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 found 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.

The digestion of human fibrinogen by plasmin results in five terminal digestion products identified as Fragments A, B, C, D, and E (1–5). Recently the appearance of these fragments have been correlated with changes in the subunit structure of fibrinogen and fibrin during plasmin digestion (1, 2). Fragments A, B, and C are derived from the α(A) chains of fibrinogen while both Fragments D and E contain polypeptide chains derived from each of the three subunit chains of fibrinogen. Fragment E is derived from the NH2-terminal region of the molecule and contains three pairs of low molecular weight peptides held together by disulfide bonds (1, 2). The three chains composing Fragment D are also held together by disulfide bonds, although fewer are probably present than in Fragment E since the NH2-terminal region is particularly rich in disulfide bonds (6). In Fragment D the subunit derived from the α(A) chains is designated as the α′′ chain and has a molecular weight of about 15,000; that derived from the β(B) chains is called the β′′ chain and has a molecular weight of 44,500. Finally, depending on the extent of proteolysis by plasmin, any one of three types of degraded γ chain may be present in different species of Fragment D and designated as the γ′, γ′′, and γ′′′ chains with molecular weights of 42,000, 37,000, and 27,000, respectively. The corresponding Fragment D species are called Fragments D1, D2, and D3, respectively.

Recent studies of fibrin digestion by plasmin (2) suggest that Fragment E from fibrinogen and Fragment E from cross-linked fibrin are identical in structure. However, Fragment D from fibrinogen and cross-linked fibrin differ since the latter contains intermolecular COOH-terminal cross-links between its γ chains. The formation of these cross-links prevents cleavage in this region of the γ dimers. We now wish to report additional data on the structures of Fragment D derived from digests of fibrinogen or highly cross-linked fibrin. Highly purified Fragment D from both sources was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis before and after reduction. Moreover, the ability of various types of fibrinogen Fragment D to cross-link with itself or native fibrinogen was also examined. The results of these studies support models previously presented for the digestion of fibrinogen and highly cross-linked fibrin (1, 2).

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 so-
dium carbonate, pH 8.9. At the end of this time the pH and
conductivity of the solution were identical with the sodium car-
bonate buffer. Purification of the fibrinogen and fibrin digest
products was achieved by elution from a DEAE-cellulose col-
umn, 50 × 1.5 cm, equilibrated with 0.01 M sodium carbonate,
pH 8.9. Following the application of the sample, a linear gra-
dient of 0 to 0.2 M sodium chloride in 0.01 M sodium carbonate
buffer, pH 8.9, was used to develop the column. After collecting
about 800 ml, the gradient was discontinued since no more pro-
tein appeared in the effluent, and a final wash of 0.5 M sodium
chloride in 0.01 M sodium carbonate, pH 8.9, was used to re-
move 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 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 gra-
dient 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 in this experiment. Samples were pooled as
indicated, dialyzed, and lyophilized as described above, fol-
lowing which SDS-gel electrophoresis was again used to identify
the products. Moreover, standard electrophoresis at pH 3.2 in
the presence of urea (9) was used to examine these peaks.

Fibrinogen Fragment D which was found mainly in Peak 4
was further purified by gel filtration as follows. A 25-mg sam-
ple was dissolved in 10 ml of 0.2 M sodium phosphate, pH 7.2,
and applied to a Sephadex G-100 column, 100 × 1.5 cm, equil-
ibrated in the same buffer. The first peak was pooled, dialyzed
against distilled water, and lyophilized as described above; a
final yield of 15 mg was obtained. 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 Frag-
ment D by microimmunodiffusion in 1% agarose (10), using
antiserum 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—Purified
fibrinogen (1, 2) and Fragment D1 (Fig. 3) were each made up
to a concentration of 10 mg per ml in 0.05 M Tris-Cl 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 Frag-
ment 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 solu-
tion 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 D1 to fibrinogen molar ratio of 40:1, the clot was
extremely small and fragmented. Thrombin and fibrin-stabi-
lizing 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-β-mercaptoethanol. Following overnight incubation at room temperature, these samples were then ex-
amined 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 Fragments 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 puri-
fication 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 3) 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 sig-
nificantly greater than that of unreduced fibrin Fragment D on
Gel b. Moreover, as shown on Gel c, the reduced subunit pat-
ters differ in that the γ' chains of fibrinogen Fragment D have
undergone extensive degradation to give γ" chain of 37,000
molecular weight and γ' 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 es-
tablished by comparison to pure soybean trypsin inhibitor sub-
jected 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 D1 and D2, 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 5 was the parent Fragment D1; Peak 4 contained primarily Fragment D1 as well as a small amount of Fragments D2 and D3; Peak 3 contained mainly Fragments D2 and D3. 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 42,000 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 S, 4, and 5 of Fig. 3. Peak S 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 3 from the elution of fibrinogen digestion fragments described in Fig. 2 contained Fragment D1 contaminated by a small amount of Fragments D2, D3, and D5. Fragment D species that is carbohydrate positive by the periodic acid-Schiff method (11). The fate of the α" chain of ~15,000 molecular weight is not certain; it appears to persist at least through the formation of Fragment D3. In Fragment D4, it probably has been cleaved to a lower molecular weight and hence is hypothetically designated as α"" chain.

Immunodiffusion Studies—Fig. 6 shows the comparison of purified fibrinogen Fragment D1 with purified fibrin Fragment D by microimmunodiffusion against anti-fibrinogen serum and anti-fibrinogen Fragment D serum. The latter was prepared against the Fragment D species recovered from a terminal fibrinogen digest; however, the relative amounts of the different species of Fragment D in the digest were not known. As is evident, a line of identity was obtained for the fibrinogen Fragment D1 and fibrin Fragment D antigens when either the anti-fibrinogen or anti-fibrinogen Fragment D serum was used.

Cross-Linking of Fibrinogen Fragment D with Fibrin—Fig. 7 shows the reduced SDS-gel electrophoretic patterns of Fragment D1, which was cross-linked with fibrin under conditions listed in Table I. The fibrinogen Fragment D1 used in this study was from the preparation shown in Fig. 3 and thus contains primarily Fragment D1 contaminated with very small amounts of Fragments D2 and D5. The first reaction mixture contained only fibrinogen and thus serves as a control by providing a normal pattern of fibrin cross-linking. As can be seen on Gel a, this fibrin is highly cross-linked and both γ dimer and α polymer are present (8). Gel b shows the subunit pattern of fibrin formed when the molar ratio of Fragment D1 to fibrinogen is 6:1. Below the γ dimer area, there are two bands visible on this gel which are absent on the control gel. As shown on Gel c, d, and e, the intensity of these two species becomes more pronounced as the molar ratio of Fragment D1 to fibrinogen increases. In addition, as these higher ratios are approached, the amount of α polymer progressively decreases until only a small amount is apparent on Gel e when the molar ratio of Fragment D1 to fibrinogen is 40:1. Finally, Gel f shows the cross-linking of Fragment D1 in the absence of fibrinogen. Although no precipitate or other visible evidence of cross-linking was observed, examination of the electrophoretic pattern for this sample shows the formation of a γ′ dimer and the disappearance of about 50% of the γ′ monomer. The origin of the minor band, which has a lower mobility than the γ′ dimer on Gel f, is uncertain. There was no indication that the γ″ chain or the γ′′ chain of Fragments D2 and D3, respectively, cross-linked with either intact γ chains, γ′ chains, or themselves, as can be seen by the constant intensity of these two species on Gels b through f.

**DISCUSSION**

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). Microimmmodiffusion 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 \( \gamma \) chains and cross-linking of most of the \( \alpha \) chains. Gel b 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 \( \gamma \) dimer which represent an intact \( \gamma \) chain cross-linked to a \( \gamma' \) chain, and two \( \gamma' \) chains cross-linked together. These bands increase in intensity in going from left to right. Gel f shows only the \( \gamma' \) dimer since no intact \( \gamma \) 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 \( \gamma'' \) or \( \gamma''' \) chains can participate in cross-linking, either with themselves or with intact \( \gamma \) chains. Furthermore no evidence was found to suggest that the \( \alpha'' \) chains can cross-link with themselves or with intact \( \alpha \) chains.

TABLE I

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<th>Cross-linking of purified fragment D1 with and without fibrinogen</th>
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* Based on an assumed molecular weight of 80,000 for Fragment D1 and 330,000 for fibrinogen.

size of which largely depends on the extent of \( \gamma \) 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 \( \alpha'' \) chain of about 15,000 molecular weight, a \( \beta'' \) chain of 44,500 molecular weight, and a \( \gamma' \) 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 and D3 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 \( \gamma'' \) chain of 37,000 molecular weight instead of the \( \gamma' \) chain; and Fragment D3 which contained the \( \gamma''' \) chain of 27,000 molecular weight instead of the \( \gamma'' \) or \( \gamma''' \) chains. At still longer digestion times than shown in this study Fragment D1 can be completely digested with a concomitant increase in Fragment D2. 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 \( \alpha'' \) chain digestion, since there appears to be no change in molecular weight or decrease in intensity of the \( \beta'' \) or \( \gamma''' \) chains (2). Following even the most prolonged digestion, no species of fibrinogen Fragment D ever showed evidence of \( \beta'' \) chain digestion. For convenience of the reader, Table II summarizes the subunit structures of the various

TABLE II

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<th>Polypeptide chain composition of Fragment D species</th>
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<td>Fragment D species</td>
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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 γ' chains. Hence, the 81,000 molecular weight subunit found in fibrin Fragment D must result from the partial digestion of γ 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. Cutanzaro 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 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 antiserum to fibrinogen Fragment D. Thus the presence of intermolecular cross-links in the COOH-terminal portions of the γ' chains does not alter the reactivity of fibrin Fragment D with an antibody to fibrinogen Fragment D1. Therefore attempts to use such antisera to distinguish between fibrinogen Fragment D1 and fibrin Fragment D2 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 42,000 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 D1. 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 data 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, 29). 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 D1. 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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