Primary Structure of Human Fibrinogen and Fibrin

ISOLATION AND PARTIAL CHARACTERIZATION OF CHAINS OF FRAGMENT D*

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SUMMARY

Fragment D has been isolated as an apparently single molecular weight species (molecular weight about 100,000) from plasmin digests of human fibrinogen, using a combination of affinity chromatography on insolubilized “fibrin monomer” and gel filtration. This fragment consists of three chains with molecular weights of 45,000 (Dβ), 42,500 (Dγ1), or 39,500 (Dγ2), and 14,000 (Da) held together by disulfide bonds. The S-carboxymethyl derivatives of the chains have been separated by gel filtration and ion exchange chromatography, and their identity has been confirmed by peptide mapping and immunological analysis. The chain with a molecular weight of 45,000 is a fragment of the Bβ chain of fibrinogen. The chain derived from the γ chain of fibrinogen occurred in two molecular forms having molecular weight 42,500 and 39,500. The chain derivative with molecular weight 14,000 is most likely derived from the Aα chain of fibrinogen. The chains were characterized by NH₂-terminal sequence analysis, amino acid composition, and carbohydrate staining. The two molecular forms of the γ chain appeared to be identical except for an NH₂-terminal peptide extension of 23 amino acid residues in the longer chain. The latter has sequences in common with the COOH-terminal part of the γ chain of the NII-terminal disulfide knot (Blomback, B., Gröndahl, N. J., Hessel, B., Iwanaga, S., and Wallén, P. (1973) J. Biol. Chem. 248, 5806-5820); its NH₂-terminal residue being Ala-63 of the γ chain of fibrinogen.

Studies from this laboratory are aimed at the elucidation of the primary structure of human fibrinogen and fibrin. Cleavage of fibrinogen with CNBr was selected as a degradation method in order to obtain fragments suitable for structural analysis. Thus the primary structure of the CNBr-derived NH₂-terminal disulfide knot (N-DSK) has been established (1-3). The structures of the other CNBr-derived fragments of fibrinogen are under investigation (4-6). In order to align the latter fragments in the structure of fibrinogen, digestion with plasmin was carried out. The investigation of the structural relationship between the plasmin-derived Fragment E and the CNBr-derived N-DSK has resulted in the elucidation of the major portion of the primary structure of Fragment E and has provided overlaps between CNBr-fragments of both the Aα and the Bβ chains of fibrinogen (7, 8). Furthermore, it has been demonstrated (4, 6) that the plasmin-derived Fragment D contained three of the disulfide-containing CNBr fragments of fibrinogen accounting for all except two of the disulfide bridges of fibrinogen located outside N-DSK.

Several studies have shown that Fragment D is heterogeneous with respect to charge as well as to size (4, 9-17). Plasmin digests of fibrinogen always contain several D fragments with a molecular weight usually varying between 80,000 and 100,000. These fragments appear to be composed of three chains held together by disulfide bonds (17, 18). It was suggested that one of the chains, with a molecular weight of about 14,000, is derived from the Aα chain, and one with a molecular weight of 42,000 to 45,000 from the Bβ chain of fibrinogen. The γ chain derivatives with molecular weights varying between 42,000 and 25,000 were also reported.

In an effort to position the disulfide-containing CNBr fragments in the structure of Fragment D an investigation of its primary structure was undertaken. In the present report, the largest component (molecular weight about 100,000) of the D family has been characterized.

EXPERIMENTAL PROCEDURE

Human Fibrinogen, Enzymes, Other Reagents, and Apparatus

Fibrinogen—Fibrinogen of 96 to 99% purity was prepared as previously described (19).

Enzymes—Human plasmin (EC 3.4.21.7) (Lot Pli 45) containing about 10 CTA (Committee on Thrombolytic Agents) caseinolytic units per mg of protein was kindly supplied by Dr. P. Wallén.

1 The disulfide-containing fragments are provisionally classified into two groups, namely hydrophobic and hydrophilic disulfide knots (DSK), based on their partition coefficient in countercurrent distribution. N DSK constitutes (Aα 51, Bβ1 118, γ 1-78). The abbreviation used is: PTH, phenylthiohydantoin.


Department of Medical Chemistry, Umeå University, Umeå, Sweden. Trypsin (EC 3.4.11.4) treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, was obtained from Worthington Biochemical Corp., Freehold, N. J. Bovine thrombin (EC 3.4.21.5, 280 NIH units per mg) was prepared as previously described (1).

Chemicals and Apparatus—Dithiothreitol came from P-L Biochemicals, Milwaukee, tritiated iodoacetic acid from The Radiochemical Centre, Amersham, England, Sephadex and Sepharose 4B from Pharmacia, Uppsala, Sweden, and CM-cellulose (Whatman CM52) from H. Reeve Angel and Co., London, England. N-O-bis-(tri-methylsilyl)-acetamide was obtained from Pierce Chemical Co., Rockford, Ill., and SP-400 from Beckman Instruments, Inc., Palo Alto, Calif. Aprotinin (Trasylol) was a gift from Baermann, Leiden, The Netherlands. All reagents used were of analytical grade. The thin layer electrophoresis equipment came from Desaga, Heidelberg, Germany, the amino acid analyzer (model available 1969) was from Technicon Instruments Corp., Chauncey, N. Y. The liquid scintillation counter (model CPM-200), the protein sequenator (model 880), gas chromatograph (model GC-65), and analytical ultracentrifuge (model E) were products of Beckman Instruments, Inc., Palo Alto, Calif. The thin layer scanner was obtained from Berthold, Wildbad, Germany. Ultrafiltration was performed with PM-30 Diallo membranes from Amicon Corporation, Lexington, Mass.

Digestion with Enzymes

Plasmic Digestion—Digestion of 1-g batches of human fibrinogen in 50 ml of 0.037 M Tris-HCl/0.1 M NaCl buffer, pH 7.6, containing 0.02% sodium azide, was performed at room temperature as previously described (4). Plasmin (0.5 mg) was added three times at 1-hour intervals and pH adjusted before each addition. The digestion was then allowed to proceed for another 20 hours, after which it was terminated by the addition of aprotinin (10,000 kallikrein inhibitor units (KIU)).

The digests were lyophilized.

Purification of Fragment D

Affinity Chromatography—Fragment D together with higher molecular weight materials (Fractions X and Y) (20) were isolated from the plasmic digest of fibrinogen by affinity chromatography on fibrinogen coupled to CNBr-activated Sepharose 4B (21-24). The conjugated gel was prepared as described previously (22) using 1 g of fibrinogen per 2 g of dry gel. The conjugate was then treated with thrombin (5 or 10 NIH units/ml of suspension) as previously described (22). After 3 hours of incubation at room temperature, the conjugate was washed with 0.05 M Tris-HCl/0.1 M NaCl/0.025 M e-aminoacproic acid/6 M urea, pH 4.1, to remove thrombin.

For the present experiments, a column (5 cm x 70 cm) of the conjugate containing 7 g of insolubilized protein was equilibrated at room temperature with 0.05 M Tris-HCl/0.1 M NaCl/0.025 M e-aminoacproic acid buffer, pH 7.6, containing 0.02% sodium azide. The solution was designated as preservative. Of the plasmic digests, 50 ml (20 mg/ml) were applied to the column and nonadsorbed protein was eluted with the equilibrium buffer at a flow rate of 60 ml/hour. Elution of absorbed proteins was then performed with a linear urea gradient using 500 ml of starting buffer containing 4 M urea in the mixing chamber and 500 ml of starting buffer containing 4 M urea in the reservoir. After use, the column was regenerated by washing first with 50 to 100 ml of 6 M guanidine HCl and then re-equilibrated with buffer. The affinity columns were used repetitively during several months. Fractions containing the largest Fragment D (cf. Fig. 1) were pooled, dialyzed against distilled water, and lyophilized. Alternatively, the dialyate was concentrated by ultrafiltration.

Gel Filtration—In order to remove the larger degradation products (Fractions X and Y) from the Fragment D preparations, gel filtration was performed at room temperature on Sephadex G-150 columns (20 cm x 89 cm) equilibrated and eluted with 0.1 M NH₄HCO₃.

Fig. 1. Polyacrylamide gel electrophorogram of intact plasmic digests. Of the samples, 10 ~1 (200 mg of protein) mixed with 20 ~1 of 10 M urea/5% acetic acid were applied to the gel. The electrophoresis was performed on 7.5% gels in 5% acetic acid for 45 min. Under the conditions of digestion used, the polyacrylamide gel patterns varied between extremes, depicted as A and B. D, Fragment D; E, Fragment E.

Isolation of S-Carboxymethyl Derivative Chains of Fragment D

A two-step procedure was adopted to separate the chains of Fragment D.

Gel Filtration—Gel filtration was performed on Sephadex G-100 (20 cm x 90 cm) columns in 10% acetic acid at room temperature. The eluates were monitored by measurement of absorbance at 280 nm and by radioactivity measurements.

Ion Exchange Chromatography—The main protein fraction from the eluate of the Sephadex column was adsorbed on a CM-cellulose column (5 cm x 40 cm) equilibrated with 0.04 M sodium acetate/4 M urea, pH 4.3, containing 8 M urea. The column was developed with a gradient of increasing pH. The protein fractions were pooled, desalted on Sephadex G-25 in 50% acetic acid, and lyophilized.

Isolation of S-Carboxymethyl Derivative Chains of Fibrinogen

The chains of fibrinogen were separated essentially according to Murano et al. (24), but using a more shallow, slightly convex pH gradient to obtain better resolution.
The manual phenylisothiocyanate method of Edman was used (27). Qualitative NH₂-terminal analyses were made as described elsewhere (28). Quantitative NH₂-terminal analyses using [³⁵S]-phenylisothiocyanate were performed essentially as previously described (2, 29-30). The modified methods for soluble and conjugated proteins are described below.

**Soluble Proteins**—Between 0.2 and 1.0 mg of protein were dissolved in 200 μl of 10 M urea in 0.15 M Tris-HCl, pH 9.5; subsequently 400 μl of pyridine were added. After coupling with 5 to 10 μl of [³⁵S]phenylisothiocyanate (specific activity 20 μCi/μmol) for 1 hour (40° excess reagents were removed by repeated (five times) washing with 1.2 ml of benzene. Bovine serum albumin (4 mg) was added as carrier, after which all proteins were precipitated by adding 2 ml of acetone. The precipitate was removed by centrifugation and washed twice with aqueous acetone. (The precipitate was first stirred with 0.8 ml anhydrous acetone, whereupon 0.2 ml of water was added and stirring continued.)

The phenylisocarbamyl protein was dried over P₂O₅ and KOH in vacuo. Cleavage and cyclization were performed by adding 300 μl of 1 N HCl and heating on a boiling water bath for 1 hour. Phenylthiohydantoins were secured by three successive extractions with 0.6 ml of ethyl acetate containing a mixture of non-radioactive PTH-derivatives (0.05 μmol of each per ml). The extracts were pooled and evaporated. The nitrogen at 40° was replaced by 20 to 220 μl of ethyl acetate. Recovery of PTH-derivatives was calculated on the basis of radioactivity determination of the ethyl acetate solution. The PTH-derivatives were identified by thin layer chromatography and paper electrophoresis (28). The plates were scanned for radioactivity, the radioactive spots were eluted with 2 to 3.5 ml of 95% ethanol, and the radioactive PTH-derivatives were quantitated by liquid scintillation counting.

**Conjugated Proteins**—Between 1 and 15 mg of Sepharose-conjugated protein were used. The dry conjugates were suspended in 200 μl of 0.15 M Tris-HCl buffer, pH 9.5, and allowed to swell in vacuo. Cleavage and cyclization were performed by adding 300 μl of 1 N HCl for 60 min at 80°.

**Sequence Analysis**

Degradation of 0.2 to 0.43 μmol of the isolated S-carboxymethylated chains of Fragment D was performed with a Beckman Sequencer using the protein program D. The PTH-derivatives were identified by thin layer chromatography (28, 30), paper electrophoresis (28), and gas chromatography. Identification by gas chromatographic analysis on SP-400 was performed essentially as described by Hermanson et al. (31). Trimethylsilylations were performed in 0.6% agar in 0.04 M Tris-HCl buffer, pH 7.2, containing 0.11 M NaCl and 0.1% NaOH (32). Solutions of S-carboxymethylated chains were made by first dissolving 1.0 mg of each in 250 μl of 0.04 M Tris-HCl buffer containing 0.11 M NaCl and 8 M urea, pH 7.2. After dissolution 750 μl of the same buffer without urea were added slowly with vigorous stirring. This procedure allows for the chains to remain in solution with only minimal precipitation. Direct dissolution of chains in the same buffer containing 2 M urea is not possible.

**Additional Analysis**

Polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate was as previously described (36, 37). Gels were stained for carbohydrate with basic fuchsin after oxidation with periodic acid (38).

**RESULTS**

**Preparation of Fragment D and Isolation of Its S-Carboxymethylated Chains**

Preparation and Characterization of Fragment D—Under the conditions of plasmid digestion the polyacrylamide gel pattern of different digests varied between the two extremes shown in Fig. 1. With regard to Fragment D, in the one case (Fig. 1, Gel A) the slowest Fragment D band is predominant over the other members of the same family. Such preparations also contain other degradation products (presumably X and Y). In the other case (Fig. 1, Gel B) faster moving components are present in significant amounts together with slower moving Fragments D. The main electrophoretic forms of Fragment D often appeared as double bands. (This cannot be distinguished in Fig. 1.)

The plasmid digests were subjected to chromatography on fibrinogen-Sepharose which had been treated with thrombin. Quantitative NH₂-terminal analysis of the conjugated gels revealed a glycine to tyrosine ratio varying between 1 and 1.95. In addition to glycine and tyrosine valine was also found (valine to tyrosine between 0.05 and 0.2) (23).

The results of affinity chromatography of the plasmid digests are summarized in Fig. 2. In the digests in which the persistence of Fragments X and Y indicated a less complete digestion, the nonadsorbed protein (Fig. 2A, 1) represented about 45% of the material applied to the column and the adsorbed material (the peak eluted with the urea gradient) represented about 53% (Fig. 2A, 2). In the more complete digests the nonadsorbed protein (Fig. 2B, 1) represented 74% of the material and only 19% molecular weights were determined at 20° using the meniscus depletion technique at 14,290 rpm (33). The runs were for about 18 hours. The samples (1 mg/ml) were dissolved in 0.1 M Tris, pH 9.0, containing 0.1% EDTA. The concentration was determined with interference optics. The partial specific volume was calculated from the amino acid composition, as previously described (2).
Fig. 2. Purification of large molecular weight Fragment D from plasmin digests of fibrinogen by affinity chromatography and gel filtration. A, affinity chromatography on a thrombin-modified fibrinogen-substituted agarose column (5 cm² x 70 cm) equilibrated with 0.05 M Tris-HCl/0.1 M NaCl/0.025 M e-aminocaproic acid/0.02% NaN₃ buffer, pH 7.6 (containing 5 KIU of Trasylol/ml). Of the plasmin digest shown in Fig. 1A, 50 ml were applied and nonadsorbed proteins washed out for 12 to 15 hours at room temperature at a flow rate of 60 ml/hour (first peak). Elution was then performed with a linear gradient consisting of 500 ml of equilibration buffer (starting buffer) and 500 ml of equilibration buffer containing 4 M urea (limiting buffer) at the same flow rate (second peak). Subsequently the column was regenerated by washing with 6 M guanidine hydrochloride, and re-equilibrated with starting buffer (third peak). The protein peak eluted with the urea gradient was concentrated by dialysis against distilled water and lyophilization or by ultrafiltration. —, ultraviolet absorbance; —-—, urea gradient. Insets, polyacrylamide gel electrophoretograms using 50 to 100 µg of the whole digests (gels marked sm) and of subfractions from the eluted peaks. B, plasmin digest shown in Fig. 1B was used. Otherwise the same as under A. C, gel filtration on a column (20 cm² x 90 cm) of Sephadex G-150 equilibrated with 0.1 M NaHCO₃. The protein material (225 mg) contained in Peak 2 in A was dissolved (15 ml/ml) in the equilibration buffer and chromatographed at a flow rate of 50 ml per hour. Inset, polyacrylamide gel electrophoretograms at pH 2.5 using 50 to 100 µg of protein. The fractions were pooled as indicated and lyophilized. D, protein material (130 mg) contained in Peak 2 in B was chromatographed as described under C.

was adsorbed (Fig. 2B, 3). Subsequent washing of the columns with 6 M guanidine HCl resulted in additional elution of smaller amounts of protein (Fig. 2, A, 3 and B, 3).

Fragments D with faster mobility on polyacrylamide gel electrophoresis (having a lower molecular weight as determined by Na dodecyl-SO₄ polyacrylamide gel electrophoresis) were found in the nonadsorbed fraction (Fig. 2B, 1). However, they appeared to have some affinity for the gel since 2- to 3-column volumes of buffer were required for their elution. This agrees with previous findings (23). The adsorbed material consisted of larger molecular weight degradation products (Fragments of X and Y) and Fragment D of high molecular weight (Fig. 2A, 2 and B, 2). In the beginning of the gradient, small amounts of lower molecular weight Fragment D were also demonstrated. The fractions containing Fragments X and Y and Fragment D of high molecular weight were pooled and subsequently gel filtered on Sephadex G-150 (Fig. 2, C and D). This resulted in a separation of Fragment D from larger degradation products. In some instances (Fig. 2C, 2), small amounts of material with mobility of Fragment E were eluted before the main Fragment D peak. Probably this
FIG. 3. Na dodecyl-\(\text{-SO}_4\) polyacrylamide gel electrophoresis of purified Fragment D and of the isolated \(S\)-carboxymethylated chains of Fragment D. About 20 \(\mu\)g of protein were applied. Electrophoresis was run for 23/4 hours in 7% gels. 1, Fragment D from a less complete digest (cf. Fig. 1A). 2, Fragment D from a less complete digest after reduction with dithiothreitol. 3, Fragment D from a complete digest (cf. Fig. 1B). 4, Fragment D from a complete digest after reduction. \(D_a\), \(D_b\), and \(D_y\) are the isolated \(S\)-carboxymethylated chains of Fragment D shown in 2.

material was gel filtered in the form of a Fragment D–E complex, which is known to occur at neutral or slightly alkaline pH (39). The material in the main protein peak, representing the largest molecular weight Fragment D was dialyzed and lyophilized. The yield of this Fragment D species varied between 175 and 350 mg/g of fibrinogen (or 0.8 to 1.2 mol/mol of fibrinogen). Lower yields were recorded for the more extensive digests.

Polyacrylamide gel electrophoresis at pH 2.5 of the high affinity Fragment D from the different types of plasmic digests revealed two closely migrating bands with the same mobilities as the slowest doublet bands observed in the original digests. Na dodecyl-\(\text{-SO}_4\) polyacrylamide gel electrophoresis of this material revealed one main band with an estimated molecular weight between 105,000 and 115,000 (Fig. 3, 1 and 3). Smaller amounts of higher molecular weight material were also discernible. Ultracentrifuge analysis of Fragment D showed a main component with an \(S\) value of 5.0. Small amounts (about 5 to 10\%) of material with a higher \(S\) value (7.0) were also detected. In equilibrium centrifugation Fragment D also showed signs of heterogeneity and therefore calculation of discrete molecular weights was not possible. However, the analysis showed that the molecular species are distributed in the range \(M_e = 81,000\) to 115,000.

Na dodecyl-\(\text{-SO}_4\) polyacrylamide gel electrophoresis in the presence of dithiothreitol (Fig. 3, 2 and 4) showed the presence of three or four bands. The bands with molecular weights of 45,000 and 39,000, respectively, occurred in constant amounts in all digests. Two intermediate bands with molecular weights of 42,500 and 39,500, respectively, occurred in variable ratios. Retrospectively it appeared that in Fragment D, derived from less complete plasmic digests (as in Fig. 3, 2), the band with a molecular weight of 42,500 was predominant, whereas in Fragment D, derived from more extensively digested fibrinogen (as in Fig. 3, 4), the band with a molecular weight of 39,500 was predominant. Carbohydrate staining with fuchsin sulfite of polyacrylamide gels before and after reduction and alkylation, showed initially a positive staining reaction for Fragment D and for all chains of Fragment D. However, only the color associated with the band corresponding to 45,000 molecular weight species persisted during destaining.

Quantitative NH\(_2\)-terminal amino acid analysis of Fragment D is shown in Fig. 4. Aspartic acid and alanine are the main NH\(_2\)-terminal amino acids in Fragment D. Small amounts of other amino acids were also detected. Fragment D derived from digests depicted in Fig. 1A revealed 1.4 mol of aspartic acid and 0.6 mol of alanine per mol of Fragment D (Fig. 4a). Fragment D preparations derived from more extensively degraded fibrinogen (cf. Fig. 1B) had a lower alanine content (Fig. 4b). Retrospectively this proved to be due to conversion of the chain with NH\(_2\)-terminal alanine to a chain with NH\(_2\)-terminal serine. The PTH-derivative of serine is to a large extent degraded during the analysis, which may explain our failure to demonstrate serine as NH\(_2\)-terminal residue in Fragment D.

The amino acid composition of Fragment D is shown in Table...
TABLE I
Amino acid composition of Fragment D and its isolated S-carboxymethylated chains

The samples were hydrolyzed in 5.7 N HCl at 110°. The figures are derived from 22-, 48-, and 72-hour hydrolysates and expressed as moles of amino acid per mol of the fragment.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Fragment D (MW 101,500)</th>
<th>Dα (MW 40,000)</th>
<th>Dβ (MW 45,000)</th>
<th>Dγ (MW 42,500)</th>
<th>Dβ (MW 39,500)</th>
<th>Sum of Dα, Dβ, and Dγ</th>
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<td>Aspartic acid</td>
<td>111.2</td>
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<td>49.3</td>
<td>44.3</td>
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<td>38.5</td>
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<td>Total residues</td>
<td>708.6</td>
<td>94.0</td>
<td>299.9</td>
<td>340.9</td>
<td>303.8</td>
<td>734.8</td>
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^a Figures obtained by extrapolation to zero time.
^b Determined as cysteic acid.
^c Determined as S-carboxymethylysteine.
^d Determined by ultraviolet analysis (cf. Ref. 1).

I. The Fragment D used in this analysis was derived from a digest similar to that shown in Fig. 1A. Fragment D appeared not to contain any free -SH groups as determined with Ellman's reagent. Antisera raised to Fragment D gave only one precipitin line on immunodiffusion against the immunizing antigen (Fig. 5). The same results were obtained in immunoelectrophoresis.

Isolation of S-Carboxymethylated Chains of Fragment D—The chains of reduced and alkylated Fragment D were separated in a two-step procedure involving Sephadex G-100 gel filtration in 10% acetic acid and CM-cellulose chromatography. Fig. 6 shows the separation of the chains of Fragment D derived from a digest as shown in Fig. 1A (less complete). On Sephadex gel filtration several peaks were obtained (Fig. 6A). Small amounts of material (less than 5% of the total) were eluted close to the void volume of the column. Most of this material is likely to represent incompletely hydrolyzed chains of Fragment D. On CM-cellulose chromatography (Fig. 6B), two major peaks were obtained (Fig. 6B, a and b). One of these, peak a, had a molecular weight of 42,500 and was eluted close to the void volume of the column. The other, peak b, had a molecular weight of 39,500 and was eluted in a slightly convex pH gradient. The effluent was pooled as indicated, desalted by gel filtration on Sephadex G-25 in 50% acetic acid and lyophilized. The main fraction was further fractionated by CM-cellulose chromatography, —- ultraviolet absorbance; ---, radioactivity; inset, polyacrylamide gel electrophoresis in 5% acetic acid using a 50- to 100-μg sample in each run. B, ion exchange chromatography on CM-cellulose column. The column (5 cm x 40 cm) was equilibrated with 0.04 M sodium acetate/10% acetic acid buffer, pH 4.3, containing 8 M urea. The sample (100 to 250 mg) was dissolved in 20 ml of 0.04 M sodium acetate/8 M urea buffer, pH 4.0, and was washed in with 1 column volume of equilibration buffer at a flow rate of 30 ml/hour. Elution was performed with a gradient composed of 500 ml of equilibration buffer in the mixing chamber and 550 ml of 0.04 M sodium acetate/8 M urea buffer, pH 4.9, in the reservoir. This resulted in a slightly convex pH gradient. The effluent was pooled as indicated, desalted by gel filtration on Sephadex G-25 in 50% acetic acid, and lyophilized. ---, ultraviolet absorbance; ---, radioactivity; inset, polyacrylamide gel electrophoretogram in 5% acetic acid.
completely reduced and alkylated Fragment D or aggregated car-
boxymethylated chains (Fig. 6A, inset). One protein peak (Fig.
6A, 1) contained three closely migrating bands as demonstrated by
Na dodecyl-SO4 polyacrylamide gel electrophoresis. The second peak (Fig. 6A, 2) showed only slight absorption at 280 nm, but could be localized by radioactivity measurements. Na dodecyl-SO4 polyacrylamide gel electrophoresis showed
one main component (Fig. 6A, 2). The gel filtration and Na
dodecyl-SO4 gel patterns obtained from a more complete digest
(as shown in Fig. 1B) were qualitatively similar to that described
above.

The material in the largest protein peak (Fig. 6A, 1) was sub-
jected to chromatography on CM-cellulose, which resulted in a
separation into two main peaks as shown in Fig. 6B. The first protein peak (Fig. 6B, 1) displayed one band on Na dodecyl-SO4
polyacrylamide gel electrophoresis. The elution profile of the
second peak suggested the presence of two partially separated
components (Fig. 6B, 2 and 3). By appropriate pooling these
components could be obtained in almost homogeneous forms (Fig. 6B, 2 and 3). The CM-cellulose chromatogram
of chains obtained from a more complete digest (see Fig. 1B) was
essentially the same except that the peak corresponding to that
shown in Fig. 6B, 3 was predominant. Also the Na dodecyl-SO4
polyacrylamide gel electrophorograms for the different peaks
were similar to those shown in the insets to Fig. 6B, 1 to 3.

Na dodecyl-SO4 polyacrylamide gel patterns of chains isolated
from the digests as described above are shown in close-up in
Fig. 3. The estimated molecular weights of the chain components
were: (Dp) 45,000; (Dy) 42,500; (Dyl) 39,500; and (Dx) 14,000.
NH2-terminal amino acid analysis also revealed essentially a
single amino acid for each of the separated chains. Aspartic acid,
alanine, serine, and aspartic acid were the NH2-terminal amino
acids of the chains Dp, Dx, Dy, and Dyl, respectively (Fig. 4 and Table II).

The amino acid composition of the chain components of
Fragment D is shown in Table I. The amino acid compositions of
the Dp and Dy chains are similar to one another and to that of the y chains of fibrinogen (24). The number of residues in Dy is somewhat smaller than in Dy, which is in accordance with its lower molecu-
lar weight. Likewise, the amino acid composition of Dp is similar
to that of the B3 chain of fibrinogen (24). The amino acid com-
position of the Dx chain bears little resemblance to any of the
chains of fibrinogen.

The amino acid composition of Fragment D is in good agree-
ment with that calculated as the sum of the amino acid residues
present in the three chains (Dx + Dp + Dy) of Fragment D
with the notable exception of proline and lysine. In this com-
parison the molecular weight of Fragment D was assumed to be
101,590, which is the average value for the sum of the three main
chains.

Antiserum raised to Fragment D reacted with all three com-
ponent chains (Fig. 5A). Detectable, although very weak, reac-
tions were observed between the antiserum and the B3 and y
chains of fibrinogen (Fig. 5B). No reaction was observed with
anti-Fragment D and the Ax chain of fibrinogen. In addition it
was found that another antiserum, anti-N-DSK (2), in immuno-
electrophoresis gave one single anodal precipitin line with reduced
and alkylated Fragment D.

These analyses show that epitopes of Fragment D are present
in all three chains and that very likely segments of the B3 and y
chains of fibrinogen are represented in the molecule, which is in
agreement with the amino acid analysis of these chains. Since
the Ax chain of fibrinogen did not react with anti-Fragment D
it must be concluded that these epitopes are either hidden in the
intact chain or that they belong to a different chain structure in
fibrinogen. The results with anti-N-DSK would indicate that
N-DSK and Fragment D have structures in common.

Identification of Isolated S-carboxymethylated Chains of Fragment
D with Respect to Chains of Fibrinogen by Peptide Mapping

Peptide maps of tryptic peptides of the chains of Fragment D were
used to confirm our preliminary identification4 as to the origin of the chains. This was particularly important for identifica-
tion of the chain designated Dx since the results of the above
analyses could not confirm that the origin was in the Ax chain.
The tryptic peptide maps of the isolated S-carboxymethylated
chains of fibrinogen and Fragment D are shown in Fig. 7. A com-
parison of these maps suggested that the chain designated Dp
was derived from the B3 chain of fibrinogen. The chain designa-
ted Dy (Dy1) appeared to belong to the y chain. This interpre-
tation was further substantiated by comparison of the amino
acid composition of corresponding, well separated peptides in
the maps of these chains with those derived from the intact chains of
fibrinogen (Fig. 7, a and b, insets). The peptide map of the chain
designated Dx showed a striking resemblance with the peptide
map of the Ax chain of fibrinogen. Also in this case correspond-
ing peptides were eluted for amino acid analysis. The identity of
these peptides could, however, not be established because the
peptides derived from the peptide map of the intact Ax chain
were either to a great extent overlapping with other peptides in
the map or they were obtained in too low yield.

These analyses show, beyond any reasonable doubt, that the
chains designated Dy and Dy1 originate in the B3 and y chains of
fibrinogen, respectively. The fact that Dy has an amino acid
composition similar to Dy1 must mean that they have the same
chain origin. The analyses also suggest that Dx originates in the
Ax chain of fibrinogen.

Partial Amino Acid Sequence of Chains of Fragment D

The partial amino acid sequence of the chains of Fragment D
is shown in Table III. For Dx 12 residues and for Dy9 residues
could be clearly identified.

Most important at this time are the results obtained with Dy1
and Dy2. For Dy1 and Dy2, 31 and 13 residues, respectively, were
deduced. As shown in the table some PTH-derivatives could not
be identified with certainty. With regard to position 24 in Dy1
trace amounts of PTH serine were identified in one solvent sys-
tem. The water phase of step Dy1 25 was accidentally lost.
However, no PTH-derivative was demonstrated in the organic

---

TABLE II

NH2-terminal analysis of Fragment D and its
S-carboxymethylated chains (Dx, Dy, and Dy1)

<table>
<thead>
<tr>
<th>PTH-derivatives</th>
<th>Fragment Dp</th>
<th>Fragment Dy</th>
<th>Dx</th>
<th>Dy</th>
<th>Dy1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.81</td>
<td>0.19</td>
<td>0.04</td>
<td>0.21</td>
<td>1.00</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.00</td>
<td>2.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.17</td>
</tr>
<tr>
<td>Serine</td>
<td>0.09</td>
<td>0.08</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Fragment D obtained from the preparation shown in Fig. 1A.
b Fragment D obtained from the preparation shown in Fig. 1B.

The figures represent the relative distribution of PTH-derivatives
assuming 2.0 aspartic acid residues as integer for Fragment D,
1.0 aspartic acid residue as integer for Dx, and 0.3 alan-
ine residue as integer for Dy1.
TABLE III
Amino acid sequences of isolated S-carboxymethylated chains of Fragment D (Dα, Dβ, and Dγ1, and Dγ2)

Amino acid sequences obtained by Edman degradation in a Beckman Sequencer. Between 6 and 10 mg (Dα: 0.43 μmol; Dβ, Dγ1, and Dγ2: about 0.25 μmol) were used for coupling.

<table>
<thead>
<tr>
<th>Chain</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dα</td>
<td>Asp-Asn-Thr-Tyr-Asn-Arg-Val-Val-Glu-</td>
</tr>
<tr>
<td></td>
<td>1 5 Asp-Leu-Arg-</td>
</tr>
<tr>
<td>Dβ</td>
<td>Asp-Asn-Glu-Asn-Val-Val-Asn-Glu-Tyr-</td>
</tr>
<tr>
<td></td>
<td>1 5</td>
</tr>
<tr>
<td>Dγ1</td>
<td>Ala-Ile-Gln-Leu-Thr-Tyr-Asn-Pro-Asp-</td>
</tr>
<tr>
<td></td>
<td>10 15 Glu-Ser-Ser-Lys-Asn-Met-Ile-Asp-Ala-</td>
</tr>
<tr>
<td></td>
<td>20 25 Ala-Thr-Leu-Lys-(Ser)-Xa-Lys-Met-Leu-Xa-</td>
</tr>
<tr>
<td></td>
<td>30 Xa-Ile-Met-Lys-Tyr-</td>
</tr>
<tr>
<td>Dγ2</td>
<td>Ser-Arg-Lys-Met-Leu-Glu-Glu-Ile-Met-</td>
</tr>
<tr>
<td></td>
<td>1 5</td>
</tr>
<tr>
<td></td>
<td>Lys-Tyr-Glu-Ala-</td>
</tr>
</tbody>
</table>

* See text.

Fig. 7. Tracing of peptide maps of tryptic digest of S-carboxymethylated chains of Fragment D and fibrinogen. Of the digest, 0.5 to 1.0 mg were applied to each plate. a, Dβ and Bβ chains. Upper pattern was stained with ninhydrin and lower pattern was stained with Sakaguchi’s reagent. Inset, photograph of peptide map of Bβ chain, stained with ninhydrin, and amino acid analysis of material (from three plates) eluted from spots as indicated. b, Dγ(Dγ1) and γ chains. Upper pattern was stained with ninhydrin and lower pattern was stained with Sakaguchi’s reagent. Inset, photograph of peptide map of Dγ chain, stained with ninhydrin, and amino acid analysis of material (from three plates) eluted from spots as indicated. c, Dα and Aα chains. Upper pattern was stained with ninhydrin and lower pattern was stained with Sakaguchi’s reagent. Inset, photograph of peptide map of Dα chain.

In view of the known size heterogeneity of Fragment D an effort was made to isolate the largest member of this family from a plasmin digest of fibrinogen. Under seemingly identical conditions some variability in the extent of digestion was obtained. This may have been caused by varying amounts of plasminogen present in the fibrinogen preparations used for digestion. The two extreme cases of extent of digestion are reported in more detail in the present study. In the one case, fibrinogen was digested to a stage in which the largest Fragment D was predominant over the other members of this family. This preparation phase. Considering the sequence similarities between Dγ1 and Dγ2 it is likely that arginine occupies position 25 in Dγ1. For unknown reasons the chromatographic separation of PTH-derivatives in Solvents E and T II was not satisfactory in steps 29 and 30 of Dγ1 and glutamic acid and aspartic acid were not resolved. However, from the sequence similarity with Dγ2 it is likely that glutamic acid occupies these positions in Dγ1.

The amino acid sequence of Dγ1 shows that it has an NH2-terminal sequence of 16 residues in common with the COOH-terminal portion of the γ chain of N-DSK, i.e. γ 63 to 78 (2). Furthermore, the sequence analysis suggests that beyond residue 23 of Dγ1 the sequence is identical with that of Dγ2. Thus, it appears likely that the latter chain arises as a result of cleavage by plasmin of the Lys-85-Ser-86 bond in the γ chain of fibrinogen.

DISCUSSION

In the present study, the plasmic fibrinogen degradation product Fragment D was purified and its chains separated as a preliminary towards the elucidation of the primary structure of Fragment D. The structure of Fragment D also provides us with overlaps between CNBr-fragments that cover portions of the molecule not completely contained in Fragment D.
contained significant amounts of the larger degradation products (Fragments X and Y). In the other case, Fragments X and Y had virtually disappeared and in such preparations the smaller molecular weight species of Fragment D were present in high amounts. By affinity chromatography on fibrin monomer-Sepharose conjugates, high molecular weight degradation products including large size Fragment D and Fragments X and Y were strongly adsorbed, whereas Fragments D of lower molecular weight were not firmly bound to the column. Under our conditions of elution with urea, no separation of Fragments X and Y on the one side, and Fragment D on the other side, was obtained. Separation of these products could easily be achieved by gel filtration in bi carbonate buffer. Gel filtration in acetic acid resulted in less satisfactory separation, and furthermore, after exposure to acid the products became insoluble at neutral pH. The purified Fragment D showed essentially one band in Na dodecyl-SO₄ polyacrylamide gel electrophoresis. Ultracentrifugal analysis showed that the main component in the Fragment D preparation had an S value of 5, which is in agreement with results of other investigators (11). Small amounts of a 7 S component were also present in the preparation. The molecular weights for the different molecular species appear to be in the range 81,000 to 115,000 as determined by equilibrium centrifugation.

The amino acid composition of Fragment D described in this report is generally in good agreement with the sum of the amino acid compositions of the composite chains (Da, Dβ, Dγ) if a molecular weight of about 100,000 is assumed for Fragment D. The fact that the amounts of lysine and proline were somewhat more than could be accounted for by the isolated chains would indicate that the Fragment D preparation contains small amounts of extended chains rich in these amino acids. In fact, in reduced and alkylated Fragment D (cf. Fig. 6A) small amounts of chains with presumably higher molecular weights than Dβ and Dγ were observed.

NH₂-terminal analysis of Fragment D showed aspartic acid and alanine as NH₂-terminal residues. In agreement with our results, Gårddlund et al. (4), Marder et al. (14), and Mosesson et al. (40) also found aspartic acid to be one of the main NH₂-terminal residues isolated from a late digest. However, Marder et al. (14) and Mosesson et al. (40) also found other main NH₂-terminals in their analysis, suggesting considerable microheterogeneity in the NH₂-terminal portions of Fragment D.

In the present study the molecular weights of the three S-carboxymethylated chains add up to between 99,000 and 102,000, which is in good agreement with the result of Pizzo et al. (17) on a similar Fragment D, isolated by a different method. The minimum molecular weight is in reasonable agreement with the molecular weight range determined for whole Fragment D by ultracentrifugal analysis. This indicates that Fragment D is a monomeric structure in accordance with results of other investigators (4, 17, 18, 41).

In accordance with this is also the NH₂-terminal analysis of Fragment D. Two of the chains of Fragment D (Da and Dβ) have NH₂-terminal aspartic acid. Therefore the yield of 1.4 mol of aspartic acid per 10⁵ g of Fragment D is in agreement with the monomer concept. The yield of NH₂-terminal alanine (Dγ) in Fragment D from a less complete digest was 0.6 mol per 10⁵ g. Considering that only small amounts of serine chains (Dγ) are present in such preparations of Fragment D (cf. Fig. 6B) this again suggests that Fragment D is monomeric. These results do not support the suggestion by Mosesson et al. (40) that Fragment D is a dimeric structure. This suggestion was based on the yield of NH₂-terminal aspartic acid in the γ chain of Fragment D, which was claimed to be the only chain of Fragment D containing this NH₂-terminal residue.

The chains of Fragment D were localized in fibrinogen by comparison of the peptide maps for the separated chains, with those of intact chains of fibrinogen. Amino acid analysis of chains and eluted peptides from the maps, served to further corroborate the results. These studies unambiguously demonstrated that three of the chains of Fragment D are derived from the Bβ and γ chains of fibrinogen. Immunological analysis supports the above interpretations. Finally, the sequence analysis of the Dγ₁ and Dγ₂ definitely established their identity. The lack of reaction between anti-Fragment D and the Aα chain of fibrinogen was unexpected since all chains of Fragment D reacted with the antibody. This may indicate that the particular segment does not belong to the Aα chain or that it is hidden in the intact chain. However, the peptide map of the Da chain of Fragment D showed striking similarities with the Aα chain, but not with the other chains of fibrinogen. We therefore favor the view that the chain fragment designated Da is an Aα chain remnant. A chain of Fragment D of similar size has been assigned by other workers (16, 17) to the Aα chain from the succession of chain band patterns on polyacrylamide gel electrophoresis during plasmic digestion of fibrinogen.

Of particular interest is the occurrence in the large molecular weight Fragment D of γ chains with varying chain lengths. The precursor-product relationship and the similarity in amino acid composition between Dγ₁ and Dγ₂ suggest that Dγ₂ is derived from Dγ₁. The molecular weights of Dγ₁ and Dγ₂ (42,500 and 39,500, respectively) agree reasonably well with the values found by Pizzo et al. (17) for their γ chain components in large molecular weight Fragment D. The Dγ₂ component also agrees with the largest γ chain reported by Budzynski et al. (18). Finally, the sequence data presented here give strong support for the conclusion that the two γ chains result from cleavage by plasmin of bonds γ 62 and 63 and γ 85 and 86, respectively.

Using antiserum to N-DSK Gårddlund et al. (4) had previously observed that Fragment D appeared to have antigenic determinants in common with N-DSK. In the present report the proximity between Fragment D and N-DSK has been established on the basis of sequence data for the γ chain derivatives. Thus the results clearly show that Fragment D has sequences in common with N-DSK. This is also corroborated by our immunological results. The structure of the two γ chains shows another interesting feature, inasmuch as the sequence from Lys-85 is identical with the chain of one of the hydrophobic disulfide knots obtained by cleavage of fibrinogen with CNBr.

Consideration of the size of the chain fragments derived from fibrinogen, N-DSK, Fragment E, and Fragment D suggests that not only the γ chain but also the Bβ chain of Fragment D may be in close proximity of N-DSK. The Bβ chain with a molecular weight of 57,000 (37) must accommodate the Bβ chain portion of N-DSK with molecular weight 13,000, a small peptide segment of about M₀ = 600 (8) present in the Bβ chain of Fragment E and extending beyond the COOH-terminal residue in the Bβ chain of N-DSK, and finally the Fragment D portion of the β chain with a molecular weight of 45,000. Altogether these fragments appear to account for the molecular weight of the Bβ chain.

The fact that N-DSK and Fragment D have sequences in common is of considerable interest, since evidence for the location of complementary polymerization sites in these structures has been reported (22, 23). Thus Fragment D was found to contain a site(s) which interacts with fibrin monomer or thrombin-acti-
vated N-DSK. The question arises as to what extent the sequence in common (63 to 78) may be responsible for the affinity between these two structures. The fact that Fragment D containing Dyv, which lacks the sequence in common, also adsorbs on the affinity gel seems to rule out any major importance of the sequence between Fragment D and fibrin monomer or thrombin-activated N-DSK. In fact the NIH-terminal pattern of a mixture of Fragments D of several sizes (4) is essentially the same as found in the present report for the large size Fragment D, which would suggest that the lower molecular weight species of Fragment D are shorter at the COOH-terminal portions of their chains. Since the latter Fragments D do not firmly bind to fibrin monomer (23) the COOH-terminal segments may be of particular importance for binding. However, it is possible that the 763 to 78 sequence contains in part both of the complementary sites in N-DSK and Fragment D. This might explain why N-DSK and Fragment D-con-

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D Collen, B Kudryk, B Hessel and B Blombäck


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