Subunit Structure of Fragment D from Fibrinogen and Cross-Linked Fibrin*

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SUMMARY

The different species of Fragment D were purified from plasmin digests of either fibrinogen or highly cross-linked fibrin and examined electrophoretically in sodium dodecyl sulfate on polyacrylamide gels before and after reduction by β-mercaptoethanol. It was found that Fragment D from cross-linked fibrin is approximately twice the size of that from fibrinogen, which indicates that the COOH-terminal cross-links in the γ chain remnants are not cleaved during fibrin digestion by plasmin. The first Fragment D to appear in plasmin digests of fibrinogen is designated Fragment D1, which is composed of a β′ chain (44,500 molecular weight), a γ′ chain (42,000 molecular weight), and an α′ chain (~15,000 molecular weight) linked together by disulfide bonds. Fragment D from fibrin differs in that it has a γ′ dimer (81,000 molecular weight) in place of γ′ chains and that it contains two each of the α′ and β′ chains. Further digestion of Fragment D from fibrinogen results in cleavage of the γ′ chain to give first the γ″ chain (37,000 molecular weight) and finally the γ‴ chain (27,000 molecular weight). Neither the γ″ chain nor the γ‴ chain contained the COOH-terminal cross-links in the intact γ chain or the γ′ chain.

It was possible to show by sodium dodecyl sulfate polyacrylamide gel electrophoresis that fibrinogen Fragment D1 could be cross-linked in the presence or absence of native fibrinogen through the formation of γ-γ' or γ'-γ' dimers.

Experimental Procedure

Reagents—Purified human fibrinogen, fibrin-stabilizing factor, and plasminogen were prepared as previously described (1, 2, 7) or, as in the case of urokinase (Calbiochem), obtained from commercial sources. Purified human thrombin was kindly supplied by Dr. D. L. Aronson, Division of Biological Standards, National Institutes of Health.
**Purification of Fibrinogen and Fibrin Degradation Products—**

Fibrinogen as well as fibrin degradation products were purified by a modification of the method reported by Nussenzweig et al. (4) for the purification of fibrinogen degradation products. A 100-mg sample of fibrinogen was dissolved in 20 ml of 0.2 M sodium carbonate, pH 8.9. At the end of this time the pH and conductivity of the solution were identical with the sodium carbonate buffer. Purification of the fibrinogen and fibrin digest products was achieved by elution from a DEAE-cellulose column, 50 × 1.5 cm, equilibrated with 0.01 M sodium carbonate, pH 8.9. Following the application of the sample, a linear gradient of 0 to 0.2 M sodium chloride in 0.01 M sodium carbonate, pH 8.9, was used to develop the column. After collecting about 300 ml, the gradient was discontinued since no more protein appeared in the effluent, and a final wash of 0.5 volumes of distilled water, and then lyophilized. SDS-polyacrylamide gel electrophoresis was used to identify the digestion products (1-5).

Since it was found that the Fragment D obtained from the 18-hour fibrinogen digest already showed extensive degradation of the γ chains, the purification of fibrinogen Fragment D was subsequently further modified. The digestion of fibrinogen with plasmin was allowed to proceed for only 2 hours and then soybean trypsin inhibitor was added to a final concentration of 0.3 mg per ml. This digest mixture was also purified by gradient elution from a DEAE-cellulose column, 50 × 1.5 cm, except that the linear gradient was from 0.04 to 0.4 M sodium chloride in 0.01 M sodium carbonate, pH 8.9. No attempt was made to recover Fragment E from the column. Protein peaks were pooled as indicated, dialyzed for 24 hours at 4° against three changes of 40 volumes of distilled water, and then lyophilized. SDS-gel electrophoresis was used to identify this fraction as Fragment D.

**Immunodiffusion Studies**—The above preparation of fibrinogen Fragment D was also compared with the purified fibrin Fragment D by microimmunodiffusion in 1% agarose (10), using antisera against human fibrinogen or fibrinogen Fragment D. Both antisera were prepared and kindly supplied to us by Dr. J. Gormsen, Sundby Hospital, Copenhagen, Denmark.

**Cross-Linking of Fibrinogen Fragment D with Fibrin—**

Fibrinogen (1, 2) and Fragment D (Fig. 3) were each made up to a concentration of 10 mg per ml in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M sodium chloride. Purified fibrin-stabilizing factor (9) was dissolved in 0.5 M sodium citrate-0.16 M potassium chloride, pH 7.1, to give an absorbance at 280 nm of 0.5 per ml. A series of mixtures of native fibrinogen and Fragment D1 were prepared as shown in Table I. In each case 5 μl of human thrombin solution, 25 NIH units per ml (2), 5 μl of 0.8 M calcium chloride, and 5 μl of fibrin-stabilizing factor solution were added and the samples were then incubated at room temperature for 4 hours. Visible clot formation occurred in all tubes except that containing Fragment D1 only, however, at a Fragment D to fibrinogen molar ratio of 40:1, the clot was extremely small and fragmented. Thrombin and fibrin-stabilizing factor activities were stopped in each reaction mixture by the addition of 5 μl of 0.5 M EDTA, pH 7.1, and 0.9 ml of 2% SDS-5 M urea-2% β-mercaptoethanol. Following overnight incubation at room temperature, these samples were then examined by SDS-gel electrophoresis.

**SDS-Gel Electrophoresis—**Polyacrylamide gel electrophoresis in SDS was performed as previously described (1, 2). In all cases 7.5% polyacrylamide gels were used.

**EXPERIMENTAL RESULTS**

**Purification of Fragment D from Fibrinogen and Fibrin—**Fig. 1A shows the purification of the fragments from fibrinogen digests by DEAE-cellulose chromatography. As previously reported by Nussenzweig et al. (4), Peak 1 contains Fragment A; Peak 2, Fragments B and C; Peak 3, Fragment D; and Peak 5, Fragment E. In the present study an additional peak, labeled 4, is soybean trypsin inhibitor which is present in a much higher concentration than originally used (4). Fig. 1B shows the purification of the fragments from a digest of fibrin by the same method. Peaks 1 and 2 which contained Fragments A, B, and C in the case of fibrinogen digests are not observed. Otherwise, the patterns are very similar except that fibrin Fragment D (Peak 5) eluted at a higher salt concentration. Fig. 2 shows the gel electrophoretic patterns of fibrinogen Fragment D and fibrin Fragment D before and after reduction. As can be seen the mobility of unreduced fibrinogen Fragment D on Gel a is significantly greater than that of unreduced fibrin Fragment D on Gel b. Moreover, as shown on Gel c, the reduced subunit pattern differs in that the γ chains of fibrinogen Fragment D have undergone extensive degradation to give γ2′ chain of 37,000 molecular weight and γ′2 chain of 27,000 molecular weight, respectively. On the other hand, Gel d shows that reduced fibrin Fragment D contains an 81,000 molecular weight degradation product of the γ dimer (2). Even in prolonged digests of fibrin, only the one species of fibrin Fragment D (Fig. 4) was produced.

The identity of Peak 4 as soybean trypsin inhibitor was established by comparison to pure soybean trypsin inhibitor subjected to electrophoresis under the same conditions. The identification of Peak 5 as Fragment E was determined by comparison to the Fragment E seen on electrophoresis of terminal fibrinogen and fibrin digests (1, 2) and by comparison to purified Fragment E prepared by a different method (2).
Fibrinogen Fragment D appeared to consist of several species which had slightly different mobilities on SDS-gel electrophoresis. The lower molecular weight species were formed as a function of digestion time and were arbitrarily designated as D1, D2, D3, and D4 according to their order of appearance. Because the fibrinogen Fragment D species isolated in the above experiment consisted mainly of D2 and D3, the method was modified by allowing plasmin to digest fibrinogen for only 2 hours before the addition of soybean trypsin inhibitor and subsequent purification by DEAE-cellulose chromatography. It should also be noted that the concentration of soybean trypsin inhibitor was reduced 7-fold since the higher concentration was found unnecessary. Fig. 3 shows the chromatographic pattern for this preparation. Once again Peaks 1 and 2 contain Fragments A, and B and C, respectively. The soybean trypsin inhibitor was present in Peak 6. As can be seen by examining the electrophoretic patterns shown in Fig. 4, Fragment D species were found in Peaks 3, 4, and 5. The species present in Peak 3 was the parent Fragment D1; Peak 4 contained primarily Fragment D1 as well as a small amount of Fragments D2 and D3; Peak 5 contained mainly Fragments D3 and D4. In addition to SDS-gel electrophoretic analysis, acrylamide gel electrophoresis at pH 3.2 in urea (9) was used to examine the fibrinogen Fragment D species contained in Peaks 3, 4, and 5. As can be seen on the middle three gels in Fig. 4, at least five fibrinogen Fragment D species are resolved on the basis of charge by this method. The gel pattern for Peak 3, which essentially consists of Fragment D1, reveals two major and three minor bands. The gel for Peak 4, which contains primarily Fragment D1 and much smaller amounts of Fragments D2 and D3, shows the same two major bands and an increased amount of three minor bands. Thus, the two major bands observed by this electrophoretic method must correspond to Fragment D1 while the three minor bands apparently represent Fragments D2 and D3.

As shown in Fig. 5, Peak 4, which contained the largest amount of the parent fibrinogen Fragment D1 species, was subsequently rechromatographed on Sephadex G-100 to remove small amounts of low molecular weight contaminants. The subunit structure of purified fibrinogen Fragment D1, which is known to be composed of an α'' chain, a β'' chain, and the γ'-chain, all held together by disulfide bonds (1), is shown on Gel a in Fig. 4. The subunit structures of Fragments D2 and D3 are shown on Gels c and d, in Fig. 2, and in place of the γ' chain of 27,000 molecular weight as found in Fragment D1, the γ'' chain of 37,000 molecular weight is found in Fragment D2, and the γ'' chain of 27,000 molecular weight is present in Fragment D3. Finally, it should be noted that Gel b of Fig. 4 shows that the β'' chain of 44,500 molecular weight is the only subunit of any of the Frag-
FIG. 4. The first three gels show the nonreduced SDS-gel electrophoretic patterns of the fibrinogen Fragment D species contained in Peaks 3, 4, and 5 of Fig. 3. Peak 3 contained Fragment D1; Peak 4 contained primarily Fragment D1, with a small amount of both Fragments D2 and D3; Peak 5 contained only a small amount of Fragment D1 and primarily Fragments D2 and D3. The middle three gels are standard pH 3.2-urea polyacrylamide gels of Peaks 3, 4, and 5. Peak 3 appears to show two major species and three minor species. The concentrations of the minor species were increased in Peaks 4 and 5 while the major species were decreased as indicated by staining intensity. Gels a and b show the reduced SDS-gel electrophoretic patterns of Fragment D1. Gel a was stained for protein and Gel b for carbohydrate. Following reduction, a comparison of Gel a, primarily fibrinogen Fragment D1, with Gel c of Fig. 2, primarily fibrinogen Fragments D2 and D3, shows that the γ' chain is the major γ chain degradation product in Fragment D1.

FIG. 5. Sephadex G-100 chromatogram of Fragment D1. Peak 4 from the elution of fibrinogen digestion fragments described in Fig. 2 contained Fragment D1 contaminated by a small amount of Fragments D2 and D3.

Purification of Fragment D from Plasmin Digests of Fibrinogen and Cross-Linked Fibrin—Previous studies of the digestion of fibrinogen and fibrin by plasmin have resulted in models which predict the subunit structure of the various intermediate and terminal degradation products of both proteins (1, 2). The data in the present study provide additional evidence that further substantiates these models. As has been reported previously (1), fibrinogen D exists as a population of species, the
FIG. 6. (left). Microimmnodiffusion comparing fibrinogen Fragment D1 to fibrin Fragment D. Well a contained antiserum to human fibrinogen and Well d, antiserum to fibrinogen Fragment D. In both cases, Well b contained fibrinogen Fragment D1 and Well c, fibrin Fragment D.

FIG. 7. (right). The reduced SDS-gel electrophoretic patterns for the cross-linking of fibrinogen Fragment D1 with fibrinogen. Gel a shows the electrophoretic pattern of highly cross-linked fibrin formed in the absence of Fragment D1. This gel shows a normal cross-linking pattern with virtually complete cross-linking of the y chains and cross-linking of most of the o chains. Gel b through e shows the fibrin formed from a molar ratio of Fragment D1 to fibrinogen of 6:1; Gel c, a molar ratio of 10:1; Gel d, a molar ratio of 20:1; and Gel e shows a molar ratio of 40:1. Gel f shows the cross-linking of Fragment D1 in the absence of fibrinogen. Gel b through e shows two bands below the y dimer which represent an intact y chain cross-linked to a y' chain, and two y' chains cross-linked together. These bands increase in intensity in going from left to right. Gel f shows only the y' dimer since no intact y chains were present in this sample. The origin of the minor high molecular weight band is uncertain. Gels b through f show no evidence that y' or y'' chains can participate in cross-linking, either with themselves or with intact y chains. Furthermore, no evidence was found to suggest that the o' chains can cross-link with themselves or with intact o chains.

TABLE I

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<thead>
<tr>
<th>Cross-linking of purified fragment D1 with and without fibrinogen</th>
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<td>Volume</td>
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TABLE II

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<th>Polypeptide chain composition of Fragment D species</th>
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* Based on an assumed molecular weight of 80,000 for Fragment D1 and 330,000 for fibrinogen.

The size of which largely depends on the extent of y chain digestion. As shown in Fig. 3, the first species of Fragment D to appear in plasmin digests is Fragment D1, which has the following subunit structure: an o'' chain of about 15,000 molecular weight, a y'' chain of 44,500 molecular weight, and a y' chain of 42,000 molecular weight. However, as seen in Fig. 4, by lengthening the period of digestion, further cleavage of Fragment D1 results in the formation of Fragments D2, D3, and D4, which increase in intensity as Fragment D1 gradually disappears. In the chromatogram shown in Fig. 1A, the majority of the Fragment D species existed as Fragment D2 which contained the y'' chain of 37,000 molecular weight instead of the y' chain; and Fragment D3 which contained the y'' chain of 27,000 molecular weight instead of the y' or y'' chains. At still longer digestion times than shown in this study Fragment D4 can be completely digested with a concomitant increase in Fragment D5. In addition, with further degradation, another lower molecular weight species of Fragment D, D4, can be isolated whose subunit structure is thought to vary as a result of o'' chain digestion, since there appears to be no change in molecular weight or decrease in intensity of the y'' or y''' chains (2). Following even the most prolonged digestion, no species of fibrinogen Fragment D ever showed evidence of y'' chain digestion. For convenience of the reader, Table II summarizes the subunit structures of the various
fibrinogen Fragment D species. As indicated in the accompanying paper, the species of Fragment D derived from non-cross-linked fibrin had identical subunits to those of Fragment D from fibrinogen (2).

In contrast to the multiple forms of fibrinogen Fragment D, cross-linked fibrin Fragment D (Fig. 1B) was isolated as only a single species, even after prolonged digestions. As shown in Figs. 4 and 5, fibrin Fragment D showed a much lower mobility than fibrinogen Fragment D when the two proteins were compared by SDS-gel electrophoresis. The difference in mobilities is easily explained, since fibrin Fragment D is essentially composed of two fibrinogen Fragment D1 molecules held together by cross-links in the COOH-terminal portion of the η' chains. Hence, the 81,000 molecular weight subunit found in fibrin Fragment D must result from the partial digestion of a dimer. As a result of this cross-linked subunit species, the molecular weight of fibrin Fragment D would therefore be twice that reported for fibrinogen Fragment D (5). Additional support for this conclusion is provided by our observation that fibrin Fragment D contained one pair of ε-(γ-glutamyl)lysyl cross-links as opposed to fibrinogen Fragment D1 which, as expected, contained none (12). These findings are in disagreement with the report by Dudek et al. that fibrinogen Fragment D and fibrin Fragment D have virtually identical molecular weights (13). Conceivably their fibrin Fragment D could have been recovered from a digest of non-cross-linked fibrin, thus explaining the discrepancy between their results and ours.

When the species of fibrinogen Fragment D were examined for charge heterogeneity as shown in Fig. 5, at least five different forms were identified. It appeared that Fragment D1 itself contained at least two major species by charge and that Fragment D2 or D3 also contained more than one species of charge. Catanazzo et al. (14) have recently reported the identification of eight bands on standard polyacrylamide gel electrophoresis at pH 8.6 for a Fragment D species which contained three subunits of molecular weights of 44,500, 25,000, and 10,000. This Fragment D species presumably corresponds to our Fragment D2 in light of the subunit molecular weights and the fact that an 18-hour digestion time was used. These results would seem to indicate that all or, at least several, of the Fragment D species may be very similar in size but different in charge.

**Immunodiffusion Studies**—The immunodiffusion studies in Fig. 6 show that Fragment D1 from fibrinogen and Fragment D from fibrin gave a line of identity when diffused against antisem to fibrinogen Fragment D. Thus the presence of intermolecular cross-links in the COOH-terminal portions of the η' chains does not alter the reactivity of fibrin Fragment D with an antibody to fibrinogen Fragment D1. Therefore attempts to use such antisem to distinguish between fibrinogen Fragment D1 and fibrin Fragment D could lead to the erroneous conclusion that the 2 molecules are identical.

**Cross-Linking of Fibrinogen Fragment D with Fibrinogen**—The studies presented in Fig. 7 show that fibrinogen Fragment D1 can be cross-linked both to fibrin monomers and itself if fibrin-stabilizing factor is present. However, only Fragment D1 which contains γ' chains of 12,000 molecular weight is capable of forming cross-links since neither the γ'' chain nor the γ'' chain disappeared as cross-linked forms appeared. Moreover, as further confirmation of this observation, a mixture of Fragments D2 and D3, or a preparation containing primarily Fragment D2, were substituted for Fragment D1, and still no evidence of cross-link formation was noted when these species were incubated with thrombin, calcium, fibrin-stabilizing factor, and fibrinogen. Thus neither the γ' chain of Fragment D2 nor the γ'' chain of Fragment D1 can cross-link with intact γ chains to form a higher molecular weight subunit as was the case with the γ' chain of Fragment D3. However, when fibrin was formed from a mixture of Fragment D1 and fibrinogen, it was possible to identify the normal γ dimer fibrin subunit, and two new subunits consisting of a γ chain cross-linked to a γ' chain or two γ' chains cross-linked to give a γ' dimer (Fig. 6). When Fragment D1 was cross-linked in the absence of fibrin monomer, about 50% of the γ' chain disappeared with the concomitant formation of a γ' dimer. It should be emphasized that no visible precipitation or gelation occurred in this sample despite the formation of cross-linked species. The above findings indicate that γ' chains must be formed by removal of an NH₂-terminal peptide from the γ chain as previously suggested by our studies of fibrinogen and fibrin digestion by plasmin (1, 2). On the other hand, Fragments D2 and D3 which contain the γ'' chain of 37,000 molecular weight and the γ''' chain of 27,000 molecular weight, respectively, do not cross-link and hence probably do not contain the COOH terminal cross-link region (15-17) as previously suggested (1, 2).

These results also explain the reports that Fragment D is an inhibitor of fibrin formation (18-24). Previously it was speculated that cross-linking of Fragment D to fibrin monomers could account for the inhibition of clot formation (25-29). The present study confirms that this in fact is the mechanism of inhibition. More recently, data have been presented showing that a molar ratio of Fragment D1 to fibrinogen of 40:1 markedly inhibited clotting (24, 30). In the present study it was confirmed that clotting was delayed at this ratio and in fact, only a granular precipitate was observed. However, SDS-gel electrophoretic analysis of this sample (Fig. 7, Gel f) showed that while α polymerization was only partial with some α chains remaining non-cross-linked, all of the intact γ chains of fibrinogen became cross-linked to another intact γ chain or to a γ' chain of Fragment D3. All of the γ' chains did not become cross-linked, possibly as a result of the conformation of Fragment D1 being altered in such a way that the rate of cross-linking was decreased. It must be kept in mind that at high ratios of fibrinogen Fragment D to fibrinogen, the γ chain of a fibrin monomer will more likely encounter a γ' chain of a Fragment D1 species than a γ chain of another fibrin monomer, thus markedly slowing the rate of fibrin polymerization.

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Subunit Structure of Fragment D from Fibrinogen and Cross-Linked Fibrin
Salvatore V. Pizzo, Lloyd M. Taylor, Jr., Martin L. Schwartz, Robert L. Hill and Patrick A. McKee


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