Structure of Plasmin Degradation Products of Human Fibrinogen

FIBRINOPEPTIDE AND POLYPEPTIDE CHAIN ANALYSIS*

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SUMMARY

Direct determination of fibrinopeptides A and B in purified plasmin degradation products of human fibrinogen shows that fibrinogen and Fragments X and Y contain 2 moles of fibrinopeptide A per mole; Fragment E has approximately 0.3 mole of fibrinopeptide A per mole. Fibrinopeptide B is present in only trace amounts in some Fragment X preparations and is absent from Fragments Y and E. Fragment D contains neither fibrinopeptide A nor B. These data and the results on molecular weight, carbohydrate content, and reactivity with thrombin of polypeptide chains of the purified degradation products were used to assign these chains to the Aα, Bβ, or γ chains of the original fibrinogen molecule. Fragment X has altered Aα chains with a molecular weight of 25,000, Bβ chains that are either intact (58,000) or cleaved at the NH₂-terminal end (53,000), and γ chains that are either intact (48,000) or cleaved at the COOH-terminal end (40,000). Fragment Y has two distinct sets of polypeptide chains: the heavier ones are almost the same as those of the smallest Fragment X species. The lighter chains of Fragment Y are similar to those of Fragment E, which has three major components of 13,000, 8,000, and 10,000 assigned tentatively as Aα, Bβ, and γ fibrinogen chain remnants, respectively. The chains of Fragment D have been classified as a 13,000 molecular weight Aα chain remnant, a 47,000 β chain remnant and a heterogeneous group of fibrinogen chain remnants. The presence of 2 moles of fibrinopeptide A per mole of Fragment Y and the demonstration of the heavy and light sets of polypeptide chains in Fragment Y provide substantial evidence for its asymmetric structure.

A complex proteolytic partition of fibrinogen molecule by plasmin was inferred from the multiple chain structure of degradation product, Fragment D, as shown by starch gel electrophoresis (1, 2). Polyelectrolyte gel technique revealed that of the three polypeptide chains of fibrinogen,† the Aα chain is most susceptible to degradation (4). Cleavages first occur at the COOH-terminal end of this chain since the resultant degradation products retain alanine as an NH₂-terminal amino acid (5, 6), fibrinopeptide A is not cleaved (7, 8), and the Aα chain remnant yields fibrinopeptide A after exposure to thrombin (9). Partial degradation of the Aα chain is followed by Bβ chain cleavage at the NH₂-terminal portion, at which time a polypeptide chain fragment containing fibrinopeptide B is split (7, 8). These proteolytic cleavages yield a heterogeneous group of fibrinogen degradation products which are coagulable by thrombin, called here the Fragment X family (10). According to an asymmetric scheme of degradation (11–13), plasmin splits the smallest of the Fragment X molecules (mol wt approximately 240,000) into unequal parts: a Fragment Y molecule of approximately 135,000 and a Fragment D molecule of approximately 83,000. With continued plasmin action, Fragment Y would be split into a second Fragment D molecule and a single Fragment E molecule of approximately 50,000. This scheme was initially based upon data on molecular weight, antigenic determinants, and yield of the degradation products from fibrinogen, and is supported by recent SDSpolyacrylamide gel electrophoresis studies (9, 14–21).

In addition, the determination of the NH₂-terminal amino acids of the degradation products indicates that the NH₂-terminal portion of fibrinogen is present in Fragments X, Y, and E but not in Fragment D (9). This observation further supports the concept of an asymmetric splitting of Fragment X, specifically on either side of a junction center encompassing the NH₂-
Fragments D and E (11). This contains Fragments X, Y, I>, and E; and Stage 3 contains mainly the terminal portion of the molecule. In the present report, fibrinogen degradation products (Fragments X, Y, D, and E) are studied by quantitative measurement of fibrinopeptides A and B and by determination of polypeptide chain molecular weight, PAS stainability, and thrombin susceptibility. The structure of Fragment Y receives special emphasis, since it must be an unbalanced molecule which contains the NH2-terminal region of both halves of the parent fibrinogen substrate, if the postulated asymmetric scheme of lysis is valid.

MATERIALS AND METHODS

Preparation of Fibrinogen Degradation Products—Human fibrinogen (Grade L, A. B. Kabi, Stockholm) dissolved in water was 95% clottable after addition of thrombin (bovine, Parke-Davis, Detroit, Mich.) and contained enough plasminogen to cause extensive proteolytic degradation after addition of streptokinase (Varidase, Lederle Laboratories, Pearl River, N.Y.). Technical details of the digestion have been described previously (11, 12). Degradation was terminated by the addition of soybean trypsin inhibitor (Worthington, Freehold, N.J.) or N-acetyl-L-cysteine (Sigma, St. Louis, Mo.) (12). Fragment X was purified from Stage I and Stage 2 digestes and Fragment Y from Stage 2 digestes by gel filtration on Sephadex G-200 (Pharmacia, Piscataway, N.J.) columns (11, 12). Thrombin clotting time (12, 22) of Fragment X from Stage 1 digest was 65% and from Stage 2 digestes 64%. Fragments D and E were prepared from Stage 3 digestes by block electrophoresis (23) on Pevikon (C. 870, Mercer, N.J.).

Reduction of Disulfide Bonds—Reduction of disulfide bonds was achieved by the incubation of 0.1 ml of protein (6 mg per ml) with 0.4 ml of a solution containing 9 m urea, 3% SDS, and 3% \( \beta \)-mercaptoethanol for 24 hours at 37°.

Polyacrylamide Gel Electrophoresis—Three different systems were used. Nonreduced proteins were analyzed in 5% gels (0.5 × 7 cm) at pH 2.7 in 2 M urea (24). Electrophoresis of approximately 10-μg samples was toward the cathode at a constant current of 3 ma per tube for 60 min at room temperature. The gels were stained in Coomassie brilliant blue for 2 hours and destained electrophoretically in a transverse field (Quick Gel Destainer, Canaco, Toockville, Md.). Reduced proteins were tested in 7% gels (0.5 × 7 cm) containing 0.1% SDS (25, 26). Approximately 10-μg samples were applied and electrophoresis was conducted toward the anode at a constant current of 2 ma per tube for 20 hours at room temperature. The gels were stained in Coomassie brilliant blue for 4 hours, soaked overnight in a 5% methanol-7% acetic acid solution, and destained electrophoretically using fresh methanol-acetic acid solution. Polypeptide chains of molecular weight below 20,000 were separated best in 12.5% gels (0.5 X 13 cm) containing 0.1% SDS (27) using a similar electrophoretic procedure as for the 7% gels. Destaining was achieved by diffusion alone using 25% methanol-7% acetic acid solution which was changed frequently over a period of 60 hours.

Fig. 1. Standard lines used for the calculation of molecular weights of polypeptide chains of the fibrinogen (FBG) degradation products. The electrophoretic mobilities in SDS polyacrylamide gels of the reduced polypeptides and proteins of known molecular weight are indicated relative to that of lysozyme. The lines are calculated by the method of least squares from the relationship between electrophoretic mobility and logarithm of molecular weight of standard proteins. The horizontal bars show the lowest and highest relative mobilities of each standard protein in all experiments. Left, separation of standard proteins of molecular weight between 14,300 and 130,000 in 7% polyacrylamide gel. Right, the separation of standard peptides and proteins of molecular weights between 2,550 and 14,300 in 12.5% polyacrylamide gel. The symbols \( \beta \)-Lys, \( \gamma \)-Lys, \( \beta \)-Lyc, and \( \gamma \)-Lyc denote the polypeptides derived from egg white lysozyme and sperm whale myoglobin, respectively, after reaction with cyanogen bromide. \( \beta \)-Laetoglobulin, myoglobin, and Lys-3 do not fit the calculated linear plot, limiting the molecular weight determination in this system.

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chain of human fibrinogen, 96,000; bovine serum albumin, 68,000; Aβ, Bβ, and γ chains of human fibrinogen, 70,000, 58,000, and 48,000, respectively; heavy and light chains of human IgG, 50,000 and 25,700, respectively; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,000; myoglobin, 17,200; and lysozyme, 14,500. Other standards of molecular weight below 20,000 utilized sperm whale myoglobin and egg white lysozyme degraded with cyanogen bromide (29). The three peptides obtained from myoglobin have molecular weights of 8,270, 6,420, and 2,650; those obtained from lysozyme have molecular weights of 10,200, 2,850, and 1,300, as calculated from amino acid sequence data (30).

Quantitative Determination of Fibrinopeptide Content of Fibrinogen Degradation Products—The samples of degradation products were supplied frozen in Tris-buffered saline at pH 7.4 and tested without knowledge of their content. The 5-mg samples were diluted with buffer at a volume of 2.0 ml and divided into 2.5-mg portions. One sample was treated at room temperature with 2 p-tosyl-L-arginine methyl ester units of chromatographically purified bovine thrombin for 1 hour and the other with 20 units for 4 hours. Clots were present in the fibrinogen and Fragment X preparations isolated from both Stage 1 and Stage 2 digests; no clot formed in the reaction mixtures containing Fragments Y, D, or E. After thrombin treatment, 0.1 ml of 10% monochloroacetic acid was added to dissolve the coagula or precipitates, following which the protein was precipitated by adding 0.22 ml of 30% trichloroacetic acid. The precipitate was collected by centrifugation, washed twice with about 10 ml of 5% trichloroacetic acid, and subjected to Kjeldahl digestion to determine nitrogen content. The protein content was calculated using a factor of 6.25 mg of protein per mg of nitrogen, and it was in agreement with that determined by direct ultraviolet spectrophotometry. A 1.0-ml portion of the supernatant fluid was applied directly to a 20-ml column of Sephadex G-10 equilibrated with 1% formic acid. The portion of eluent containing the fibrinopeptides (between 10 and 17 ml) was collected and divided in half. Each half was concentrated and transferred quantitatively to paper for electrophoretic analysis at pH 2 (1.25% formic acid and 4% acetic acid) and at pH 8.7 (0.045 M potassium orthoborate). Quantitation of the separated fibrinopeptide was based on densitometric integration of the separated fibrinopeptide, was obtained from lysozyme have molecular weights of 10,200, 2,850, and 1,300, as calculated from amino acid sequence data (30).

RESULTS

Purity of Fibrinogen Degradation Products—Fig. 2 shows the polycrylamide gel electrophoretic patterns of fibrinogen and purified preparations of the four major groups of plasmic degradation products. The Fragment X preparation was obtained from a stage 1 plasmin digest of fibrinogen and has the same electrophoretic mobility as Fragment X isolated from Stage 2 digests. An excess of Fragment Y was applied to the gels in order to demonstrate the absence of Fragments X and D in this preparation. All samples except Fragment D migrate as single bands in 5% polyacrylamide gel at pH 2.7 in the presence of 2 M urea. During prolonged incubation of Fragment D with plasmin, the slower electrophoretic bands decrease in concentration and are gradually replaced by the faster migrating bands. No attempt was made to separate the three electrophoretic varieties seen in this Fragment D preparation obtained from a Stage 3 digest.

Molecular Weight and PAS Staining of Polypeptide Chains of Fibrinogen Degradation Products—Fig. 3 illustrates the electrophoretic patterns of the polypeptide chains of fibrinogen and Fragments X, Y, D, and E, and shows the mean values of molecular weights. The values have been calculated from multiple reduction and electrophoretic experiments, the results of which are presented in Table 1. Fibrinogen shows three bands corresponding to the Aα, Bβ, and γ chains, which are considered to have molecular weights of 70,000, 58,000, and 48,000, respectively, according to published data using SDS polyacrylamide gel electrophoretic techniques (4, 14, 16, 24). Only the Bβ and γ chains stain with PAS reagents (14), although all three chains contain carbohydrate (32, 33). Fragment X of Stage 1 digest has five major bands corresponding to molecular weights of 58,300, 53,200, 48,000, 39,800, and 26,800, and a minor band of approximately 35,000 (Fig. 3). All bands stain with PAS reagent except that of 26,800 molecular weight. The electrophoretic bands of Fragment X of the Stage 2 digest show a similar pattern to that of Fragment X from Stage 1. Four major bands were observed with molecular weights of 58,800, 47,800, 40,800, and 25,000, corresponding to four of the six bands in the Stage 1, Fragment X preparation. The differences between these two fragments are the increased amount of the 40,800 band, the absence of the 58,300 and 35,000 bands, and the faster electrophoretic mobility of the 25,000 band in the Fragment X (Stage 2) sample. All of the bands in Fragment X (Stage 2), except that of 25,000, stain with PAS reagent.

The electrophoretic pattern in 7% polyacrylamide gels of the polypeptide chains of Fragment Y is very similar to that of Fragment X from the Stage 2 digest. Four bands were seen with molecular weights of 50,700, 47,600, 40,800, and 25,000, of which only the last does not stain with PAS reagent. In addition to the bands noted in 7% polyacrylamide gels (Fig. 3), other bands of molecular weight between 15,000 and 7,000 can be demonstrated using electrophoretic separation in 12.5% polyacrylamide gels. These bands were found in the Fragments Y and E preparations and were absent in the Fragment X samples (Fig. 4).
FIG. 3. The electrophoretic patterns in SDS polyacrylamide gels of reduced human fibrinogen and the reduced Fragments X, Y, D, and E. Cathode and origin at the top, anode at the bottom of each gel. The experimental conditions for fibrinogen (F), Fragment X obtained from Stage 1 and Stage 2 digests (St. 1, St. 2) and Fragment Y (in 7% gel) utilized a simultaneous electrophoretic run in 7% gels for 20 hours at 2 ma per gel. The mobility of individual chains can be directly compared with that of the α, β, and γ chains of fibrinogen. Fragment D was tested in 7% gels for 8 hours at 2 ma per gel together with a mixture of standard proteins (STND. PROT.) which contained in order of increasing mobility: bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin, and lysozyme. Fragment Y (12.5% gel) and Fragment E (12.5% gel) were run for 20 hours at 2 ma per gel, together with standards containing in order of decreasing molecular weight: bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin, and lysozyme. In addition to these reduced proteins, two lysozyme polypeptide fragments (Lys-1, Lys-2) were added to standards run simultaneously with Fragment E. The mean molecular weight of individual chains (Table I) was calculated from the results of multiple experiments, using the standard lines shown in Fig. 1.

Table I

Major electrophoretic bands of fibrinogen and fibrinogen degradation products

<table>
<thead>
<tr>
<th></th>
<th>Fragment X (Stage 1)</th>
<th>Fragment X (Stage 2)</th>
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<tbody>
<tr>
<td>Fibrinogen</td>
<td>70,000</td>
<td>52,800</td>
</tr>
<tr>
<td></td>
<td>58,000 (PAS+)</td>
<td>47,800 (PAS+)</td>
</tr>
<tr>
<td></td>
<td>48,000 (PAS+)</td>
<td>40,800 (PAS+)</td>
</tr>
<tr>
<td>Fragment Y</td>
<td>42,500 ± 4,780 (PAS+)</td>
<td>13,400 ± 740</td>
</tr>
<tr>
<td></td>
<td>38,600 ± 3,000</td>
<td>10,300 ± 1,680</td>
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<tr>
<td></td>
<td>32,400 ± 3,200</td>
<td>7,700 ± 780</td>
</tr>
<tr>
<td></td>
<td>26,800 ± 3,480</td>
<td>12,600 ± 450</td>
</tr>
<tr>
<td></td>
<td>12,600 ± 1,780</td>
<td>10,400 ± 710</td>
</tr>
<tr>
<td></td>
<td>8,600 ± 640</td>
<td>5,350 ± 1,970</td>
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</table>

In the Fragment Y preparations, 2 to 6 such bands were identified and assigned to three groups of molecular weights, 12,600, 10,400, and 8,600. Only the 10,400 molecular weight band stains positively with PAS.

The polypeptide chains of reduced Fragment E were best resolved in 12.5% polyacrylamide gels (Figs. 3 and 4). Three predominant bands have molecular weights of 13,100, 10,300, and 7,700. Some samples of reduced Fragment E show a small amount of material close to the top of the gel, probably the result of aggregation of chains, and a variable amount of material of approximate molecular weight 15,000. Only the band of molecular weight 10,400 stains with PAS reagent. The band of molecular weight 7,700 stains with Coomassie blue with a distinctive bluish hue instead of the usual purple color. The same characteristic staining is observed with the band of molecular weight 8,000 from reduced Fragment Y.

Fragment D shows five electrophoretic bands in 7% polyacrylamide gels (Fig. 3) with molecular weights of 42,500, 38,600, 32,400, 26,400, and 12,600. A sixth band of approximately 5,000 molecular weight is sometimes present in 12.5% polyacrylamide gels. The intensity of staining of the 38,600 and 32,400 bands varies considerably in the Fragment D preparations obtained from different batches of Stage 3 digests. Only the slowest moving band of molecular weight 42,500 stains with PAS reagent.
The electrophoretic patterns of reduced Fragments X (Stage 1), Y, and E in 12.5% polyacrylamide gels. The electrophoretic conditions were the same as those given in the legend to Fig. 3. The heavy polypeptide chains of Fragment Y have similar electrophoretic mobilities as the chains of Fragment X and remain near the top of the gel. The light polypeptide chains of Fragment Y migrate in the approximate position of those of Fragment X. The standards are in order of decreasing molecular weight, bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, and the three myoglobin polypeptide fragments (Myo-1, Myo-2, Myo-3).

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Content of fibrinopeptides in Fragments X, Y, D, and E—The fibrinopeptides recovered from the thrombin-treated degradation products are illustrated in Fig. 5, which shows the scans of paper electropherograms stained with Sakaguchi reagent. The fibrinogen preparation contains fibrinopeptides A and B and small amounts of the fibrinopeptide A derivatives, AP and AY. Fibrinopeptide B was not recovered from any of the fibrinogen degradation products, except for trace amounts in Fragment X preparations from Stage 1 digests. Fibrinopeptide A and small amounts of fibrinopeptides AP and AY were found in Fragments X, Y, and E. Preparations of Fragment D contain neither fibrinopeptide A nor B.

Quantitative analysis of the fibrinopeptide content in fibrinogen and the fibrinogen degradation products is summarized in Table II. The total amount of fibrinopeptides A, AP, and AY recovered from two samples of fibrinogen was 2.0 and 1.9 moles per mole of protein. Only 1.4 and 1.7 moles of fibrinopeptide B were recovered from each mole of this commercial fibrinogen. Fragment X preparations obtained from Stage 1 and from Stage 2 digests showed no loss of fibrinopeptide A content, with an average of 1.9 and 1.8 moles per mole of protein. Trace or barely measurable amounts of fibrinopeptide B were found in Fragment X preparations isolated from Stage 1 digests, but were not detected in those obtained from Stage 2 digests. Fragment Y contained approximately 2 moles of fibrinopeptide A and no fibrinopeptide B, just as was observed for Fragment X of Stage 2 digests. Fragment E contained less fibrinopeptide A than Fragment X or Y, about 0.3 mole per mole of protein. No fibrinopeptide B was found in Fragment E and Fragment D contained no detectable fibrinopeptide A or B.
TABLE II
Content of fibrinopeptides A and B in fibrinogen and its plasmin degradation products

Fibrinopeptide content (nmol/mg) was determined and reported for each of the indicated samples without prior knowledge of composition. Each figure represents an average of four analyses involving treatment with different concentrations of thrombin for different times and electrophoresed at two different pH values. Content of fibrinopeptides shown in moles of peptide per mole of the given protein has been calculated using following values of molecular weight: fibrinogen, 340,000; Fragment X, 260,000; Fragment Y, 155,000; Fragment D, 83,000; Fragment E, 50,000.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fibrinopeptide A</th>
<th>Fibrinopeptide B</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nmoles/mg</td>
<td>AP</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>4.2</td>
<td>1.1</td>
</tr>
<tr>
<td>X</td>
<td>4.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Stage 1</td>
<td>4.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Stage 2</td>
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<td>Stage 2</td>
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<tr>
<td>Y</td>
<td>8.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Stage 2</td>
<td>0.2</td>
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</tr>
<tr>
<td>E</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Stage 2</td>
<td>6.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* These preparations were purified from a digest obtained in the presence of hirudin and heparin.

To test the possibility that contaminating thrombin caused the loss of fibrinopeptide A from Fragment E and the loss of both fibrinopeptides A and B from Fragment D, plasmin digestion of fibrinogen was performed in the presence of hirudin (Sigma, St. Louis, Mo.) (5.0 units per ml final concentration) and heparin (Lipo-Hepin, Riker, Northridge, Calif.) (0.5 unit per ml final concentration). Fragments D and E were purified as described under “Materials and Methods,” except that hirudin and heparin were added to all reagents. Electrophoretic analyses of the purified fragments showed the same patterns as in Figs. 2 and 3. Fragment E contained 0.4 mole per mole of fibrinopeptide A and no fibrinopeptide B; Fragment D lacked both fibrinopeptides (Table II). Thus, the presence of less fibrinopeptide A in Fragment E than in Fragment X or Y is not due to the action of contaminating thrombin.

The presence of fibrinopeptides in individual polypeptide chains of the degradation products was analyzed by SDS polyacrylamide gel electrophoresis before and after thrombin treatment (Fig. 6). After reaction with thrombin, two polypeptide chains in Fragment X of Stage 1 digests showed increased electrophoretic mobility. The band with molecular weight of 58,300 moves slightly faster and is contiguous with the band of 53,200, suggesting the loss of a fibrinopeptide. The polypeptide chain of molecular weight of 20,800 also increases its mobility after thrombin action, suggesting the loss of a fibrinopeptide from this chain as well. In the Fragment X preparation from Stage 2 digest, only the 25,000 molecular weight polypeptide chain migrated faster after thrombin exposure. Thrombin action on Fragment Y caused an increase in electrophoretic mobility of the 25,000 molecular weight band (Fig. 6, 7% gel). The mean decrease in molecular weight of the fibrinogen Aα chain remnants in Fragments X and Y after thrombin exposure in 12 paired experiments was 1,890, with a p value of <0.01 as calculated by the paired t test. In contrast, measurement of the Bβ and γ chain remnants before and after thrombin exposure showed no consistent differences in molecular weight (p values >0.20). In addition, there was a definite change in the pattern of light chains of Fragment Y (Fig. 6, 13.5% gel). Although the heterogeneity of these chains precludes a precise determination of the changes, part of the material in the region of molecular weight of 12,600 and 10,400 was not demonstrable in the thrombin-treated sample.

No consistent changes in polypeptide chain pattern were observed in the Fragment D preparations. Although the fastest moving band of molecular weight 12,600 appears to be retarded after thrombin action (mol wt 13,400), this is apparently due to an experimental error since the other four major bands visible in the thrombin-treated preparation also migrate slightly slower than their counterparts in the untreated sample. The change in staining intensity of the 28,600 and 32,400 bands after thrombin was not a consistent finding in repeated study. Fragment E showed no change in polypeptide chain mobility after thrombin exposure, but only 15% of the Aα chain remnant would be expected to change mobility and these fibrinopeptide-free species might be obscured by other bands.

DISCUSSION

Among the five major electrophoretic bands present in Fragment X preparations, those with molecular weight in the range of 25,000 to 26,800 (Fig. 3 and Table I) can be identified with the greatest confidence. These bands migrate faster after thrombin treatment (Fig. 6), indicating the loss of a fibrinopeptide. Since these bands do not stain for carbohydrate (Table I), and since fibrinopeptide A but not fibrinopeptide B is recovered in significant quantity from thrombin-treated Fragment X (Fig 5 and Table II), these chains must derive from the NH2-terminal portion of the Aα chain. The Fragment X polypeptide chains of molecular weight 58,300 and approximately 53,000 stained with PAS reagent (Table I), suggesting their origin from either the Bβ or γ chains. Since the molecular weight of these chains is higher than that of intact γ chains, they are considered to be either intact Bβ chains or derivatives of it. The band of 58,300 was observed in only small amounts in Fragment X preparations from Stage 1 digests, consistent with the presence of only a small amount of fibrinopeptide B in this Fragment X preparation (Table II). The species of approximately 53,000 molecular weight probably represent the remainder of the Bβ chain after detachment of a 5,000 molecular weight NH2-terminal segment containing fibrinopeptide B (8, 15). This is consistent with the almost complete absence of fibrinopeptide B in Fragment X of Stage 2 digests (Fig. 5 and Table II).

Recent quantitative measurements of NH2-terminal amino acids in clots of coagulable derivatives in Stage 1 plasmin digests, here called the Fragment X family, have shown that more than 2 moles per mole of alanine and less than 2 moles per mole of glycine are present (35). Since the content of NH2-terminal tyrosine was the same as that of fibrinogen and since 2 moles of
FIG. 6. The effect of thrombin on the electrophoretic mobility of the polypeptide chains of fibrinogen degradation products. The arrows indicate bands affected by thrombin. Each pair of gels represents a nontreated (left) and a thrombin-treated sample (right). The electrophoretic conditions were the same as given in the legend to Fig. 3. In 7% polyacrylamide gels: Fragments X from Stage 1 (X, St. 1) and Stage 2 (X, St. 2) digests and Fragment Y (separate experiment) were run for 20 hours and Fragment D for 8 hours at 2 mA per gel. In 12.5% polyacrylamide gels: Fragments Y and E were run for 20 hours at 2 mA per gel in separate experiments. The migration of individual bands in these electrophoretic systems may vary (25-27) and a change in mobility shown in the experiment in this figure may not correspond exactly to the mean change calculated from a group of such experiments.

Fibrinopeptide A are recovered from each mole of Fragment X (Table II), the NH₂-terminal parts of the Aα and the γ chains of this family of derivatives are intact. Therefore, it seems likely that the additional alanine residues are the result of the cleavage of the Bβ chain. Knowing the precise amino acid sequence of the NH₂-terminal portion of the Bβ chain of fibrinogen (30), this cleavage could occur at the Arg-Ala at position 42 to 43, the Lys-Ala at position 47 to 48, or the Lys-Ala at position 58 to 59. These postulated Bβ chain cleavages of 42, 47, or 58 residues would remove fragments of molecular weight of approximately 5,000 to 6,500, corresponding to the difference between the 58,300 and approximately 53,000 polypeptide chains of Fragment X (Fig. 3 and Table I). The polypeptide chain of approximately 48,000 molecular weight, which stains with PAS reagent (Table I), is considered to be an intact γ chain. Since the NH₂-terminal portion of the γ chain remnant of Fragment X is intact (6), the band of molecular weight 40,000 may result from a cleavage of the COOH-terminal region of the γ chain. However, a Bβ chain derivation of this 40,000 molecular weight band cannot be ruled out. These data are consistent with the view that Fragment X consists of a heterogeneous population of thrombin-clottable molecules which possess (a) an NH₂-terminal portion of the fibrinogen Aα chain of molecular weight down to 25,000, (b) a Bβ chain that is either intact (mol wt 58,300) or that lacks the NH₂-terminal 42, 47, or 58 residues (mol wt approximately 53,000), and (c) a γ chain that is either intact (mol wt 48,000) or shorter at the COOH-terminal portion (mol wt approximately 40,000). Thrombin-clottable fibrinogen derivatives with Aα chain remnants greater than 26,800 found in Fragment X (Stage 1) result from the COOH-terminal cleavages of the Aα chain noted in degraded fibrinogen prepared from human plasma (37). Fibrinopeptide A is present in the same amount as in fibrinogen (2 moles per mole), and even the Fragment X preparations obtained from Stage 2 digest exhibit the ability to form clots after exposure to thrombin.

The electrophoretic pattern of reduced Fragment Y shows seven predominant bands (Figs. 3 and 4 and Table I), the four largest of which bear a striking similarity to those of Fragment X obtained from Stage 2 digests. These remnants are assigned to the same Aα, Bβ, and γ chain origins as those of the Fragment X components. The 25,000 molecular weight component is thrombin-sensitive (Fig. 6) and corresponds to the 25,000 chain remnant of Fragment X (Stage 2). In support of this conclusion, thrombin treatment of Fragment Y releases 2 moles of fibrinopeptide A per mole (Fig. 5 and Table II), the same as was obtained from fibrinogen and from Fragment X (Stage 2). As expected, Fragment Y does not contain fibrinopeptide B, just as Fragment X (Stage 2) lacks this peptide. The 50,700 molecular weight chain is too large to derive from the Aα or γ chain remnants of Fragment X, and appears to be a slightly degraded chain of the 52,800 Bβ chain remnant of Fragment X (Stage 2). The polypeptide chains of 47,600 and 40,300 probably correspond to those of similar size in Fragment X (Stage 2) and are therefore assigned to the γ chain.

The three remaining bands in Fragment Y have molecular weights of 12,600, 10,400, and 8,600 (Fig. 3 and Table I). These remnants are absent in Fragment X (Stage 1) preparations (Fig. 4); on the other hand, they are similar, although not identical, to the polypeptide chains of Fragment Y. This similarity is
interpreted to reflect a true structural relationship, in which the lighter remnants of Fragment Y correspond to the polypeptide chains of one-half of the Fragment E molecule (see Fig. 7).

The polypeptide chains of molecular weights 19,400 (Fragment Y) and 18,300 (Fragment E) are assigned to a γ chain origin from fibrinogen. This conclusion is based on the staining reaction with PAS reagent (Table I) and the molecular similarity of Fragment E with the NH-terminal disulfide knot (6, 38, 39), which has a single carbohydrate moiety in position 52 of the γ chain remnant (40), just as in fibrinogen (41). The polypeptide chain of molecular weight of 12,600 (Fragment Y) is assigned to an Αα chain origin. This is based upon an increase in electrophoretic mobility after incubation with thrombin and apparent decrease in molecular weight compatible with the loss of a peptide (Fig. 6). The Fragment E band of molecular weight 13,400 corresponds closely with the 12,600 Αα chain remnant of Fragment Y and is therefore also assigned to an Αα chain origin. The chains of molecular weight of 8,600 (Fragment Y) and 7,700 (Fragment E) have the same distinctive bluish hue after staining with Coomasie blue and are assigned by elimination to a Bβ chain origin. These data are in agreement with the primary structure of Fragment E (42), indicating molecular weights of approximately 10,000, 7,000, and 9,000 for the Αα, Bβ, and γ chain remnants, respectively. The higher molecular weight found in the present study for the Αα remnant of Fragment E probably reflects the error of the SDS gel technique.

The Fragment Y family of derivatives could thus be described as a heterogeneous group of molecular species with (a) two sets (heavy and light) of polypeptide chain remnants, (b) 2 moles per mole of fibrinopeptide A, presumably on both the heavy and light chain remnants of the fibrinogen Αα chain, and (c) NH₂-terminal tyrosine on both the heavy and light remnants of the fibrinogen γ chains.

The electrophoretic patterns indicate that our preparations of Fragment D contain six predominant bands (Table I). The largest has a molecular weight of 42,500 and stains with PAS reagent. It is considered a derivative of the PAS⁺ Bβ chain of fibrinogen, since Fragment E appears to contain the PAS⁺ remnant of the fibrinogen γ chain. Fragment D bands of 38,600, 32,400, and 26,400 do not stain with PAS reagent, are too large to derive from the Αα chain remnant of Fragment Y, and are considered to be the carbohydrate-free remnants of the fibrinogen γ chain. The Fragment D chain of 12,600 would be an Αα chain remnant by exclusion and the 5,350 piece is unassigned at present.

The heterogeneity of the Fragment X and E preparations implies that these derivatives may not have two identical sets of polypeptide chains in the molecule. However, the differences between the sets are relatively minor, in comparison with their over-all symmetry, especially in contrast to Fragment Y, which appears to have a striking disproportion between its two sets (heavy and light) of polypeptide chains (Fig. 7). Since Fragment Y originates from a symmetrical precursor (Fragment X), the presence of light and heavy groups of polypeptide chains indicates that Fragment X is split "off center" and that Fragment Y is an asymmetric molecule. The heavy remnants of Fragment Y would correspond to the chains of the Fragment X family, on either side of the NH₂-terminal junctional center of the molecule (6). The light remnants of Fragment Y represent the residual of a set of Fragment X chains, originating after Fragment X has been split asymmetrically into Fragment Y and Fragment E moieties (11-13). Implicit in this scheme is the origin of a second Fragment D molecule from the intact, heavy chain portion of Fragment Y, cleavage of which leaves behind Fragment E (Fig. 7). The light remnants of Fragment Y correspond to one-half of Fragment E, the second half appearing after the splitting of the second Fragment D molecule from the heavy chain region by plasmin. This results in the appearance of a molecule which consists of three pairs of light chains linked by disulfide bonds, recognizable now as Fragment E. Fig. 7 shows the formation of the second Fragment D (from Fragment Y), the first Fragment D having already been released from the other set of heavy chains of Fragment X, when it was cleaved by plasmin. Fragment D lacks fibrinopeptides (Table II and Figs. 5 and 7), consistent with its origin from the COOH-terminal region of Fragments X and Y.

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