Interaction of Fibrinogen and Its Derivatives with Fibrin*

(Received for publication, May 13, 1988)

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The binding between complementary polymerization sites of fibrin monomers plays an essential role in the formation of the fibrin clot. One set of polymerization sites involved in the interaction of fibrin monomers is believed to pre-exist in fibrinogen, while the complementary set of binding sites is exposed after the cleavage of fibrinopeptides from fibrinogen. The polymerization sites present in fibrinogen and its derivatives mediate their binding to fibrin. Although the binding of fibrinogen and its derivatives to fibrin have been qualitatively studied, there has been no systematic, quantitative investigation of their interaction with forming or preformed clots. In the present study, the binding of fibrinogen and fragments DD, D1, and E1 was measured using a sonicated suspension of plasminogen- and thrombin-free human cross-linked fibrin as a model of a preformed clot. Dissociation constants of 0.056, 0.19, and 2.44 μM, and the number of binding sites corresponding to 0.10, 0.21, and 0.13/fibrin monomer unit of fibrin polymer were found for fibrinogen, fragment DD, and fragment D1, respectively. Fragment E1 did not bind to sonicated noncross-linked or cross-linked fibrin suspensions. However, it was bound to forming fibrin clots as well as to fibrin-Celite, suggesting that the binding sites on fibrin involved in the interaction with fragment E1 may have been altered upon sonication. Affinity chromatography of various fibrinogen derivatives on a fibrin-Celite column showed that only part of the bound fragment DD was displaced by arginine, whereas fragments D1 and E1 were completely eluted under the same conditions. The results indicate that interaction of fibrinogen with the preformed fibrin clots is characterized by affinity in the nanomolar range and that binding between fibrin monomers, in the process of clot formation, could be characterized by even a higher affinity.

Fibrin constitutes a network of polymers that bind together platelets and several proteins in blood clots. Thrombin catalyzes the conversion of fibrinogen to fibrin monomer which spontaneously polymerizes in a half-staggered manner to form a three-dimensional network of fibers (1). Electron micro-

* This work was supported by Grants HL36221 (to A. Z. B.) and HL30954 (to J. W. W.) from the National Heart, Lung and Blood Institute, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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scope data provide evidence that initial polymers consist of double-stranded protofibrils (2). The latter are kept together by interaction of complementary polymerization sites on fibrin monomer molecules that are spatially organized in a face-to-face manner (3–6). One of the polymerization sites involved in the formation of fibrin protofibrils pre-exists in fibrinogen near the ends of the molecule, while the complementary binding site in the central region is exposed after the cleavage of fibrinopeptides by thrombin. The initial formation of protofibrils is followed by their spontaneous lateral association, resulting in thickening to yield fibers. The coalescence of protofibrils seems to occur via a different set of polymerization sites than those involved in the generation of protofibrils from fibrin monomers.

A terminal degradation of fibrinogen or noncross-linked fibrin by plasmin in the presence of calcium ions yields fragments D1 and E0, which constitute the end and central regions of the molecule, respectively. However, degradation of cross-linked fibrin results in the (DD)E complex in which two covalently cross-linked D1 moieties are bound noncovalently to fragment E1 (7). Fragments D1 and DD retain affinity for fibrin monomer as evidenced by binding to the immobilized protein (8–10) and by inhibition of the rate of fibrin monomer polymerization (11). Fragment E0, but not fragment E0, has been shown to bind to cross-linked fibrin polymers (6), to blood clots formed in vitro as well as in animals (12), and to thrombi in humans (13). However, binding of fibrinogen and its derivatives to the fibrin clot has not been investigated systematically, and the respective dissociation constants and number of moles bound/mole of fibrin monomer unit of fibrin polymer are not known.

The research described in this paper is one approach to the determination of the fibrin-binding properties of fibrinogen and its derivatives, fragments DD, D1, and E1, all of which contain binding sites with affinity for a preformed fibrin clot. The quantification of binding is important in understanding how fibrin in blood clots contributes to the growth of a thrombus in vivo and to elucidate the regulatory role of fibrinogen and fibrin fragments on the propagation of the fibrin network. In addition, the data provide the quantitative basis for the use of fragments with affinity for fibrin as thrombus-specific molecular markers.

EXPERIMENTAL PROCEDURES

Materials

Human fibrinogen (grade L) was obtained from Kabivitrum, Stockholm, Sweden. The material was depleted of contaminating plasminogen (14) by two passages of the protein (1 g, dissolved in 100 ml of water) through a 120-ml column of lysine-Sepharose (Pharmacia, Uppsala, Sweden), equilibrated with 0.05 M sodium citrate, containing 0.1 M NaCl and 25 μIU/ml of traspoyl (Aprotinin, Mobay Chemical Corp., New York, NY), adjusted to pH 7.4. Human α-thrombin was a generous gift from Dr. John W. Fenton, II, New York State Department of Health, Albany, NY. The product had a specific activity of 3315 units/mg. Human fibrinogen (Band 1), (18), fragment
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D, (16), fragment DD (7), and fragment E, (7) were purified as described in previous reports. D-Phe-Pro-Arg-chloromethylketone was a product of Behring Diagnostics. a-Aminocaproic acid and Tween 80 were obtained from Sigma. Other reagents were of analytical-grade purity.

Methods

Radioiodination—Radioiodination of fibrinogen (Band 1) and fragment DD with iodine-125 was performed using the sodium monochloride technique of McFarlane (17) with modifications described previously (18).

Preparation of Noncross-linked and Cross-linked Fibrin Suspensions—A homogeneous suspension of fibrin, free from plasminogen and thrombin, was prepared by a modification of a procedure described by Lucas and colleagues (19).

Noncross-linked fibrin was prepared using 50 ml of plasminogen-free human fibrinogen (7 mg/ml) containing 50 mM sodium citrate. This fibrinogen solution was diluted with 70 ml of 0.05 M Tris-HCl buffer containing 0.15 M NaCl, pH 7.4, and 70 µl of human a-thrombin (350 units total) was then added. The formed clot was incubated at 37°C for 2 h. It was then cut in small pieces and suspended in 1000 ml of 1 M NaCl and 0.1 M a-aminocaproic acid, adjusted to pH 7.4. The suspended clot was stirred on a magnetic stirrer in a cold room overnight to remove protein impurities, in particular any trace of plasminogen that may have been left after affinity chromatography. The suspension was then filtered using a Buchner funnel with 0.05 M Tris-HCl buffer, containing 0.15 M NaCl and 0.01% Tween 80, pH 7.4. The clot suspension was sonicated in a Heat Systems-Ultrasonics cell disruptor, equipped with a microtip, at maximum power (setting 6) for periods of 5 min with ice cooling. The protein concentration was determined after dissolving it in 2% acetic acid and reading the absorbance at 280 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) was performed on the unfractionated clot suspension. The fibrin suspension contained on the average 8 mg/ml of protein, as determined after dissolving it in 2% acetic acid and reading the absorbance at 280 nm versus the control that did not contain fibrinogen. The clot suspension was stored at 4°C for up to 1 week; for longer storage, it was frozen at -20°C. Before use, the fibrin suspension was sonicated for 1 min.

Cross-linked fibrin suspension was prepared in the same way as above, except that before addition of thrombin to fibrinogen, 100 mM CaCl2 was added to the solution. To determine protein concentration, 0.2 ml of sonicated fibrin suspension was added to 1.8 ml of 8 M urea containing 10 mM dithiothreitol and incubated at 37°C for 2 h. The absorbance was read at 280 nm versus the control that did not contain fibrin. Sodium dodecyl sulfate-polyacrylamide gel analysis (20) of the reduced fibrin preparation showed that the preparation was noncross-linked. The suspension was stored at 4°C for up to 1 week; for longer storage, it was frozen at -20°C. Before use, the fibrin suspension was sonicated for 1 min.

Electron Microscopy—Sonicated fibrin preparations were applied to carbon-coated Formvar grids, negatively contrasted with 1% uranyl acetate and observed at a variety of magnifications with a Philips 400 electron microscope (Philips Electronic Instruments, Mahwah, NJ). The microscope magnifications were calibrated with tropomyosin paracrystals with a repeat of 39.5 nm.

Preparation of Noncross-linked and Cross-linked Fibrin-Celite—Noncross-linked fibrin-Celite was prepared essentially as described earlier (21) with the following modifications. After removing fine particles from the starting Celite (Hyflo Super Cel, Fluka, Ronkonkoma, NY) by repeated suspension in water, the material was washed twice, each time by gently stirring with 1 liter of 1 M NaCl for 2 h. This treatment removes material present in some batches of Celite that otherwise retards clotting of fibrinogen. To obtain fibrin-Celite, fibrinogen was clotted in the presence of Celite and then washed on a Buchner funnel with 0.05 M Tris-HCl buffer, containing 0.15 M NaCl, pH 7.4. Cross-linked fibrin-Celite was prepared by suspending washed, noncross-linked fibrin-Celite in Tris buffer, containing 5 mM CaCl2, and 1 mM mercaptoethanol and incubating at 20°C for 15 h. Cross-linked fibrin-Celite was subsequently washed as above. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) showed complete cross-linking of the a and y chains.

Binding Studies with Fibrin Suspensions—Two methods were used for measuring the binding of fibrinogen or fibrin derivatives to fibrin. 1) Precipitation of fibrin suspension (100 µl of 0.5-0.6 mg/ml) in 0.05 M Tris-HCl buffer, containing 0.15 M NaCl and 0.01% Tween 80, pH 7.4, was mixed with radiolabeled ligand, and the volume was adjusted to 200 µl with the buffer. The suspension was incubated at room temperature for 15 min and then centrifuged in an Eppendorf centrifuge for 5 min. An aliquot of the supernatant was withdrawn and counted for radioactivity to determine the concentration of unbound ligand. In a parallel sample, buffer (100 µl) was used instead of fibrin suspension and counted (total ligand). The difference between total and unbound radioactivity gave bound ligand. In competition experiments, radiolabeled ligand was incubated with fibrin suspension, followed by addition of nonradioactively labeled competing ligand. The binding constant for the ligand-fibrin interaction, incorporation of radioactive ligand into the clot, was followed directly by measuring radioactivity in the clot. For this purpose, fibrin suspension in buffer (100 µl) containing 0.05 mg/ml of fibrin was mixed with 125I-labeled ligand at the desired concentration range, and the volume was brought up to 200 µl. The suspension was incubated at room temperature for 15 min with occasional vortexing and centrifuged in an Eppendorf centrifuge for 5 min. The entire supernatant was carefully removed with a microsyringe, leaving the clot undisturbed at the bottom of the conical Eppendorf tube. The supernatant was then collected by centrifugation. The upper quarter of the tube was cut off with scissors, and the bottom portion containing the clot was counted for radioactivity.

In a parallel control experiment, 125I-heparin, a nonradioactively labeled fragment E, that did not bind to fibrin was added, using the same number of counts as those used in the ligand binding experiments. The control experiments gave the amount of nonspecifically trapped radioactivity contained in the fibrin clot. Subtraction of this figure from radioactivity of labeled ligand incorporated into the clot gave the specific binding of the ligand to fibrin.

Dissociation Constants—The dissociation constants of ligand-fibrin interaction and the number of ligand molecules bound/molecule of fibrin monomer unit of fibrin polymer were determined by Scatchard analysis of the binding data (22). The best fit of lines was obtained by least square analysis using the StatView 512+ program (Brainpower, Calabassa, CA) on a Macintosh Plus computer.

Binding Experiments with Fibrin-Celite—Binding of fibrinogen and its various derivatives to noncross-linked or cross-linked fibrin-Celite was investigated by affinity chromatography. Fibrin-Celite (1.5 ml) was packed in a column (0.8 x 3.5 cm) and equilibrated with 0.05 M sodium phosphate buffer, containing 0.3 M NaCl and 0.05% Tween 80, pH 7.4. Radiolabeled fibrinogen or fibrin fragment (4-20 µg) was applied to the column. The nonbound protein was washed with 8 column volumes of the equilibration buffer. The bound ligand was then eluted with a solution containing buffer containing 0.5 M arginine. The radioactive ligand not eluted from the column by arginine was determined by counting the contents of the column.

RESULTS

Characterization of Sonicated Fibrin Preparations—Electron microscopy of negatively contrasted, sonicated cross-linked fibrin preparations revealed somewhat irregular, elongated nodular structures that varied in length from about 40 to more than 300 nm with an average of 200 ± 100 nm (Fig. 1). The average diameter of the elongated structures was about 70 nm. Many branched fibrous networks consisting of these nodular elements were also observed. These networks could represent either aggregates of the smaller subunits or an incompletely fragmented clot. They usually appeared to consist of subunits similar in size to the individual elongated structures. Neither protofibrils nor normal fiber networks, consisting of cylindrical, branched fibers, were observed. In addition, the characteristic cross-striations of fibrin (23) were completely absent. From these results it may be concluded that the fibers had been sheared so that there were periodic breaks in the protofibrils and that the precise longitudinal alignment of monomers in protofibrils had been distorted. Since two-stranded protofibrils are made up of fibrin monomer molecules 45 nm in length, it can be calculated that each protofibril in a nodular structure with a length of 200 nm would consist of about 9 monomer molecules; depending
on the density of packing of protofibrils, each nodular structure may contain a total of about 1000 monomer molecules.

To determine whether sonication of the fibrin suspension affected the binding properties of fibrin, sonicated noncross-linked fibrin was dissolved in 0.05 M acetic acid. After pH adjustment to 7.4, the fibrin solution repolymerized and formed a clot that appeared normal. The repolymerized sonicated fibrin clot also incorporated $^{125}$I-labeled fragment E$_1$. This result showed that sonicated fibrin, after its dispersion into monomers, retained the ability to assemble to form functional clots.

Interaction of Fibrinogen with the Preformed Fibrin Clot—Addition of a constant concentration of $^{125}$I-fibrinogen (0.03 $\mu$M) to variable amounts of fibrin (0.05-3.2 mg/ml) showed that the fibrinogen was depleted from the solution until a plateau was reached above a fibrin concentration of 1.5 mg/ml (Fig. 2, top). To exclude any possibility that traces of thrombin contaminating fibrin may have caused clotting of the $^{125}$I-fibrinogen, binding studies were performed in the presence of D-Phe-Pro-Arg-chloromethylketone (10 $\mu$M), a potent inhibitor of thrombin. To minimize nonspecific binding of fibrinogen to fibrin, Tween 80 (0.03%) was included in the binding medium.

To determine the dissociation constant of the fibrinogen-fibrin complex, binding studies were performed at a constant concentration of fibrinogen and variable concentrations of $^{125}$I-fibrinogen. The amount of $^{125}$I-fibrinogen bound to fibrin was determined from the counts incorporated into the clot after centrifugation and removal of the supernatant. Under these conditions, fibrinogen binding to fibrin was also saturable (Fig. 2, bottom). Scatchard analysis of the binding data demonstrated that fibrin possesses a single type of binding site for fibrinogen with a dissociation constant of 56 nM (Fig. 2, bottom inset and Table I). The number of binding sites present in the cross-linked fibrin suspension was 0.10/fibrin monomer molecule. This number does not represent all the potential binding sites for fibrinogen present in fibrin, but only the sites that are not blocked by interaction with the complementary binding sites present on other molecules of fibrin monomer already incorporated into the fibrin network.

![FIG. 1. Electron microscopy of sonicated fibrin.](image)

![FIG. 2. Binding of $^{125}$I-fibrinogen by sonicated cross-linked fibrin.](image)

**TABLE I**

| Fragment | Fibrin type       | $K_d$, $\mu$M | No. |
|----------|------------------|---------------|-----|
| Fibrinogen| Cross-linked     | 0.056         | 0.10|
| DD       | Cross-linked     | 0.19          | 0.21|
| DD       | Noncross-linked  | 0.19          | 0.14|
| $D_1$    | Cross-linked     | 2.44          | 0.15|

**Binding of Fragment DD**—At a constant concentration of $^{125}$I-labeled fragment DD (0.18 $\mu$M and variable concentrations of fibrin) the interaction was concentration-dependent (Fig. 3, top). Scatchard analysis of the binding data obtained at constant concentration of fibrin and variable concentrations of $^{125}$I-fragment DD showed that the fragment bound to cross-linked fibrin with a dissociation constant of 0.19 $\mu$M (Fig. 3, bottom inset and Table I). The number of binding sites present in cross-linked fibrin was 0.21. The binding of fragment DD to sonicated noncross-linked fibrin suspension (data not shown) indicated that the affinity was the same as for cross-linked fibrin, but the stoichiometry was lower (Table I).

To investigate whether the binding of $^{125}$I-fragment DD to fibrin was specific, the effect of unlabeled fragment DD (0.09 $\mu$M) on the interaction of the radiolabeled fragment with fibrin was studied. As shown in Fig. 4, unlabeled fragment DD reversed the binding of the radiolabeled fragment in a dose-dependent manner, suggesting that the interaction between the fragment and fibrin was specific. To find out whether fragment DD and fibrinogen bind to the fibrin clot through a common polymerization site, competition of fibrinogen with the binding of $^{125}$I-fragment DD by fibrin was investigated.
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FIG. 3. Binding of $^{125}$I-labeled fragment DD by sonicated cross-linked fibrin. Top, binding of fragment DD to variable concentrations of fibrin. Binding studies were performed using method 1. Fragment DD concentration was 0.18 μM. Bottom, Scatchard analysis of fragment DD-fibrin interaction. Binding experiments were performed and the data analyzed as described in method 2. B and B/F were calculated as described in Fig. 1.

Like unlabeled fragment DD, fibrinogen also reversed the binding of fragment DD by fibrin. This finding supports the hypothesis that fibrinogen and fragment DD share a common binding region associated with polymerization site "a."

Binding of Fragment D1—Since fragment D1 corresponds structurally to half of fragment DD, its fibrin-binding ability was compared with that of the dimeric fragment. Studies at constant fragment D1 concentration (0.03 μM) and variable cross-linked fibrin concentrations showed that depletion of the fragment by fibrin occurred under the experimental conditions. Scatchard analysis of binding data obtained at constant fibrin concentration and variable fragment D1 concentration gave a dissociation constant of 2.44 μM and the number of fragment D1 molecules bound/fibrin monomer of 0.13 (Fig. 5, bottom inset and Table I).

To investigate further the differences in the fibrin binding properties of fragments DD and D1, affinity chromatography on cross-linked fibrin-Celite was performed with $^{125}$I-labeled fragments. An excess of labeled fragment DD or D1 was applied to the affinity column to saturate the fibrin-binding sites. After washing with the equilibration buffer, the bound fragments were eluted with 0.5 M arginine hydrochloride in the buffer (Fig. 6). The portion of the fragment that was not eluted with arginine and remained bound to the column amounted to 11 and 41% of the total bound radioactivity of fragments D1 and DD, respectively. This result showed that fragment D1 was displaced by arginine from fibrin-Celite more easily than fragment DD.

Interaction of Fragment E1—Contrary to the expectation of finding a strong binding between fragment E1 and sonicated fibrin, experiments evaluating the interaction of noncross-linked and cross-linked fibrin suspensions with radiolabeled fragment E1 at constant ligand concentration and variable fibrin concentrations, showed that there was no measurable affinity between this ligand and the fibrin matrix, even at high concentrations of fibrin (Fig. 7, top). To investigate the possibility that the fibrin binding affinity of fragment E1 may have been impaired during radioiodination, incorporation of radiolabeled fragment E1 into forming clots was tested. For this purpose, $^{125}$I-fragment E1 was mixed with Kabi fibrinogen, purified plasminogen-free fibrinogen, or normal human plasma and the solutions were clotted with thrombin. After 1

FIG. 4. Competition of unlabeled fibrinogen and fragment DD with labeled fragment DD binding. For binding to fibrin, radiolabeled fragment DD (4 μg) was mixed with a suspension of cross-linked fibrin (0.8 mg/ml) in 0.05 M Tris-HCl buffer containing 0.15 M NaCl and 0.01% Tween 80, pH 7.4. Variable amounts of unlabeled fragment DD or fibrinogen were added. After incubation at room temperature for 15 min, binding of radiolabeled fragment DD to the clot was determined by method 1. The amount of $^{125}$I-fragment DD bound in the absence of competing proteins is expressed as 100% bound.

Fig. 5. Binding of $^{125}$I-fragment D1 by sonicated cross-linked fibrin. Top, fibrin dependence of the binding of fragment D1. Binding studies were performed at constant concentration of radiolabeled fragment D1 (0.03 μM) and variable concentrations of cross-linked fibrin suspension as described in method 1. Bottom, Scatchard analysis of fragment D1-fibrin interaction. Binding studies were performed and the data was analyzed by method 2.
to fibrils or may function cooperatively with the protein binds to fibrin (Refs. 8, 9, respectively. The site a is exposed on fibrinogen since this number of the unpaired complementary binding sites, "B" and "b," are less well defined by some investigators polymers into a clot postulates at least four different complementary polymerization sites. Two complementary primary sites, "A" and "a," responsible for linear polymerization and formation of protofibrils (2), are located in the E and D domains of fibrin, respectively. The site a is exposed on fibrinogen since this protein binds to fibrin (Refs. 8, 9, 24 and Fig. 2). Two complementary binding sites, "B" and "b," are less well defined but are believed to mediate lateral coalescence of protofibrils or may function cooperatively with the A and a sites (6, 25). A preformed fibrin clot should contain only a limited number of the unpaired A and a sites at the ends of long fibrin filaments. Although the number of potential lateral binding sites available in a clot must be much greater than that of the free A and a sites, still the majority of the lateral sites are masked by being utilized to keep the protofibrils together. Disruption of the original fibrin clot network by sonication should separate fibrils from each other and cleave them to shorter species exposing many lateral and some primary polymerization sites. For this reason, sonicated fibrin was used in this work as a model of a preformed clot with an increased number of binding sites. It should be noted, however, that at the present time there are no reliable techniques available that can clearly distinguish between primary and lateral polymerization sites (6).

Measurements from electron micrographs of sonicated fibrin (Fig. 1) have revealed that an average fibrin nodule derived from the disrupted clot contains about 9 fibrin monomers/each protofibril. Thus, one free end appears per approximately 5 fibrin monomer molecules. This result is in agreement with the determined n values from Scatchard plots. It is of interest to note that repeated sonication of the same fibrin suspension did not markedly decrease the size of the fibrin nodules and did not affect the number of exposed binding sites present in the preparation, suggesting resistance of nodules against disruption and demonstrating some stability of nodular structures. It is interesting to note that a uniform 70-nm diameter of nodules is very similar to the 80-nm diameter of fibers formed by lateral aggregation of normal, nonsonicated protofibrils (6). These results, together with the absence of protofibrils in sonicated preparations, suggest that the main effect of sonication is lateral shearing of the fibers, although the organization of protofibrils is also disrupted.

Binding of Fibrinogen—The present data support the concept that the addition of fibrinogen molecules to the preformed fibrin clot occurs, in the absence of thrombin, to fibrinogen, fragment DD, and fragment D1 that was measured in this study was mediated predominantly by the site a. This mechanism would require that the addition takes place at the ends of the fibrils where free complementary...
polymerization sites A are expected to be available in the E domain of the terminal fibrin monomer molecules. The number of binding sites in sonicated fibrin, as calculated from a Scatchard plot (Fig. 2 and Table I), indicated that one such site should occur per approximately 10 monomer molecules. On this basis one can conclude that fibrinogen and fragment D, as well as half of the fragment DD molecules, bind to sonicated cross-linked fibrin via primary Aa interactions, but not through lateral sites. This conclusion would imply that a thrombus formed in vivo and exposed to the circulating blood will be capped by the fibrinogen molecules attached to the ends of fibrin fibers. In spite of considerable experimental efforts, no evidence was found for two classes of polymerization sites on the fibrinogen molecule with different Kd, which would have demonstrated directly affinity differences between the a and b sites. These polymerization sites may either be characterized by the same affinity for fibrin, may be integrated within one binding domain, or have too low an affinity to be detected in the concentration range of fibrinogen used in the present study. It may be that interactions between fragments and fibrin via lateral aggregation sites do not occur at all. For example, protofibrils may be needed for lateral interactions, either for cooperative effects of a large number of weak binding sites or for new sites produced by polymerization. This hypothesis is supported by the fact that protofibrils coalesce spontaneously, without the necessity of any further cleavage once they are produced by removal of a single pair of fibrinopeptides. Thus, lateral binding of protofibrils does not appear to require the uncovering of any additional binding sites.

The measurement of mutual affinity of fibrin monomers is experimentally difficult since monomers cannot be kept in equilibrium at neutral pH, due to the phase transition associated with clot formation. One can anticipate, however, that the binding affinity will be much stronger than that expressed by fibrinogen, which can bind to the terminal fibrin molecule via only one set of polymerization sites Aa. The binding of two fibrin monomer molecules will involve at least two sets of polymerization sites, that is Aa and Bb, that will increase the binding strength.

**Binding of Fragments DD and D.**—Fragment D had about 44-fold lower affinity than fibrinogen for the fibrin clot (Figs. 2 and 5 and Table I). This difference could arise from the loss of the COOH terminus of the α-chain in fragment D. However, a contribution of the coiled-coil region, which is partly missing in fragment D, cannot be ruled out, and its cleavage may have decreased the affinity of this fragment for fibrin.

Fragment DD had approximately 13-fold stronger binding affinity for fibrin than did fragment D. The presence of cross-links either increased the binding affinity of fragment DD for fibrin or generated a new binding site with stronger affinity for fibrin. The latter possibility is in agreement with earlier findings suggesting that cross-linking of fibrin yields a new binding site for fragment E (5). Affinity chromatography of fragments DD and D on fibrin-Celite (Fig. 6) suggested that fragment DD is more strongly bound to fibrin. Whether such binding reflects an interaction through a bi-valent set of polymerization sites, or an interaction via another site, remains to be elucidated.

Interaction of fragments DD and D with fibrin would effectively regulate the growth of a thrombus, particularly in fibrinolytic states. Under such conditions, degradation of cross-linked fibrin in a thrombus and cleavage of fibrinogen in the circulation would produce fragments DD and D, respectively, that, even below micromolar concentration, impair propagation of new fibrin network formation.

**Binding of Fragment E.**—Although fragment E bound to forming and preformed purified fibrin clots and to plasma clots, it did not bind to sonicated fibrin (Fig. 7, top). It is interesting to note that sonicated fibrin retained the property of binding not only fibrinogen, fragments DD and D, (Figs. 2, 3, and 5), but also plasminogen (19), single-chain urokinase (26), and tissue plasminogen activator. The absence of binding between fragment E and sonicated fibrin may possibly be explained by a model involving the two types of binding sites proposed earlier (5). The first type of binding involves Aa interaction between fragment E and fibrin, respectively. This mode of binding of fragment E, which can take place through the unoccupied terminal a sites of fibrin, can only be monomeric and, therefore too weak to be observable. This conclusion is supported by the lack of binding between fragments E and D. The second type of binding, much stronger than the first type, involves cooperative interaction of the A and B sites of fragment E with a and b sites of fibrin. It is possible that upon sonication of fibrin there is a distortion of either polymerization site a or b in the D domain. A lack of cross-striation in sonicated fibrin nodules observed in the electron microscope supports this possibility. Yet, polymerization sites A and B were functional, as demonstrated by binding of fibrinogen and fragments DD and D to sonicated fibrin. Since noncross-linked fibrin was not converted to monomeric units upon sonication, either sites a or b had to be functional to keep fibrin monomers together.

To reconcile the lack of binding of fragment E to sonicated fibrin with the expression of binding toward fibrin-Celite, forming fibrin clots, and preformed clots (12), the involvement of a second set of binding sites, B and b, in fragment E, should also be considered. From a number of considerations, it has been proposed that juxtaposition of two D domains in two different fibrin monomers and stabilization by cross-linking, yields a new site, b, for interaction with the E domain of another fibrin monomer (5). The nature and function of this new site is not fully understood, although its absence has been demonstrated in abnormal fibrinogen Tokyo II (27). This binding site may possibly be involved in the lateral interaction of protofibrils. Cross-linked fibrin probably interacts with fragment E via the cross-link-dependent site b. It is possible that upon sonication of fibrin there is a distortion of the b polymerization site in the D domain with concomitant loss of affinity for fragment E. This phenomenon is consistent with a hypothesis that fibrin polymerization sites involve amino acid residues located in non-adjacent sequences of the α, β, and perhaps γ chains and, in addition, require a specific conformation of that region (28, 29). In contrast to denaturants that cause an irreversible loss of functional polymerization sites, sonication appears to be a mild treatment. First, there was no loss of polymerization sites A in the E domain of sonicated fibrin. Second, dissolved, sonicated noncross-linked fibrin did repolymerize and formed a clot, indicating that its specific conformation had not been altered irreversibly.

**Acknowledgment.**—We greatly appreciate the help of Dr. Edward P. Kirby of Temple University for critical comments and discussions.

**REFERENCES**

1. Doolittle, R. F. (1984) Annu. Rev. Biochem. 53, 185-229
2. Hantgan, R. R., Fowler, W., Erickson, H., and Hermanns, J. (1980) Thromb. Haemostasis 44, 119-124
3. Blomback, B., Hessel, B., Hogg, D., and Therkildsen, L. (1978) Nature 275, 501-505
4. Shainoff, J. R., and Dardik, B. N. (1979) Science 204, 200-202
5. Eley, S. A., and Bodzynski, A. Z. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1374-1378
Interaction of Fibrin Derivatives with Clots

6. Weisel, J. W. (1986) Biophys. J. 50, 1079–1093
7. Olexa, S. A., and Budzynski, A. Z. (1979) Biochemistry 18, 991–995
8. Matthias, F. R., Heene, D. L., and Konradi, E. (1973) Thromb. Res. 3, 657–664
9. Kudryk, B. J., Collen, D., Woods, K. R., and Blomback, B. (1974) J. Biol. Chem. 249, 3322–3328
10. Olexa, S. A., and Budzynski, A. Z. (1979) J. Biol. Chem. 254, 4925–4932
11. Budzynski, A. Z., Olexa, S. A., and Brizuela, B. S. (1979) Biochim. Biophys. Acta 584, 264–287
12. Knight, L. C., Olexa, S. A., Malmud, L. S., and Budzynski, A. Z. (1983) J. Clin. Invest. 72, 2007–2013
13. Knight, L. C., Maurer, A. H., Robbins, P. S., Malmud, L. S., and Budzynski, A. Z. (1985) Radiology 156, 509–514
14. Deutsch, D. G., and Mertz, E. (1970) Science 170, 1095–1096
15. Pandya, B. V., and Budzynski, A. Z. (1984) Biochemistry 23, 460–470
16. Marder, V. J., James, H. L., and Sherry, S. (1969) Thromb. Diath. Haemorrh. 22, 234–239
17. McFarlane, A. S. (1963) J. Clin. Invest. 42, 346–361
18. Knight, L. C., Budzynski, A. Z., and Olexa, S. A. (1981) Thrombo. Haemostasis 46, 593–596
19. Lucas, M. A., Fretto, L. J., and McKee, P. A. (1983) J. Biol. Chem. 258, 4249–4256
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Husain, S. S., Lipinski, B., and Gurewich, V. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4265–4269
22. Scatchard, G. (1949) Annu. N. Y. Acad. Sci. 51, 660–672
23. Weisel, J. W. (1987) J. Ultrastruct. Mol. Struct. Res. 96, 176–188
24. Shainoff, J. R., and Page, I. H. (1960) Circ. Res. 8, 1013–1022
25. Shainoff, J. R., and Dardik, B. N. (1983) Ann. N. Y. Acad. Sci. 408, 254–267
26. Husain, S. S. (1985) Thromb. Haemostasis 54, 122
27. Matsuda, M., Baba, M., Morimoto, K., and Nakamikawa, C. (1983) J. Clin. Invest. 72, 1034–1041
28. Cierniewski, C. S., Kloczewiak, M., and Budzynski, A. Z. (1986) J. Biol. Chem. 261, 9116–9121
29. Budzynski, A. Z. (1986) CRC Crit. Rev. Oncol./Hematol. 6, 97–146