formation of Fragments DD and E, and α polymer remnants as the major degradation products (2-9).

In plasmic digests of cross-linked fibrin, the antigenic determinants of Fragment E appear in association with Fragment D determinants. This association, first observed by Gorosn and Feddersen (9), and identified later as the DD-E complex (4, 7), is quite stable, suggesting a unique molecular arrangement between Fragments DD and E. Observations by Hudry-Clergeon and colleagues (7) suggested that, upon dissociation of the DD-E complex, the resulting Fragment E had a slower electrophoretic mobility in SDS gels than the uncomplexed Fragment E present in the digest.

Fragment E is a dimeric molecule derived from the NH2-terminus of the fibrinogen molecule (10). Fragments E isolated from terminal digests of fibrinogen, noncross-linked, and cross-linked fibrin do not differ significantly in molecular weight (11, 12). The molecular weight of Fragment E has been reported between 40,000 and 50,000 from analytical ultracentrifugation (12-15) and in the range of 50,000 to 60,000 from polyacrylamide gel electrophoresis (11, 15, 16); the amino acid sequence gives a molecular weight of 44,000 to 50,000 (17). Purified Fragment E has been shown to contain three distinct components by isoelectric focusing (18). Garlund and colleagues (17) suggested that heterogeneity of Fragment E is due to multiple cleavages by plasmin.

We have noticed in plasmic digests of human cross-linked fibrin a heterogeneity of the Fragment E electrophoretic band on SDS-polyacrylamide gels. It appeared that the increase of the Fragment E band with the fastest mobility was concurrent with the decrease of the DD-E complex. This observation suggested that not all Fragment E species participate in the complex.

The purpose of the present work was to isolate the three species of Fragment E present in a plasmic digest of cross-linked fibrin and to characterize them by their ability to associate with Fragment DD forming a DD-E complex. The specificity of this phenomenon was analyzed by testing for complex formation between Fragment DD and Fragment E with a variety of plasmic degradation products of fibrinogen or noncross-linked fibrin. A better understanding of the structure and function of the DD-E complex can be approached by studying its components and the intermolecular arrangement of the subunits (19). This information can be used in the development of an assay to detect the DD-E complex in vivo, since the complex appears to be a specific molecular marker of fibrinolysis. Moreover, the data contribute to the clarification of the mechanism of fibrin monomer polymerization by localization of the complementary binding sites between the Fragment DD and E domains.

**MATERIALS AND METHODS**

Reagents were of analytical grade obtained from commercial sources.

**Preparation of Cross-linked Fibrin**—Human fibrinogen (grade L; A. B. Kabi, Stockholm, Sweden) was enriched with Factor XIII, clotted and freeze-dried as described (5); noncross-linked fibrin was obtained similarly except that EDTA replaced calcium chloride, Factor XIII, and β-mercaptoethanol.

**Digestion of Cross-linked Fibrin**—One gram of freeze-dried cross-linked fibrin was suspended in 20 ml of prewarmed (37°C) 0.15 M Tris-HCl buffer, pH 7.8, containing 5 mM calcium chloride. An aliquot of 0.6 ml of human plasmin (10.2 CTA units/ml; 12.7 CTA units/mg of protein) which was kindly provided by Dr. David L. Aronson

1 The abbreviations used are: CTA, Committee on Thrombolytic Agents (20); SDS, sodium dodecyl sulfate; KIU, kallikrein inhibitor units; NIH, National Institutes of Health units; ATU, antithrombin units; PAGE, polyacrylamide gel electrophoresis; NDSK, NH2-terminal disulfide knot.
(Bureau of Biologics, Food and Drug Administration, Rockville, Md.) was added and the mixture was incubated with gentle magnetic stirring at 37°C for 24 h, at which time the particulate material was completely dissolved. To stop the reaction, 1.0 ml of Trasylol (aprotinin, 10,000 KIU/ml, Mobay Chemical Corp., New York) was added.

**Preparation of Degradation Products of Fibrinogen, Cross-linked and Non-cross-linked Fibrin**—Fragment DD from a cross-linked fibrin digest was prepared by gel filtration on Sepharose CL-6B of a cross-linked fibrin digest containing primarily this species, as outlined in Fig. 1A. Fragments D (Stage 2), E (Stage 2), and E (Stage 3) were obtained from Stage 2 and Stage 3 digests of fibrinogen or non-cross-linked fibrin, respectively, by preparative electrophoresis on a Pevikon (C-870; Mercer, N. J.) block (22). The NH₂-terminal disulfide knot (NDSK) was isolated from cyanogen bromide-degraded fibrinogen according to the method of Bloombach and colleagues (23). Purification was done with two modifications: 0.1 M sodium chloride was added to 10% acetic acid (10) to improve the gel filtration pattern on Sephadex G-100 and an additional gel filtration step was done on Sepharose CL-6B (1 x 95 cm) in 0.05 M Tris-HCl buffer, pH 7.4, containing 1 M sodium chloride, to remove any aggregated material. Fragments X from Stage 1 and Stage 2 digests of fibrinogen and Fragment Y from the latter digest were purified according to the method of Murano and colleagues (27). Molecular weights of the polypeptide chains were determined by SDS-PAGE (28).

**Polyacrylamide Gel Electrophoresis**—Two systems were used with acrylamide (Eastman) recrystallized from chloroform. Tris/glycine-PAGE was used to test proteins in a nondissociating medium (29). Minor modifications were introduced: 9% gels (0.5 x 9 cm) were used; the upper and lower reservoir buffer contained 0.27 M Tris and 0.036 M glycine, pH 8.6, and sample and stacking gels were not used. Approximately 10 μg of protein/gel was electrophoresed at a constant current of 4 mA/gel for 2 h. Staining was performed by the method of Fairbanks and colleagues (25). SDS-PAGE was done in 7% gels (0.5 x 9 cm) containing 0.1% SDS using the method of Weber and Osborn (29). For routine protein analysis and to assess the amount of contamination in purified species of Fragment E, approximately 10 μg of sample was applied/gel. However, in order to align the purified preparations of Fragments E with the Fragment E varieties present in the unfractionated digest, these gels were underloded, using approximately 1.0 μg of sample/gel.

**Molecular Weight Determination**—Four preparations of purified Fragments E were reduced and carboxymethylated by the method of Murano and colleagues (27). Molecular weights of the polypeptide chains were determined by SDS-PAGE (28).

**Double Immunodiffusion**—Immunodiffusion was done in 1% agarose gel (SeaKern, Bausch and Lomb) according to the method of Ouchterlony (29). Slides were stained with Coomassie brilliant blue by the method of Fairbanks and colleagues (25). Antisera were raised in white New Zealand rabbits against fibrinogen Fragments D (Stage 3) and E (Stage 3), absorbed with normal human serum and then with appropriate fragments to remove any cross-reactivity.

**Binding Experiments**—Purified fragments (1 mg in 1 ml) were dialyzed against three changes of 100 ml of 0.15 M Tris-HCl buffer, pH 7.4, containing 0.15 M Tris-HCl buffer, pH 7.4, containing 0.028 M sodium citrate, 0.2 M L-α-amino acid, and 0.02% sodium azide. Approximately 500 mg of a cross-linked fibrin digest was diluted with equal volume of double concentration buffer and chromatographed at a flow rate of 60 ml/h. Fractions (3 ml) were collected, assayed for absorbance at 280 nm, and pooled as indicated, and the solution was concentrated by ultrafiltration. B, Peak 1 was diluted with equal volume of 6 M urea, 0.05 M sodium citrate, pH 5.5, and incubated at 37°C for 4 h; then 100 μg of this sample was rechromatographed on a Sepharose CL-6B column (25 x 170 cm) in the same buffer as in A. Fractions (3 ml) were collected and pooled as indicated. Flow rate was 60 ml/h. C, Peak 1B was dialyzed against 500-fold volumes of 0.01 M sodium carbonate buffer, pH 8.9, then 10 mg of the preparation was applied on a DEAE-cellulose (D502 Whatman, Clifton, N. J.) column (0.6 x 6 cm) and eluted at a flow rate of 90 ml/h with a linear gradient of 0 to 0.5 M NaCl in 0.01 M sodium carbonate buffer, pH 8.9. Fractions (1 ml) were collected and pooled as indicated and the solution was dialyzed in 3 500-fold volumes of 0.15 M Tris-HCl buffer, pH 7.8. D, Peak 1P material was dialyzed against 5 500-fold volumes of 0.01 M sodium carbonate buffer, pH 8.5. About 100 mg was applied on a DEAE-cellulose column (1 x 50 cm) and eluted stepwise with 0.2 and 0.5 M NaCl washes in the same buffer. Fractions (3 ml) were collected and pooled and the solution was dialyzed in 3 500-fold volumes of 0.15 M Tris-HCl buffer, pH 7.8.

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2 Stage 1 digest of fibrinogen contains mainly Fragment X, a Stage 2 digest is characterized by the presence of Fragments X, Y, D, and E, while a Stage 3 digest is characterized by the presence of Fragments D and E only (21).
Fragment E (2 mg in 0.5 ml) were dialyzed against three changes of 50 ml of 0.15 M Tris-HCl buffer, pH 7.8, for 24 h and diluted to 1 mg/ml in the same buffer. Human plasmin (10.2 CTA units/ml) was added in the ratio of 0.008 CTA units/mg of Fragment E and incubated at 37°C for 4 h. Digestion was stopped by the addition of 0.01 ml of Trasylol and the products were analyzed by SDS- and Tris/glycine-PAGE.

**RESULTS**

**Purification of Fragments E**—The digestion of cross-linked fibrin by plasmin resulted in the formation of the DD.E complex and Fragments DD and E as the major high molecular weight products. In the experimental conditions used, both Fragment DD and the DD-E complex were present as shown in Tris/glycine-PAGE (Fig. 2, upper panel). In SDS-PAGE (Fig. 2, lower panel) the complex dissociated and Fragment DD migrated as one homogeneous band. Three bands of Fragment E were distinguished. The symbols E1, E2, and E3 were assigned to the molecules in order of increasing electrophoretic mobility (Fig. 2). Their molecular weights, approximately 60,000, 55,000, and 50,000, respectively (Table I), were calculated from molecular weights of reduced polypeptide chains determined on SDS-PAGE.

Cross-linked fibrin digest chromatographed on a Sepharose CL-6B column was separated into four major peaks (Fig. 1A). Peak I contained DD.E complex, Fragment DD, and Fragments E1 and E2. Both Fragment E species participated in the DD.E complex since no free Fragment E was seen on Tris/glycine-PAGE (Fig. 2). Peak II (Fig. 1) had primarily Fragment E3, contaminated with a small amount of Fragment DD. There was no indication of the presence of a DD complex in this fraction (Fig. 2). Peak III (Fig. 1) contained heterogeneous material of $M_r = 21,000$ which was not resolved into discrete bands in either SDS- or Tris/glycine-PAGE. The pattern in SDS gels did not change after reduction and since this material reacted with antiserum against Aa chain of human fibrinogen, it may represent a chain remnants (33, 34) designated as Fragment A (35). Low molecular weight peptides were present in Peak IV (Fig. 1). Gel filtration of a cross-linked fibrin digest on Sepharose CL-6B did not adequately separate Fragment DD and the complex; however, it did provide complete separation of Fragments E1 and E2 from Fragment E3. This observation allowed the conclusion that Fragments E1 and E2 were involved in the DD.E complex while Fragment E3 was not (Fig. 2 lower panel).

Peak I from the Sepharose CL-6B column (Fig. 1A), which contained the DD.E complex and Fragment DD, was diluted with an equal volume of 6 M urea in 0.05 M sodium citrate, pH 5.5, and incubated at 37°C for 4 h. This treatment dissociated the DD.E complex and caused aggregation of Fragment DD.

**TABLE I**

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<tr>
<td>$M_r$</td>
<td>58,900 ± 1,200</td>
<td>55,500 ± 1,700</td>
<td>49,500 ± 700</td>
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* The molecular weight of dimeric Fragment E was obtained by addition of the molecular weights of the three polypeptide chains and doubling this figure.
(19). The sample was then rechromatographed on a Sepharose CL-6B column, completely separating Fragment DD from Fragments E (Figs. 1B and 2). Tris/glycine-PAGE of Peak IA (Fig. 2) showed that Fragment DD was aggregated and did not enter the gel. Peak IB contained two species of Fragment E, that is Fragments E₁ and E₂ (Fig. 2).

In order to separate Fragments E₁ and E₂, approximately 10 mg of the mixture from Peak IB was rechromatographed on a DEAE-cellulose column with a linear gradient of sodium chloride (Fig. 1C). SDS- and Tris/glycine-PAGE (Fig. 2) demonstrated that DEAE-cellulose chromatography separated Fragments E₁ and E₂, Peaks IB' and IB'', respectively, with less than 1% cross-contamination as determined using overloaded Tris/glycine-PAGE.

Peak II from the original Sepharose CL-6B column, which contained Fragment E₂ contaminated with Fragment DD, was rechromatographed on a DEAE-cellulose column developed with a stepwise gradient of sodium chloride (Fig. 1D). The 0.2 M sodium chloride wash, Peak II', eluted Fragment DD while the 0.5 M sodium chloride wash, Peak II, eluted Fragment E₂ (Fig. 1D). SDS- and Tris/glycine-PAGE (Fig. 2) showed that Peak II contained a pure preparation of Fragment E₂.

Comparison of the Tris/glycine gel patterns of Peaks IB', IB'', and IIB, containing Fragments E₁, E₂, and E₃, respectively (Fig. 2), demonstrated that the three species of Fragment E differed not only in molecular weight but also in charge.

All three purified species of Fragment E gave a continuous immunoprecipitation line with a monospecific anti-E antiserum in double immunodiffusion (Fig. 3). No immunoprecipitation was observed with anti-D antiserum.

The Binding of Fragments E₁, E₂, and E₃—The DD-E complex is a unique soluble degradation product of cross-linked fibrin (30) and it was of interest to see whether the complex could be re-formed from its components. Thus, the three purified species of Fragment E were incubated with Fragment DD, which was isolated by ion exchange chromatography under nondenaturing conditions (19), and monitored for the formation of the DD-E complex. The binding was visualized in Tris/glycine-PAGE by the disappearance of Fragment DD and the appearance of the complex. The uncomplexed Fragments E in the mixtures are not easily quantitated due to the relatively light staining of these diffuse bands. Three different preparations of each fragment were tested and all results were consistent. Fragments E₁ and E₂ combined with purified Fragment DD, forming the DD-E complex (Fig. 4). The Fragments E also bound with Fragment DD present in the unfractionated cross-linked fibrin digest. The entire free Fragment DD in the digest was converted into the DD-E complex that on Tris/glycine-PAGE appeared identical with the complex present in the digest. This showed that the free Fragment DD in the cross-linked fibrin digest was capable of forming the DD-E complex and disruption of the complex during digestion did not adversely affect the binding function of Fragment DD.

Fragment DD incubated with ¹²⁵I-labeled Fragment E₁ in human plasma or in buffer formed the DD-E complex. In plasma milieu, 94.9 ± 6.9% of labeled Fragment E₁ was recovered in the complex as compared with 100% in buffer. Therefore, the binding of Fragments DD and E₁ can occur even in the presence of high concentrations of human plasma proteins, indicating the specificity of this reaction.

Fragments E₁ and E₂ bound only with Fragment DD from a cross-linked fibrin digest, but not with the DD-E complex, fibrinogen, or any of the plasmic degradation products of fibrinogen or of noncross-linked fibrin (Table II).

![Fig. 3. Double immunodiffusion of Fragments E₁, E₂, and E₃ with monospecific anti-E antiserum.](http://www.jbc.org/)

![Fig. 4. Demonstration of the binding of Fragments E₁, E₂, and E₃ with Fragment DD by the formation of the DD-E complex. Tris/glycine-PAGE on 9% gels at pH 8.6: 1, cross-linked fibrin digest; 2, Fragment DD; 3, Fragment E₁; 4, a mixture of Fragments DD and E₁; 5, Fragment E₂; 6, a mixture of Fragments DD and E₂; 7, Fragment E₃; and 8, a mixture of Fragments DD and E₃.](http://www.jbc.org/)
Demonstration of Binding by the Formation of Stable Complexes

The binding studies were done either in the presence of hirudin (H) at 10 ATU/mg of protein or thrombin (T) at 20 NIH units/mg or in the absence of any of these agents. NDSK = NH2-terminal disulfide knot.

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Fragment E3 did not bind to either purified Fragment DD (Fig. 4) or to that present in the digest. Evidently, the binding region in Fragment E3 was either absent or inoperative.

When Fragment DD was purified from a plasmic digest of cross-linked fibrin by DEAE-cellulose chromatography, about 5% of the Fragment DD was cleaved forming two species of $M_r = 102,000$ and $81,000$ (19). On Tris/glycine gels, these species were seen as a diffusely band of slightly faster electrophoretic mobility than Fragment DD. These species do not complex with Fragments E1 or E2 (Fig. 4), suggesting that this cleavage removes or alters the binding site present on Fragment DD.

Plasmic Degradation of Fragments E—In order to test whether the three species of Fragment E were intermediates in a stepwise degradation, the purified fragments were treated with plasmin. The electrophoretic mobility of Fragment E2 was unchanged after this treatment. However, Fragments E1 and E2 were converted to a species with the same electrophoretic mobility and antigenic specificity as Fragment E3. The resulting species were also unable to combine with Fragment DD. This demonstrated that the three species of Fragment E were not only related, but also represented stepwise plasmic degradation products.

Electrophoretic Mobility of Various Fragments E—Since Fragments E (Stage 2) and E (Stage 3) from fibrinogen or noncross-linked fibrin digest did not complex with Fragment DD, it was of interest to compare their electrophoretic mobilities with those of Fragments E1, E2, and E3 from cross-linked fibrin. In SDS-PAGE, Fragment E (Stage 2) from fibrinogen had the same electrophoretic mobility as Fragment E3; however, after thrombin treatment, the mobility resembled that of Fragment E3. Fragment E (Stage 3) from fibrinogen and Fragments E (Stage 2) and E (Stage 3) from noncross-linked fibrin migrated similar to Fragment E3. Therefore, Fragments E (Stage 2 or Stage 3) from fibrinogen or noncross-linked fibrin were similar to Fragment E3, with the exception that fibrinogen Fragment E (Stage 2) contained fibrinopeptide A (15, 36). In early or intermediate digests of fibrinogen, Fragment E species having the molecular weight or the electrophoretic mobility of Fragment E3 were not observed.

The Binding of Fragment DD—Since Fragment DD was the only product which bound with Fragments E1 and E2, the specificity of this reaction was tested. Fragment DD and also the DD.E complex were mixed with a variety of purified plasmic degradation products from fibrinogen and noncross-linked fibrin (Table II). Formation of complexes was assessed.
Fig. 5. Demonstration of the binding of Fragment DD with thrombin-treated fibrinogen and its derivatives by the formation of high molecular weight complexes. Tris/glycine-PAGE on 9% gels at pH 8.6: 1, cross-linked fibrin digest; 2, Fragment DD; 3, fibrinogen (Fbg); 4, a mixture of Fragment DD and thrombin (T)-treated fibrinogen (Fbg); 5, Fragment X (Stage 1, st. 1); 6, a mixture of Fragments DD and thrombin (T)-treated Fragment X (Fbg); 7, Fragment Y; 8, a mixture of Fragments DD and thrombin (T)-treated Fragment Y; 9, NH\textsubscript{2}-terminal disulfide knot (NDSK); 10, a mixture of Fragment DD and thrombin (T)-treated NH\textsubscript{2}-terminal disulfide knot (NDSK).}

by Tris/glycine-PAGE (Fig. 5). Fragment DD formed a complex with Fragments E\textsubscript{1} and E\textsubscript{2} from cross-linked fibrin. A complex was also formed with thrombin-treated fibrinogen and fibrinogen degradation products Fragments X (Stage 1), X (Stage 2), and Y (Table II and Fig. 5), but not with the same preparations without thrombin treatment. Also, there was no reaction between Fragment DD and Fragments E (Stage 2) or E (Stage 3) from fibrinogen (Table II). Treatment of the latter fragments with thrombin or plasmin did not induce binding. Therefore, it appeared that Fragment DD cannot bind to the intact fibrinogen molecule or its early degradation products, except after thrombin treatment. However, the Fragment E moiety released from fibrinogen was nonreactive regardless of thrombin action. Similarly, Fragment DD did not combine with Fragments E (Stage 2) or E (Stage 3) from noncross-linked fibrin. On the other hand, Fragment DD formed a complex with thrombin-treated NH\textsubscript{2}-terminal disulfide knot (NDSK) (Fig. 5 and Table II). The DD-E complex present in the unfractionated digest or re-formed from the purified components contains one Fragment DD and one Fragment E moiety (30). Since the complexes formed between Fragment DD and fibrin monomer or thrombin-treated Fragments X, Y, or NDSK have an electrophoretic mobility on Tris/glycine gels comparable to the DD-E complex (Fig. 5), it appears that the components of these complexes are also present in a 1 to 1 ratio. Fragment DD was mixed with the various species in a 1 to 2 molar ratio. In all cases, the excess uncomplexed material can be seen on the Tris/glycine gel. Excess NDSK is not easily quantitated due to the low relative protein concentration, low stainability, and diffuse nature of this band. Both the electrophoretic mobility of these complexes (Fig. 5) and the relative concentration of the excess of uncomplexed material indicate that the complexes formed consist of one molecule of Fragment DD and one molecule of the complementary fragment.

The DD-E complex present in a cross-linked fibrin digest did not combine with any of the molecules tested; thus, in the DD-E complex, the binding sites were probably mutually saturated (Table II).

**DISCUSSION**

Activation of fibrinogen by thrombin causes the release of fibrinopeptides A and B and is followed by the formation of an ordered fibrin polymer, probably due to the association of sets of complementary binding sites (37, 38). Many investigators have attempted to localize the functional groups responsible for polymerization in the fibrin monomer molecule. These studies have implicated carboxylic acid groups (39), amide groups of glutamine (40), histidine (41, 42), tyrosine (42-45), lysine (46, 47), and tryptophan (48) residues of the molecule.

Recent investigations are focused on defining areas of fibrin monomer which are active in polymerization. This was approached by identifying binding regions present on plasmic degradation products of fibrinogen or fibrin. Binding sites have been localized on Fragment D and the NH\textsubscript{2}-terminal domain of fibrinogen (52-54). Specifically, Kudryk and colleagues suggested that the COOH terminus of Fragment D \(\gamma\) chain contains a binding region (52). Localization of the NH\textsubscript{2}-terminal binding site is controversial. Kudryk and colleagues (52), using insolubilized fibrin monomer-Sepharose affinity chromatography, implied that either the NH\textsubscript{2} terminus of the B\beta chain or the COOH terminus of the \(\gamma\) chain of the Fragment E moiety are necessary for polymerization. In a later report, dealing with fibrinogen Detroit, the authors suggested that the NH\textsubscript{2}-terminal binding site is at or near residue 19 on the A\alpha chain (50). Shen and colleagues (53) indicated that the COOH terminus of the A\alpha chain facilitates polymerization. Recent evidence demonstrates that the tripeptide Gly-Pro-Arg inhibits fibrin monomer polymerization, localizing a polymerization region on the A\alpha chain of fibrinogen, very close to fibrinopeptide A (54).

The present work shows that binding sites are present on Fragments E\textsubscript{1} and E\textsubscript{2} from cross-linked fibrin digest and that the complementary sites are located on Fragment DD. The stable DD-E complex is a result of saturation of these sites on both molecules (Table II). This interaction would resemble the situation in a fibrin fiber, in which the complementary binding sites on fibrin monomer molecules are mutually saturated. Since the DD-E complex is the primary plasmic degradation product of cross-linked fibrin and is released from fibrin as an intact moiety (30), it is possible that in the DD-E complex, the spatial arrangement of the binding sites on its components, that is, Fragments DD and E, is very much the same as the spatial arrangement of these regions in polymerized fibrin. This suggests that fibrin monomer molecules polymerize by the interaction of the complementary binding sites on the Fragment D and Fragment E moieties. Conse-
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Binding Sites on Fragment E—Fragment E formed by plasmic degradation of cross-linked fibrin can exist in forms having significantly higher molecular weight than that previously reported for Fragment E isolated from terminal plasmic digests of fibrinogen (11-14), noncross-linked (12), and cross-linked (11) fibrin. Fragments E1 and E2 can combine with Fragment DD to form a DD-E complex; however, Fragment E2 cannot (Fig. 4, Table II). Fragments E1 and E3 degraded with plasmin not only have the same electrophoretic mobility as Fragment E2, but they also lose the ability to bind with Fragment DD. It appears that the plasmic digestion step converting Fragment E2 to E3 does not affect the binding sites. However, the step from Fragment E3 to E4 is very critical since the loss of peptide material of M, = approximately 5000 from Fragment E2 (Table I) prevents binding of the resulting species to Fragment DD. Therefore, the released polypeptide chains either contain sites responsible for binding, or include segments which modify these sites. Thus, Fragment E4 is the key intermediate for the isolation of these binding sites.

Fragments E from fibrinogen and noncross-linked fibrin do not bind with Fragment DD, indicating that there is a lack of functional binding sites. Since fibrinogen Fragment E (Stage 2), after thrombin treatment, has a lower molecular weight than Fragment E2, the former appears to be more degraded. Such degradation would explain the absence of binding. It is possible that the species corresponding to Fragment E2 may be either short-lived or perhaps not formed at all during plasmic digestion of fibrinogen.

The complementarity between Fragments E1 or E2 and Fragment DD is specific since it occurred in plasma. These Fragments E did not bind to fibrinogen, fibrinogen degradation products, or to Fragments D from noncross-linked fibrin (Table II). Structurally, the Fragment D (Stage 2) molecule corresponds to half of Fragment DD. One could expect that Fragments E1 or E2 would bind either one or two such Fragment D molecules. But, neither a D-E or a D-D-E complex was observed in the present work (Table II). Although there is some evidence for the existence of a D-E complex in a plasmic digest of fibrinogen (55-58), the association is weak, resulting in an unstable complex which is dissociated during PAGE in the Tris/glycine buffer system (Table II). It is plausible that the cross-linking bonds in the Fragment DD molecule stabilize the spatial arrangement of the binding sites on the two Fragment D moieties and may bring them into closer proximity. This enables the dimeric binding site of Fragment E to link two complementary binding sites on Fragment D moieties at the same time, providing the complex with great stability and mutual saturation of the binding sites on both molecules. This conclusion is supported by the fact that Fragments E1, E2, and DD do not bind with the DD-E complex (Table II).

Binding Sites on Fragment DD—Fragment DD binds not only to Fragments E1 and E2 but also to fibrinogen, and fibrinogen Fragments X (Stage 1), X (Stage 2), and Y, after thrombin treatment (Table II, Fig. 5). These results support the hypothesis that one set of binding sites involved in fibrin polymerization is located in the NH2-terminal domain of the fibrinogen molecule and it is made accessible after cleavage of fibrinopeptides (51). The binding sites on Fragment DD which mediate complex formation with thrombin-treated fibrinogen or Fragments X or Y are probably the same sites which are involved in the binding with Fragments E1 and E2. The binding of Fragment DD to thrombin-treated fibrinogen corroborates the results of Dray-Attali and Larrieu (59) who found that Fragment DD greatly increased thrombin clotting time and inhibited fibrin monomer polymerization. The binding sites on Fragment DD link to the binding sites on the NH2-terminal region of thrombin-treated fibrinogen. The formed complex has all the binding sites on the NH2-terminal region occupied, but the binding sites on the Fragment D moieties of fibrin monomer molecules are still available. Polymerizing fibrin may incorporate this complex but only through the latter sites. For this reason, the Fragment DD-fibrin monomer complex incorporates into fibrin less efficiently than fibrin monomer, thereby inhibiting the polymerization reaction. This observation also suggests that Fragment DD, per se, may not be present in vivo during fibrinolysis but may be linked to fibrin monomers or its fragments. On the other hand, the DD-E complex probably circulates as a free species.

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