Localization of the α-Chain Cross-link Acceptor Sites of Human Fibrin*

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The potential cross-link acceptor sites of fibrin were specifically labeled with the fluorescent, substitute cross-link donor monodansyl cadaverine (MDC). Several fluorescent α-chain peptides generated from enzymatic and cyanogen bromide (CNBr) cleavage of the labeled fibrin were identified by sodium dodecyl sulfate disc gel electrophoresis; they were isolated and then characterized by amino acid analysis, NH₂-terminal sequence analysis, and chromatographic and electrophoretic analyses of their digestion products. Ancrod cleavage of MDC-labeled fibrin produced a series of six α-chain peptides of molecular weights 34,000 to 12,000, each of which contained an MDC-labeled acceptor site, and an NH₂-terminal α-chain derivative of molecular weight 37,500. The latter remains disulfide bound in the residual fibrin and has two MDC-labeled sites which are separable by CNBr cleavage. Mild plasmin digestion of MDC-labeled fibrin generated fluorescent α-chain peptides of molecular weights 45,000, 42,000, 35,000, 23,000, 21,000, and 2,500 in the supernatant and a nonfluorescent NH₂-terminal α-chain derivative of molecular weight 25,000 which remained in the insoluble residual fibrin. The alignment of these plasmic superantenn peptides was determined from NH₂-terminal sequence analyses which indicated that an MDC acceptor site was located at approximately residue 255 of the α-chain. Cleavage of the MDC-labeled α-chain by CNBr, however, localized most of its fluorescence (~80%) to a fragment of molecular weight 29,000 which had the same NH₂-terminal sequence as the labeled plasmic peptide of molecular weight 21,000. Both peptides were cleaved by ancrod into two acceptor site-containing peptides of approximately equal fluorescence. The preliminary NH₂-terminal sequence analyses of these peptides, when combined with the above findings, indicated that these two other cross-link acceptor sites are in a peptide segment which comprises the middle 17% of the α-chain.

Human fibrinogen (Mₙ = 340,000) is composed of three pairs of polypeptide subunits (αA, βB, and γ) which are joined by disulfide bonds located mainly in their NH₂-terminal regions; the biochemistry of the conversion of fibrinogen to fibrin has been recently reviewed (1, 2). Cleavage of the αA- and βB-subunits by thrombin releases the NH₂-terminal fibrinopeptides A and B and forms the α- and β-chains of fibrin monomer. Fibrin monomers then associate into an ordered matrix of uncleaved fibrin. In the presence of activated fibrin-stabilizing factor and calcium, two intermolecular ε-(γ-glutamyl)lysine cross-links are rapidly formed between lysyl donor groups and glutaminyl acceptor groups in antiparallel γ-chains to give the γ-dimer subunit of partially cross-linked fibrin. Additional cross-linking occurs more slowly between α-chains to produce the α-polymer subunit of stabilized, fully cross-linked fibrin. The β-chains of fibrin do not cross-link. Each fibrin monomer is reported to contain four to six cross-link acceptor-donor pairs; each γ-chain has one such pair and each α-chain has either one or two such cross-link pairs. In the γ-chain it was shown that both the acceptor and donor cross-link sites are located within 15 residues of the COOH terminus.

In the case of the α-chain, previous reports have proposed that both the COOH- and NH₂-terminal halves may contain cross-link sites (3-5). Earlier studies from this laboratory demonstrated that the α-chain is clearly the first of the subunits to be cleaved by plasmin and that, with the degradation of fibrinogen to Fragment X, the cross-linking potential of the α-chain is lost while that of the γ-chain remains (6). This suggested that the acceptor and donor cross-link sites probably reside in the COOH-terminal two-thirds of the α-chain, which is released to give Fragment X. We previously confirmed this localization for the acceptor sites by utilizing the observations that monodansyl cadaverine is a site-specific fluorescent label for the glutaminyl cross-link sites of fibrin (7, 8). The location of one of these sites, which was labeled with a different substituent donor compound, was proposed to be near the middle of the α-chain (9). However, Doolittle and co-workers have recently indicated that this glutaminyl residue was not cross-linked in fibrin prepared from purified fibrinogen (10). The purpose of this communication is to
provide evidence for the specific location in the α-chain of three potential cross-link acceptor sites. We report the results of time course digestions of MDC-labeled α-chains of monomeric fibrin by plasmin, ancrod, CNBr, and trypsin. Fluorescently labeled peptides from such digestions were identified, isolated, and partially characterized by sodium dodecyl sulfate-gel electrophoresis, amino acid compositions, and NH₂-terminal sequence analyses.

**Materials and Methods**

Reagents—All reagents were analytical grade and were obtained from commercial sources. Tris/NaCl buffer refers to 0.15 M NaCl, 0.05 M Tris/HCl, pH 7.4. Ammonium bicarbonate buffer refers to 0.1 M ammonium bicarbonate, pH 7.9. MDC (Cyto Chemical Company) was made 7.5 mm in Tris/NaCl buffer and stored at 4°C. Iodoacetamide was recrystallized twice from hexane. Pervidine was redistilled prior to use.

Fibrinogen—Human fibrinogen (Grade L, Kabi, Stockholm) was -95% clottable and contained sufficient fibrin-stabilizing factor to catalyze highly cross-linked fibrin formation. The plasmin formed from urokinase activation of the contaminant plasminogen in this fibrinogen was adequate to fully digest the fibrinogen α-chain fibrin formed from this preparation.

Enzymes—Human urokinase (Leo Pharmaceuticals, Copenhagen) was dissolved in Tris/NaCl buffer to a concentration of 5000 Ploug units/ml and stored at -20°C. Human thrombin (from Dr. D. L. Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) was dissolved immediately before use in Tris/NaCl buffer at 90 NIH units/ml. Human plasminogen was isolated and partially characterized by sodium dodecyl sulfate; CNBr, cyanogen bromide; NPGB, p-nitrophenyl glycine borohydride; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide; NPGB, p-nitrophenyl glycine borohydride; SDS, sodium dodecyl sulfate; HCl, hydrochloric acid; NaCl, sodium chloride.

**Digestion by Trypsin and Chymotrypsin**

Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone and α-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) were each dissolved in 0.001 N HCl, 0.02 M CaCl₂, before use.

*Polyacrylamide Gel Electrophoresis*—Electrophoresis in the presence of SDS was performed on 5, 7.5, and 12.5% polyacrylamide gels which were examined for fluorescence and stained for protein or carbohydrate as previously described (7). Also SDS 20% gels were prepared according to Laemmli (12, 13); SDS, 6 M urea, 10% gels containing 1.2% bisacrylamide were used to enhance resolution of peptide in the range of 8,000 to 20,000 molecular weight. Certain isolated peptides were examined for purity by polyacrylamide gel electrophoresis at pH 3.2 and 8.9 (14, 15).

**This Layer Chromatography**—Ascending chromatography in piperidine-acetic acid/water (10:15:3:12) on plastic-backed cellulose (20 × 20 cm, 0.1 mm, MN 300, Brinkmann) was frequently used to separate the peptides purified by column chromatography and to isolate small MDC-labeled peptides. These were then eluted from cellulose scrapings with 50% acetic acid.

Preparation of MDC-labeled Fibrin—Incubation of MDC into the cross-link acceptor sites of fibrin monomer was based on the method of Lorand et al. (8). The fibrin was formed by combining 1 volume of 1% fibrinogen solution, 2 volumes of 7.5 mm MDC, 0.1 volume of freshly prepared 0.5% s-cysteine (adjusted to pH 7.4), 0.1 volume of 0.8 M CaCl₂, and 0.65 volume of thrombin (90 NIH units/ml). The mixture began to clot at about 30 s; it was covered and left at 37°C. CNBr cleavage was complete after 2 h. After 7 h the remaining supernatant was removed by filtration through Whatman No. 1-MM paper and was combined with the 250-ml filtrate recovered from washing the residual ancrod-treated fibrin. This plasmin was finely minced, resuspended to 20 mg/ml in ammonium bicarbonate buffer, and reincubated with 40 units of ancrod/ml for 5 h at 37°C. Following this second digestion, the supernatant was removed as before, and the fibrin was washed, lyophilized, and subjected to CNBr cleavage as described below. The lyophilized supernatant protein from the initial (11 h) and the second (16 h) ancrod digests of MDC-labeled fibrin was combined and dissolved at 20 mg/ml in ammonium bicarbonate buffer and incubated at 37°C with 60 units of ancrod/ml which were added at 0 h and again at 3 h. After 5 h the digest was lyophilized, analyzed by SDS-gel electrophoresis and stored for use in other studies as described above.

Ancrod digests of MDC-labeled α-chain and several purified fluorescent α-chain peptides were performed at 37°C in ammonium bicarbonate buffer. The substrate and enzyme concentrations were in the range of 2 to 5 mg/ml and 5 to 70 units/ml, respectively.

**Plasmin Digestion**—MDC-labeled fibrin was minced, suspended at 20 mg/ml in ammonium bicarbonate buffer, and an aliquot of the stock urokinase solution was added to give a final concentration of 10 Ploug units/ml. The mixture was incubated at 37°C with constant shaking for 6 h by which time the fibrin had completely lysed. To obtain larger molecular weight α-chain peptides, two limited digestions were performed at 23°C with stirring for 2 h or less and then terminated with NPGB. Following centrifugation (3500 × g, 10 min) of the mixture, the supernatant fluid was frozen immediately, lyophilized, and stored at -20°C until it could be further purified.

Plasmin digests of fibrinogen were prepared by incubating 1 g of fibrinogen at 5.7 mg/ml with urokinase at 5.7 Ploug units/ml in ammonium bicarbonate buffer at 37°C with constant shaking. Depending on the size of the peptides sought, the reaction was terminated at 45 min or 3 h by adding 1.2 mg of NPGB and then immediately freeze drying.

The particulate phase of ancrod-digested MDC-labeled fibrin was incubated at 10 mg/ml with 1 mg of plasminogen and 150 Ploug units of urokinase in 15 ml of ammonium bicarbonate buffer at 25°C with constant stirring. Digestion was terminated at 90 min by adding 0.3 mg of NPGB; at this time only a small amount of precipitate remained.

MDC-labeled α-chain peptides, isolated from CNBr or enzymatic cleavages, were each dissolved in an appropriate volume of ammonium bicarbonate. Plasmin digests were prepared by incubating 5 g/ml in 0.05 M Tris, 0.05 M lysine/HCl, pH 8, with urokinase at 120 Ploug units/ml for 20 min at room temperature; the plasmin was then diluted to 1 mg/ml with cold ammonium bicarbonate buffer containing 20% glycerol and kept at 4°C. About 2 to 5 mg of each peptide were incubated with three to four serial additions of 1 to 3% (w/w) plasmin at 25°C or 37°C for up to 24 h.

**CNBr Cleavage**—One gram of MDC-labeled fibrin was dissolved in 100 ml of 70% formic acid by vigorous stirring for 1 h at room temperature and dialyzed against three changes of 75-fold volumes of 70% formic acid at 4°C. Solid CNBr was added at room temperature with stirring to the final diisolate to make the solution 3% CNBr, which was a 150-fold molar excess of CNBr relative to the methionine content. SDS-gel electrophoretic analysis of aliquots at various times indicated that CNBr cleavage was complete after 2 h. After 7 h the reaction was terminated by diluting the mixture 12-fold with water and lyophilizing. Cleavage of 5 g of fibrinogen was performed as for labeled fibrin, except that fibrinogen was dialyzed against water at 4°C and lyophilized before being made 1% in 70% formic acid.

Ancrod-treated MDC-labeled fibrin remaining from the 16-h ancrod digest was dissolved at 10 mg/ml in 70% formic acid (85 ml) and mixed with 3.5 g of solid CNBr for 10 h at 23°C; the reaction was terminated as before.

Approximately 45 mg of MDC-labeled S-sulfotyrosyl α-chain was reacted with 140 mg of CNBr in 5 ml of 70% formic acid for 9 h at 25°C. CNBr cleavages of isolated peptides were performed at concentrations of 5 to 9 mg/ml in 70 to 80% formic acid and molar ratios of CNBr/methionine in the range of 200 to 350.

**Digestion by Trypsin and Chymotrypsin**—Digestion of certain acceptor site-containing peptides was usually performed in ammonium bicarbonate buffer at 37°C with three serial additions of 0.02 mg of...
enzyme/mg of peptide. In some cases the substrate solutions, which ranged in concentration from 2 to 20 mg/ml, were placed in a boiling water bath for 5 min or more initiating the flocculation. Trypsin digestion was performed at pH 1.5 (2.5 ml) containing CNBr and moderate amounts of Hol-DSK was lyophilized on the same column. The fractions (0.4 to 0.6 V) were pooled and each fraction was exhaustively dialyzed against 0.01 M NH₄HCO₃, 0.07 M dithiothreitol, the pool containing CNBr from Hol-DSK. The fractions were lyophilized and analyzed by SDG-gel electrophoresis. The eluant of the fluorescent peptides released into the supernatant by either plasmin or ancrod digestion of MDC-labeled fibrin were purified by gel filtration at room temperature on Ultrogel AcA44 (LKB) and on Sephadex G-50 Fine (Pharmacia Fine Chemicals) equilibrated in ammonium bicarbonate buffer at flow rates of 3 to 3.5 ml/h/cm². The eluant of these and the other chromatography experiments was continuously monitored for absorbance at 280 and 206 nm and for fluorescence (365 nm primary filter and 540 nm secondary filter, Turner model 111 fluorometer). Aliquots from selected fractions of the chromatograms were routinely analyzed by SDS-gel electrophoresis to decide which fractions would be pooled.

The fluorescent CNBr fragment (CN29*) from the cleavage of MDC-labeled fibrin or of isolated MDC-labeled α-chain, or the analogous peptide (CN29) generated by CNBr cleavage of fibrinogen, was isolated by successive gel filtrations. Digests of 1 g or more were dissolved at 60 mg/ml in 10% acetic acid; 18-ml samples were applied to Sephadex G-100 (2.5 x 90 cm) and eluted at 18 ml/h in the same solvent. As previously reported, this step produced a broad elution profile (171, and the fluorescence was quenched by this solvent. The peak between 0.65 and 0.92 V was concentrated to 3 ml and rechromatographed on the same column. The fractions (0.4 to 0.6 V) containing CN29* and moderate amounts of Hol-DSK were lyophilized, suspended at 9 mg/ml in 0.15 M NH₄HCO₃, 0.07 M dithiothreitol, pH 7.8, and stirred overnight at room temperature in a sealed vial in the supernatant. As previously reported, the pool containing CN29* and CN29 from Hol-DSK was exhaustively dialyzed at 4°C against water adjusted to pH 4. Isolation of the MDC-labeled α-chain peptide, CNA10*, from the CNBr cleavage of ancrod-treated fibrin was achieved by gel filtration on Ultrogel AcA44 and then on Sephadex G-50 equilibrated in ammonium bicarbonate buffer. Although this was a method for pure isolation for virtually all of the CNBr fragments, CNA10* was very soluble, and this permitted its preparation in large quantities. This method was also used to purify CN29* in small quantities (5 to 10 mg); in this way reduction was unnecessary to achieve its separation from Hol-DSK.

The fluorescent α-chain derivative A38*N, in ancrod-treated MDC-labeled fibrin was isolated by electrophoresis on SDS-7.5% gels (0.5 x 10 cm). The lyophilized precipitate obtained from the ancrod digests was dissolved at 5 mg/ml in 1% SDS, 0.01 M sodium phosphate, 8 M urea, 5% β-mercaptoethanol, 0.002% bromphenol blue, pH 7.1; 55 µl of this solution was applied to each gel and electrophoresed at 45 mA/gel for 19 h. Under these conditions, the brightly fluorescent band which corresponded to the α-chain derivative was well resolved from the fluorescent γ-chain; this band was immediately sliced from the gels while viewing under an ultraviolet hand lamp. The gel slices were kept at 4°C until enough had been obtained and then they were ground to a paste in a glass mortar, suspended at 0.7 ml/slice in 0.015% SDS, 0.2% β-mercaptoethanol and stirred at room temperature for 48 h. This slurry was filtered, and the fluorescent supernatant was lyophilized and redissolved in water at 25 mg/ml to which an equal volume of 10% cold trichloroacetic acid was added while stirring. The precipitate which formed (about 0.5 mg/ml) was dissolved in 6 M guanidine HCl, 0.55 M Tris/HC1, 0.005 M EDTA, 2% β-mercaptoethanol, and carboxymethylated with 11°C iodooacetate (18).

2 All peptides referred to by abbreviations in this paper are of α-chain origin. Those labeled with MDC are designated by an asterisk following a number (M, x 10⁻²) which indicates their approximate molecular weight. The prefix indicates the agent(s) of proteolysis that generated the peptide: A, ancrod; CN, cyanogen bromide; P, plasmin; T, trypsin. The suffix "N" is used for peptides which are derived from the NH₂-terminal region of the α-chain of fibrinogen.

3 The designation Hol-DSK is according to Blomback and refers to the disulfide-bonded CNBr fragment from the Fragment D region of fibrinogen (17).

4 MDC-labeled fibrin was determined in 0.1 M ammonium bicarbonate buffer with the meniscus sedimentation equilibrium method of Yphantis (19). A Spinc model E analytical ultracentrifuge equipped with absorption optics, a scanner, an An-D rotor, and a 12-mm double-sector centriplate was used. The sample was analyzed at 18°, at a concentration of 83 µg/ml, and at rotor speeds of 40,000 and 48,000 rpm. The partial specific volume of CNA10*, as calculated from its amino acid composition (20), was 0.679 ml/g.

5 MDC Content of Labeled Peptides—The mol fraction of MDC incorporated in certain small α-chain peptides was estimated from the near ultraviolet absorption spectra of the labeled peptides in ammonium bicarbonate buffer. The molar extinction coefficients of free MDC in this solvent were determined (ε₃350 m, 4,000 M⁻¹ cm⁻¹, ε₄80 m, 1,540 M⁻¹ cm⁻¹, ε₅₄₆ m, 14,750 M⁻¹ cm⁻¹) and were assumed to be essentially the same as those of MDC. Independent estimates of MDC incorporation were made from the visible fluorescence emission spectra of the labeled peptides in ammonium bicarbonate buffer with an Amino-Bowman spectrofluorometer; 345 nm was the excitation wavelength used.

6 Amino Acid Analyses—Isolated peptides and proteins were hydrolyzed in vacuo at 110°C in 1 ml of 6 N HCl containing 0.1% (w/v) phenol for 22, 72, and occasionally 48 and 120 h. Each hydrolysate was analyzed by standard chromatographic methods (21, 22) on Beckman type AA-15 resin (0.9 x 56 cm) with a Beckman model 119 amino acid analyzer equipped with an Autolab System AA computer. Only the 570 rpm channel was integrated; praline values were computed manually. Methionine and cysteine were determined as methionine sulfoxide and cysteic acid from 20-h hydrolysates following performic acid oxidation (23). Tryptophan was determined from separate 22-h hydrolysates at 110°C with 0.5 ml of 4 n methan chloride sulfonic acid containing 0.2% 2-(2-aminoethyl)indole (24).

7 NH₂-terminal and Amino Acid Sequence Studies—NH₂-terminal analyses of 0.04 to 0.10 µmol of several isolated α-chain peptides containing a MDC-labeled cross-link acceptor site(s) were performed by the quantitative dansyl derivative labeling method (25). Certain MDC-labeled α-chain peptides (0.2 to 0.5 µmol) were subjected to amino acid sequence analysis by automated Edman degradations performed with a Beckman 890B Sequencer; a program was used which incorporated modifications of the original method of Edman and Begh (26, 27). Details of these procedures have been previously described from this laboratory (11).

RESULTS

Ancrod Digestion of MDC-labeled Fibrin—Incubation of MDC-labeled fibrin with ancrod results in selective cleavage of the α-chain into two fluorescent regions as evidenced from SDS-gel electrophoretic analyses of the digestion products. As shown in Fig. 1, ancrod rapidly released fluorescent peptides of molecular weight 34,000 to 27,000 which could be detected in the supernatant of the digest after 20 min. With time these early products were degraded to a peptide of about M = 12,000 which contained most or all of the MDC label present in each of the parent peptides. Although the apparent absence of nonfluorescent supernatant peptides in Fig. 1 could be due to their co-migration with labeled peptides, the most likely explanation is that ancrod sequentially removed only small, M = 3,000 to 5,000, nonfluorescent peptides to produce the observed digestion pattern. The fact that the sum of the molecular weights of the intermediate fluorescent peptides exceeds the molecular weight of the α-chain also supports this
human fibrin α-chain cross-link acceptor sites

FIG. 1. SDS-7.5% gel analyses of reduced (R) and nonreduced (N) samples of the supernatant removed with time from the ancrod digestion of MDC-labeled fibrin. The gels were examined for protein (P) and fluorescence (F). No fluorescence or protein was detected in the supernatant at "time zero." Mr indicates the apparent molecular weights of the reduced peptides. See text for details of incubation.

FIG. 2. SDS-7.5% gel analyses of reduced samples of particulate fibrin removed with time from the ancrod digestion of MDC-labeled fibrin. The subunits of MDC-labeled fibrin are identified in the zero time sample. The gels were examined for protein (P) and fluorescence (F). The band designated A38*N had an apparent molecular weight of 38,000 and was not present in nonreduced samples. Conditions of digestion are the same as in Fig. 1 (see text).

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explanation. The observed increase in SDS-gel electrophoretic mobility of the nonreduced relative to the reduced Mr = 34,000, 31,000, 27,000, 24,000, and 19,000 intermediates indicates that these peptides contain one or more intrachain disulfide loops (Fig. 1). This observation becomes useful later in positioning these fragments in the α-chain. Finally, confirmation of their α-chain origin was obtained from analysis of the residual insoluble ancrod-cleaved fibrin in the digestion mixture.

In Fig. 2 the time course of ancrod digestion of the MDC-labeled fibrin indicates that the β-chain, γ-chain, and γ-dimer subunits of fibrin were not detectably degraded even after incubation for 24 h; on the other hand, the α-chain was readily cleaved in half. The disappearance of the intact α-chain was noticeable after 1 h and coincided with the appearance of a brightly fluorescent Mr = 38,000 peptide observed only in the reduced SDS-gel patterns. The generation of this α-chain fragment was maximal at about 16 h, and it was resistant to further cleavage by ancrod. This peptide is designated A38*N, and it contains the second cross-link acceptor-site region of the α-chain revealed by ancrod cleavage, the first being in the nondisulfide-bound peptides released into the supernatant.

Isolation of A38*N from SDS-polyacrylamide gel slices was achieved with about 95% yield. SDS-10% gel analysis of the CNBr-cleaved A38*N revealed that (a) most of the fluorescent label was in a peptide of apparent molecular weight 17,000 which stained very poorly with Coomassie blue; (b) the remaining fluorescence appeared in a poorly staining peptide of molecular weight less than 2,000; (c) there were 3 or 4 small (Mr less than 10,000) nonfluorescent peptides detected by Coomassie stain. When examined by thin layer chromatography, the minor fluorescent peptide had a Rf of 1.0, but the major fluorescent peptide remained at the origin; the three nonfluorescent ninhydrin-positive peptides were resolved between Rf 0.25 and 0.45. Gel filtration of the CNBr digest on Sephadex G-25 gave less than a milligram of the major MDC-labeled peptide; the minor fluorescent fragment was not detected. Treatment with plasmin (5% w/w, 37°, 12 h) had no detectable effect on the peptide of apparent molecular weight 17,000 and thereby indicated that its position in the α-chain should correspond to a plasmin-resistant segment.

Plasmin Digestion of MDC-labeled Fibrin — Very early in the time course of plasmin digestion of labeled fibrin, fluorescent peptides of molecular weight 45,000, 42,000, and 35,000 were released from the clot into the supernatant. Contrary to our earlier assessment (7), these large peptides apparently contain one or more intrachain disulfide loops as evidenced by their decreased electrophoretic mobility after reduction. These peptides become degraded fairly quickly to give two brightly fluorescent peptides of molecular weight 23,000 and 21,000 (designated P23* and P21*, respectively), the mobilities of which were unaffected by reduction (7). Smaller nonfluorescent peptides were also observed, one of which had a molecular weight of 18,000 and appeared to contain the disulfide loop(s), since its mobility was markedly decreased after reduction. With time P23* was degraded to P21* which then remained relatively plasmin-resistant for up to 6 h. This last cleavage generated a weakly fluorescent peptide of apparent molecular weight 2,500 as detected by SDS-12.5% gel electrophoresis (7).

The α-chain origin of these supernatant peptides was established by SDS-gel analyses of the insoluble portion of the
digestion mixture which displayed intact β-chain, γ-chain, and γ-dimer subunits for the first hour as shown in Fig. 3. The disappearance of intact α-chain during this time coincided with the appearance of fluorescent peptides in the supernatant and also with the observation of two nonfluorescent peptides of \( M_1 = 25,000 \) and \( 23,500 \) (designated P25N and P24N, respectively) in the residual insoluble fibrin after reduction. Thus, in contrast to the digestion patterns of labeled fibrin produced by ancrod, all of the α-chain cross-link acceptor sites are contained in the plasmic supernatant peptides.

After 2 h, large fluorescent fragments corresponding to fibrinogen Fragments Y and D and fibrin Fragment D dimer appeared in the supernatant of the digestion mixture. However, these derived fluorescence solely from the MDC-labeled γ-chain cross-link acceptor sites. Smaller, nonfluorescent peptides which correspond to Fragment E were also observed.

Isolation of Plasmic α-Chain Peptides which Contain Cross-link Acceptor Sites—Fig. 4 is the elution profile of 2- and 6-h plasmic digests of MDC-labeled fibrin when filtered on Ultrogel AcA44. The quantity of supernatant peptides recovered from the 2-h digestion was 140 mg per g of labeled fibrin; this represents 50% of the theoretical yield which was computed by assuming that two \( M_1 = 45,000 \) segments per fibrin monomer were released into the supernatant. SDS-gel analysis of the unfraccionated supernatant is shown in the upper panel of Fig. 4, gels a and b. The major peak in the chromatogram of the 2-h digest contained approximately equal amounts of P23* and P21* and also smaller nonfluorescent peptides as demonstrated by SDS-7.5% gel analysis of Pool III (Fig. 4, gels c and d). Purification of P42* (Pool I) and P35* (Pool II) was achieved by rechromatography of each pool on the same Ultrogel column; the final yields were 10 to 12 mg of each per g of labeled fibrin. The peptides generated by the digestion of P42* and P35* to P23* were detected in the elution profile by absorption at 206 nm and displayed weak absorption at 280 nm and slight, if any, fluorescence. The weakly fluorescent peaks observed near the column volume (Pool V) probably contained the labeled peptide cleaved from P23* when P21* is formed.

When the 6-h digest was filtered on Ultrogel AcA44 (Fig. 4, lower panel), P21* was well resolved from Fragments D and E which eluted in a broad peak (Pool I) just after the void volume; it should be noted that considerable overlap occurred between P21* and Fragment E when filtered on Sephadex G-100 whether or not Fragment D was removed by prior heat precipitation. In contrast to the 2-h digest, P21* was not detected in the chromatography of the 6-h digest. Approximately 65% of the maximum theoretical yield of P21* was recovered in Pool II and after rechromatography on Sephadex G-50 (2.7 \( \times \) 120 cm), the overall yield of P21* was 50%. This preparation was homogeneous by NH₂-terminal sequence analysis and had an \( E_{280}^{\text{nm}} \) (280 nm) of 18.0. Identical chromatographic results were obtained when unlabeled P21 was prepared from fibrinogen.

CNBr Fragments of MDC-labeled α-Chain—SDS electrophoresis of the CNBr fragments from MDC-labeled fibrin showed two regions of fluorescence, a discrete protein band of molecular weight 29,000, the mobility of which was slightly decreased after reduction, and a more diffuse band of about \( M_1 = 8,000 \) (Fig. 5). The fluorescent intensity of this smaller fragment on SDS-12.5% polyacrylamide gels appeared to be

![Fig. 3. SDS-7.5% gel analyses of reduced samples of particulate fibrin removed with time from the plasmin digestion of MDC-labeled fibrin which was suspended at 10 mg/ml in 0.1 M ammonium bicarbonate, pH 7.8, at 37°. Plasmin added was 1% (w/w) of substrate. Gels were examined for protein (P) and fluorescence (F). The nonfluorescent band designated P25N is the NH₂-terminal \( M_1 = 25,000 \) α-chain derivative.](https://www.jbc.org/)

![Fig. 4. Gel filtration on Ultrogel AcA44 (6 \( \times \) 90 cm) of plasmin digests of MDC-labeled fibrin. The column was equilibrated and developed at 23° in 0.1 M ammonium bicarbonate, pH 7.9; flow rate = 3.0 ml/h/cm². Upper panel, elution profiles of 2-h digest (75 mg) applied in 41 ml; 12-ml fractions were collected. Absorbance was measured in a 5-mm path flow cell. Inset, SDS-7.5% gel analyses of the reduced whole digest (gels a and b) and of unreduced Pool III (gels c and d). Peptide designations are defined in text. P, protein; F, fluorescence. Lower panel, elution profile of 6-h digest (1.6 g) applied in 41 ml; 17-ml fractions were collected. Absorbance values are for a 1-cm path length. Pool II was ~85% homogeneous. Inset, elution profile of 6-h digest on Sephadex G-100 obtained under equivalent conditions.](https://www.jbc.org/)
As demonstrated in Fig. 2, ancrod digestion of MDC-labeled buffer was less than with acetic acid, the fluorescent fragment. Although the overall solubility of the CNBr digest in this which permitted the monitoring of fluorescence (Figs. 1s and 2s) was then cleaved with CNBr to obtain a smaller peptide which contained most (~70%) of the fluorescence in the digest. This fluorescent fragment reduces to smaller peptides which are then readily separable from CN29*

**CNBr Cleavage of Ancrod-digested MDC-labeled Fibrin**

As demonstrated in Fig. 2, ancrod digestion of MDC-labeled fibrin produced an NH2-terminal fluorescent α-chain derivative which could be isolated under denaturing conditions. It was then cleaved with CNBr to obtain a smaller peptide which contained most (~70%) of the fluorescence in the digest. This acceptor site-containing CNBr fragment was more easily prepared from ancrod-digested MDC-labeled fibrin and was purified by gel filtration in 0.1 M ammonium bicarbonate, which permitted the monitoring of fluorescence (Figs. 1s and 2s). Although the overall solubility of the CNBr digest in this buffer was less than with acetic acid, the fluorescent fragment of interest was very soluble. This peptide, designated CNA10*, was recovered in 60% yield; it was homogeneous by SDS-20% gel electrophoresis and by NH2-terminal analysis (glutamic acid) and has a molecular weight of 10,150 by sedimentation equilibrium ultracentrifugation. When analyzed by SDS-gel electrophoresis, it had an abnormally low mobility and stained faintly reddish-purple with Coomassie blue, which may be related to its unusual amino acid composition (Table I). The mol fraction of MDC bound to CNA10* was estimated spectrophotometrically to be ~0.55, with the experimentally determined E1% (280 nm) of 19.2.

**Successive Enzymatic Digestions of MDC-labeled Fibrin**

MDC-labeled fibrin was digested by ancrod for 12 to 16 h and during this time fluorescent peptides were released into the supernatant. The residual particulate fibrin, now termed AFBn*, was also fluorescent. The supernatant phase was removed by centrifugation and, as indicated in the preceding section, was found to contain the fluorescent peptides A19* through A12*. The mixture of these fluorescent peptides was then incubated with plasmin for 3 h. By this time, SDS-12.5% gel analyses indicated that the peptides had been digested to a single, brightly fluorescent band of M, ~10,000. This peptide, designated PA10*, stained well with Coomassie blue and its mobility was unaltered by reduction or longer incubations with plasmin (12 h). It was purified by gel filtration on Sephadex G-50 and then on G-25; following the last chromatography step the peptide was homogeneous by SDS-20% gel electrophoresis. Its mol fraction of incorporated MDC was approximately 1.0 as estimated spectrophotometrically, using its experimentally determined E1% (280 nm) of 21.8. The solubility of PA10* in aqueous buffers was distinctly less than for most of the other isolated plasmic peptides.

The particulate fibrin, AFBn*, which was separated by centrifugation at the termination of MDC-labeled fibrin digestion by ancrod, was suspended in buffer and subjected to digestion by plasmin as described under "Materials and Methods." At 90 min SDS-7.5% gel analysis of the digestion mixture showed that two fluorescent bands of apparent molecular weight 17,000 and 8,000 had been released into the supernatant by plasmin; neither of these peptides stained well with Coomassie blue. At this stage of digestion, the supernatant also contained MDC-labeled monomeric and dimeric Fragment D species. Additional SDS-gel analyses of the particulate fibrin that remained at 90 min showed the β- and γ-subunit chains to be essentially intact. However, the NH2-terminal α-chain derivative, A38*N, was partially degraded and two nonfluorescent peptides of molecular weight 24,000 (PA24N) and 23,000 were observed after reduction. These latter peptides must be analogous to the NH2-terminal α-chain derivatives contained in the Fragment X species ordinarily generated during the early phases of fibrinogen digestion by plasmin.

The MDC-labeled peptides released into the supernatant after 90 min of plasmin digestion of AFBn* were isolated by gel filtration and ion exchange chromatography. In addition to Fragment D and trace amounts of PA21*, two fluorescent peaks of interest were eluted from Sephadex G-50 at 1.5 V0 and 2.8 V0. After rechromatography of the first pool on Sephadex G-25, SDS-20% gel analysis revealed a single band with an electrophoretic mobility and staining properties identical with CNA10*. Thin layer chromatography of the second pool revealed several nonfluorescent peptides and two MDC-labeled peptides of approximately equal fluorescent intensity. These two latter peptides were purified by Sephadex G-10 gel
tides were designated as PAIVa* and PAIVb*. The amino acid composition and NH₂-terminal analyses of these two MDC-labeled a-chains were essentially identical and also had low hydrophobic contents (2 to 6%) and were unusually rich in serine and glycine (45% of the residues); tryptophan was assumed to be 4 and 8%, respectively; valine and isoleucine values were increased by 10%.

The enzymatic digestions of P21* with CNBr produced no detectable cleavage equilibrium ultracentrifugation.

Filtration followed by CM-Sephadex C-25 chromatography and were designated as PAIVa* and PAIVb*. The amino acid compositions and NH₂-terminal analyses of these two MDC-labeled peptides (Table I) permitted their placement in the NH₂-terminal sequence of P23*.

Amino Acid Composition—Most of the α-chain peptides which were purified from digestions of MDC-labeled fibrin were subjected to thorough amino acid analyses as shown in Table I. The peptides CNA10*, PA10*, and P21* all have extremely low contents of hydrophobic residues (2 to 5%) and are unusually rich in serine and glycine (45% of the residues); none of these three peptides contain cysteine. The compositions of CN29 from fibrinogen and CN29* from MDC-labeled α-chain were essentially identical and also had low hydrophobic contents (8%). Two half-cystine residues are present in CN29 from fibrinogen and CN29* from isolated, MDC-labeled α-chain. Values were determined from quintuplicate 22-h and triplicate 72-h hydrolyses, unless noted otherwise.

Aspartic acid determined as aspartic acid (23).

Values from 22-h hydrolyses, unless noted otherwise.

Determined as methionine sulfoxide (23).

Present as homoserine and homoserine lactone.

Determined by the method of Liu and Chang (24).

Tryptophan not included.

Estimated from SDS-gel electrophoresis, except for CNA10* which was determined by ultracentrifugation.

Filtration followed by CM-Sephadex C-25 chromatography...
weight band did not stain well with Coomassie blue and probably corresponds to the CNA10* region of the α-chain. When the tryptic digestion products of P21* were compared to those of CNA10* by chromatography on Sephadex G-25, an additional fluorescent peak was observed in the elution profile. The amino acid compositions and NH₂-terminal analyses of a MDC-labeled peptide isolated from the tryptic digest of P21* and another from the tryptic digest of CNA10* are given in Table I. From those data and the amino acid composition of PA10*, it appears that there is a segment in the COOH-terminal half of CNA10* which contains a cross-link acceptor site. The fluorescent products from the tryptic digestion of P21* and CN29* appeared to be the same on SDS-10% gels; in each case two equally fluorescent bands with apparent molecular weights of 10,000 and 12,000 were observed. Finally it should be noted that the SDS electrophoretic mobilities of the tryptic digestion products of nonlabeled CN29 or P21 were identical with those of MDC-labeled CN29 or P21.

NH₂-terminal Sequence Analyses – Table II shows the results of NH₂-terminal sequence analyses for several of the peptides derived from MDC-labeled fibrin by enzymatic or chemical cleavage. The purified preparations of P21* and CN29* had identical NH₂-terminal sequences after the terminal methionyl residue in P21*. This is in agreement with and extends previously published sequence data on the analogous peptides from fibrinogen (30, 31). There were no labeled acceptor sites in the first 15 cycles of CN29* nor in the first 24 cycles of P21* which had been purified on Utrorgel AcA44 followed by Sephadex G-50 gel filtration. In the sequence analysis of the P21* preparation obtained by only Utrorgel AcA44 chromatography, definite MDC fluorescence was detected at cycle 18. Since serine was the predominant amino acid detected at this position in both P21* preparations, the fluorescence in this cycle was attributed to an MDC-labeled glutaminyl residue in the sequence of a minor component in the AcA44 pool. NH₂-terminal sequence analysis of the larger plasmic peptide P42* and P35* revealed a labeled acceptor site at cycle 7. These two peptides possessed the same NH₂-terminal regions; however, each peptide preparation contained a mixture of primarily two peptides which had overlapping sequences that were 12 residues out of phase. Assignment of the two amino acids detected at each cycle to their proper sequence would normally be very difficult since the amounts

![Diagram of peptide digestions](https://example.com/diagram)

**Fig. 6.** SDS, 5 M urea, 10% polyacrylamide gel analyses of ancrod-digested P21*. Ancrod was dialyzed into ammonium bicarbonate buffer and to this solution (70 units/ml) P21* was added to give 5 mg/ml and was incubated at 37°C. At 12 h more ancrod was added to give a final E/S ratio of 30 units/mg of P21*. After 24 h of digestion the gel patterns were essentially the same as that shown for 12 h. Mₚ designates the apparent molecular weight of the peptides. P, protein; F, fluorescence.

**Table II**

<table>
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* Discrimination between threonine versus proline and between isoleucine versus leucine was not obtained for some cycles.
were nearly equal and since quantitation by gas chromatography of certain amino acid phenylthiohydantoins is not very reliable (32). However, at the time of this endeavor, the primary structure of a 38-residue plasmic peptide from the α-chain of [14C]glycine ethyl ester-labeled fibrin was published (9). The partial sequence of this peptide, which was termed the "midsection piece," could be reconstructed from our data and was therefore used as a guide for positioning the two sequences of P23* and P21* (Table I). The yields obtained in each cycle for sequence I are not consistently greater or less than those of sequence II because of the uncertainties in quantitation. With the use of the yields of those residues which could be reliably quantitated, the relative amounts of the different NH2 termini generated by plasmin were estimated: P42* contained 40% terminal serine, 40% terminal alanine, and 20% terminal methionine; and P35* contained 60% terminal serine and 30% terminal alanine. Evidence for the lesser sequence Met-Glu-Leu-Glu in P42* established that this peptide contains the smaller plasmic peptide, P21*, in its NH2-terminal half. Furthermore, it is likely that the peptide P23* contains the labeled glutaminyl residue detected at cycle 7 because its NH2 terminus was serine and because P21* plus a small MDC-labeled peptide were generated from P23* by plasmin. It should be noted that this small MDC-labeled peptide accounted for only a fraction of the fluorescence in the α-chain; the majority of the label is contained in P21*, contrary to the finding by Takagi and Doolittle (9) of approximately equal incorporation of [14C]glycine ethyl ester into these two peptides.

Relatively intense fluorescence was detected at cycle 18 in the sequence analysis of the smallest anodic supernatant peptide (A12*), but considerable overlap between cycles and the sequence analysis of the smallest ancrodic supernatant peptides, A34* and A31*, are generated from the α-chain simultaneously. The difference of 3,000 in molecular weight between these, and similarly between P45* and P42* is therefore tentatively attributed to the COOH-terminal plasmic peptide.

The position in the α-chain of the plasmic supernatant peptides, of CN29*, and ultimately the specific locations of the acceptor sites were determined as follows. The NH2-terminal α-chain derivative A38*N resulting from ancrod digestion was cleaved by CNBr to produce the fluorescent peptide that overlaps the first cross-link acceptor site in CN29*. This isolated peptide, CNA10*, was found to have the same NH2 terminus as CN29* and, since it contained no methionine, homoserine, or homoserine lactone (Table I), its COOH terminus should be identical with that of A38*N. Using the number of amino acid residues found in A38*N and CNA10* (Table I) and the fact that ancrod cleaves the Aα-chain after residue 23 (38), the NH2 terminus of CN29* (and of CNA10*) was computed to be residue 259 of the Aα-chain. From this reference point, the locations of the plasmic peptides can be determined and the approximate locations of the three cross-link acceptor sites can be placed at residue positions 255, 310, and 385 from the NH2 terminus of the Aα-chain. The primary limitation with these calculations is the uncertainty in the molecular weight of A38*N, which was determined by SDS gel electrophoresis. An independent estimation that places the most NH2-terminal acceptor site at residue 238 in the Aα-chain can be calculated with the partial amino acid sequence of the α-chain remnant in Fragment D and the preliminary composition of the α-chain region linking this sequence with the midsection piece, both of which have been reported recently (39, 40). This estimate is in good agreement with our prediction of position 255; the difference in residues corre-
sponds to only a 5% discrepancy in the molecular weight of A38*N. The spacing between the first and second MDC-la-
beled site is based upon (a) fluorescence detected in a minor sequence of a P21* preparation containing lower molecular weight derivatives and (b) preliminary sequence analyses of two overlapping tryptic peptides from CNA10* and P21* (Table I). The location of the third site was derived from (a) the number of residues in CNA10* and (b) the detection of fluorescence in the preliminary sequence analysis of A12*, which had the same NH₂ terminus (glycine) as A19* and PA10*.

As shown schematically in Fig. 7, the above findings localize the cross-link acceptor sites to the middle 20% of the Aα-chain in contrast to the near COOH terminal location of the acceptor site in the γ-chain (41). The rationale for most of the peptide alignments shown in this model has been discussed; however, validation of certain features is obtained from examination of the amino acid compositions in Table I. For example, the proposed location of A19* predicts that its amino acid composition should be nearly identical with the difference in composition between CN29* and CNA10*. The agreement, in fact, is very good, and most amino acid values differ by less than 6%.

However, the presence of a methionyl residue and two extra residues each of leucine and phenylalanine in A19* suggests that it extends slightly beyond the COOH terminus of CN29*.

In the case of PA10*, which was derived from A19* and A12* by plasmic cleavage, its position is supported by the equivalence of its amino acid composition with the difference in composition between P21* and CNA10*. Again the agreement is good and indicates that PA10* must be shorter by only a few residues (lysine, tyrosine, threonine, and glutamic acid). Our amino acid compositions are in very good agreement with published values for peptides corresponding to P21 (31) and CN29 (30). It should be noted that our analyses of CN29*, either with or without prior performic acid oxidation, showed no evidence for incomplete CNBr cleavage as was observed in one report (42).

When used in conjunction with Fig. 7, the distribution of amino acids within certain peptides can also be calculated from Table I. For example, both halves of the strongly hydrophilic peptide, P21*, have approximately equal numbers of polar residues, while 11 of the 13 hydrophobic residues are localized in its COOH-terminal half. The 7,500-dalton COOH-terminal segment of CN29* is devoid of aromatic amino acids, derives half of its residues from lysine, threonine, and valine, and contains an intrachain disulfide loop. The latter point was deduced from the slight, but measurable decrease in mobility of reduced CN29* compared to nonreduced CN29* when analyzed by SDS-gel electrophoresis. A much larger decrease in electrophoretic mobility (corresponding to a molecular weight shift of ~5,000) after reduction was observed in SDS-gel analyses of A27* and P42*. The magnitude of this difference suggests that the two additional half-cystine residues which were detected in P42* (Table I) are also joined in an internal disulfide bond that is probably also present in A27*. Since P35* contained only one disulfide, the location of this second loop should be within the 7,000-dalton COOH-terminal segment of P42*, as shown in Fig. 7. To our knowledge this is the first indication that the COOH-terminal half of the α-chain may contain another cystine in addition to that of CN29.

The physiological significance of the three potential acceptor sites reported here could be assessed most directly by comparing these findings with the structural analyses of the α-chain cleavage products obtained from cross-linked fibrin formed in vivo. Although such studies have yet to be reported, some relevant deductions can be made from existing data. Pisano and co-workers (43) have detected by chemical and enzymic methods four to six cross-links per molecule of human fibrin, depending on the source. When whole blood was used the values averaged 4, while plasma and purified fibrinogen produced fibrin with an average of 5.4 cross-links (43). More recent data from our laboratory indicate that this value is closer to 4 when fibrin formed from whole plasma is analyzed (44). Since two cross-links per fibrin molecule are contributed by γ-chains (41), each α-chain should contribute from one to a maximum of two cross-links, depending on the clotting conditions. This indicates that possibly two of the potential acceptor sites are not required to form cross-linked fibrin. Consequently, they might represent either nonspecifically labeled glutamines or acceptor sites which in vivo participate in cross-links with another protein such as cold-insoluble globulin. This latter possibility is supported strongly by the recent reports of Mosher (45, 46). The potential acceptor site in the midsection piece may have this function since it was not involved in cross-linking of fibrin prepared from highly purified fibrinogen under conditions which caused the other two α-chain acceptor sites to become completely cross-linked (10). Complete α-polymer formation was also observed in fibrin prepared from whole plasma under conditions which gave only one cross-link per α-chain in the CN29 region.2 The specific

2 L. J. Fretto and P. A. McKee, manuscript in preparation.
acceptor site involved in this cross-link cannot be reliably predicted from this article because the relative cross-linking reactivities of the two acceptor sites in CN29 appear approximately equal when judged by MDC incorporation under the saturating conditions used in these studies. However, previous data of Lorand and co-workers (8) can be interpreted to compare the specificity of labeling in the midsection piece with that in CN29. When the CNBr fragments from fibrinogen or fibrin which had both been partially labeled with the substitute donor [*C]glycine ethyl ester were gel filtered, the only radioactive peak in the chromatograph of the fibrin digest corresponded to CN29* and it had -5 times more counts than the equivalent peak from the fibrinogen digest. Since the radioactive peak in the chromatograph of the fibrin digest was significantly larger than the equivalent peak from the fibrinogen digest corresponded to CN29* and it had 5 times more counts than the labeled midsection piece fragment (8). Apparently the release of fibrinopeptide A from fibrinogen increases the accessibility or reactivity of at least one of the cross-link acceptor sites in CN29, but does not affect pseudo-donor incorporation into the midsection piece. Taken together with the above findings, this suggests that the physiologically important cross-link acceptor site(s) for α-polymerization is located near residue 310 or 385 of the α-chain.

REFERENCES

Hunan Fibrin α-Chain Cross-link Acceptor Sites

Figure 3a. Gel filtration of CNBr-treated undigested fibrin on the Pharmacia A-540 column. The details of digestion are given in Methods. All the supernates from a suspension of 30 mg of fibrin in 2 ml of ammonium bicarbonate buffer (pH 8.2) were pooled. Postdigestion fractions were collected and those within the shaded bar were pooled for further purification (see Fig. 3b).

Figure 3b. Gel filtration of the CNBr and 6N HCl-digested fibrin on Sephacryl S-300 in ammonium bicarbonate buffer at 1 ml/30 min. Fractions were pooled and those under the shaded bar were pooled.

Table 1a:

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* Determined by the method of Gray (49).

These results indicate the presence of specific amino acids in the absorbent fibrin under various conditions.
Localization of the alpha-chain cross-link acceptor sites of human fibrin.
L J Fretto, E W Ferguson, H M Steinman and P A McKee


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