Isolation and Characterization of the S-Carboxymethyl Derivatives of Crosslinked and Noncrosslinked Human Fibrin

(factor XIII/fibrin stabilization)

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ABSTRACT The S-carboxymethyl derivative chains of crosslinked and noncrosslinked fibrins were prepared from purified human fibrinogen. For crosslinked fibrin, fibrinogen was clotted with thrombin in the presence of calcium and purified human factor XIII. For noncrosslinked fibrin, ethylenediaminetetraacetate was substituted for factor XIII and calcium. After reduction with dithiothreitol and alkylation with tritiated iodoacetic acid, the derivative chains were separated on carboxymethyl cellulose in a sodium acetate-pH gradient that contained 8 M urea. Purity of the separated chains was determined by polyacrylamide gel electrophoresis at acid and at neutral pH. The derivative chains of noncrosslinked fibrin were eluted from carboxymethyl cellulose in the order: γ -chain, β -chain, and α -chain. Each of the purified derivative chains was characterized and identified by amino-terminal amino acid analysis, amino acid composition, tryptic peptide mapping, and molecular weight estimation by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In like manner, the derivative components of crosslinked fibrin were eluted from carboxymethyl cellulose in the order: γ - γ -dimer, β -chain, and α -polymer. Application of the same analytical criteria and comparison with the derivatives of noncrosslinked fibrin confirmed the identity of these components. These data provide conclusive evidence that crosslinking of human fibrin involves formation of peptide bonds between two γ -chains to form γ - γ -dimer and between multiple α -chains to form high molecular weight polymers of α -chains.

The terminal phase of human blood coagulation involves formation of isopeptide bonds between the amide functions of certain glutamine residues and the ϵ -amino functions of certain lysine residues in fibrin (1). Fibrin that is crosslinked by such N^{ϵ} - γ -glutamyllysine $\dagger$ bridges is rendered insoluble in solvents such as 5 M urea and 1% monochloroacetic acid, and has been demonstrated to possess greater tensile strength than noncrosslinked fibrin (2). Recently, several reports have appeared on the identification of the specific chains of fibrin that are involved in the crosslinking process. Chen and Doolittle (3) have presented evidence for a crosslink between two γ -chains, involving the carboxyl-terminal moieties of the chains in a symmetrical antiparallel manner. Other suggestions for crosslinks have been of the α - γ (4) and α - α (5, 6) types, although neither of these has been well characterized. It appears that the β -chain of human fibrin is not involved in crosslinking.

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In this report we present a method for obtaining the crosslinked chains as well as the noncrosslinked chains of human fibrin and we characterize the chains of fibrin that are involved in crosslinking.

MATERIALS AND METHODS

Human Fibrinogen, Fraction I-4, was purified from Fraction I-2 (AB Kabi, Stockholm, Sweden) by described procedures (7). Fibrinogen prepared in this way was more than 96% clottable.

Thrombin (EC 3.4.4.13) was of bovine origin and had a specific activity of 250 NIH units/mg.

Factor XIII was prepared from outdated human blood by a modification of the method of Kazama and Langdell (8). The specific activity of these preparations was 25-35 μ mol of dansylcadaverine incorporation per 30 min per mg of purified factor XIII.

Fibrin was prepared by dilution of a 2% solution of Fraction I-4, dissolved in 0.3 M NaCl, 20-fold with 50 mM Tris-0.1 M NaCl, pH 7.3, containing either 10 mM CaCl_2 or 10 mM sodium ethylenediaminetetraacetate. This fibrinogen solution was then clotted in Visking dialysis casing with purified thrombin to a final concentration of 0.5 NIH units of thrombin per mg of fibrinogen. For the preparation of crosslinked fibrin, 1.0 ml of purified factor XIII (10-15 mg/ml) was added to the fibrinogen solution that contained CaCl_2 . The resulting clots were incubated in buffer at 37° for up to 24 hr and then were triturated and dialyzed at 4° against several changes of distilled water. Excess liquid was removed from the dialyzed fibrin, which was then freeze-dried.

Reduction was performed as a modification of the procedure of Murano *et al.* (9) and was done at room temperature with 1.64 mmol of dithiothreitol per g of crosslinked fibrin or one-half that amount for noncrosslinked fibrin. The freeze-dried fibrin was suspended in 8.4 M guanidine-HCl containing 10 mM Tris, pH 8.5, and was incubated for 1 hr to allow swelling of the dried material. Dithiothreitol was dissolved in 1.0 ml of distilled water and added to the fibrin suspension, and the pH was monitored and maintained at 8.5 with 1.0 N NaOH. After stabilization of the pH, the reaction vessel was flushed with N_2 and sealed. After 1 hr for noncrosslinked fibrin and 24 hr for crosslinked fibrin, 0.5-1.0 mCi of monoiodo- $[\text{^3H}]$ acetic acid (Radiochemical Centre, Amersham, England) was added in carrier iodoacetic acid to a total quantity of five times the number of moles of dithiothreitol used. Carrier iodoacetic acid was recrystallized five times

from CCl_4 and was dissolved in 1.0 ml of 1.0 N NaOH just before addition to the reaction mixture. Additional 1.0 N NaOH was added to maintain the pH at 8.5 ± 0.1 . Alkylation was allowed to proceed for 15 min at room temperature, after which a volume of glacial acetic acid equal to the total reaction volume was added to stop the reaction. The reduced, alkylated (*S*-carboxymethyl) derivatives were desalted immediately on a column ($20 \text{ cm}^2 \times 85 \text{ cm}$) of Sephadex G-25 equilibrated in 50% acetic acid at a flow rate of 20 ml/cm² per hr, and the protein peak was freeze-dried. Completion of reduction and alkylation was confirmed by amino acid analysis, which demonstrated the absence of free half-cystine.

Ion-Exchange Chromatography was performed on microgranular, preswollen, carboxymethyl cellulose (Whatman CM52). A column ($4.9 \text{ cm}^2 \times 15\text{--}20 \text{ cm}$) of CM-cellulose, precycled according to the manufacturer's recommendations, was equilibrated in starting buffer consisting of 50 mM sodium acetate containing 8 M urea, pH 4.8. After 300 ml of starting buffer had passed through the column, the pH and conductivity of the effluent were identical to those of the starting buffer. A 100–200 mg sample of freeze-dried, *S*-carboxymethyl fibrin was dissolved in starting buffer to a protein concentration of 1.5–2.0% and applied to the column. Elution was initiated by immediate application of a gradient consisting of 400 ml of starting buffer and 400 ml of 175 mM sodium acetate containing 8 M urea, pH 5.3, in chambers 1–4 and 5–8, respectively, of a 1-liter, 9-chamber, Buchler gradient device. The resulting, shallow, S-shaped gradient was run at a flow rate of 2.5–3.0 ml/cm² per hr. Material absorbing at 280 nm was monitored on an ISCO UA-2 ultraviolet analyzer. Radioactivity of the eluant fractions was determined by mixing 100- μl aliquots of each fraction with 1.9 ml of 10% acetic acid, to which 10 ml of scintillation fluid (5 g of 2,5-diphenyloxazole and 100 g of naphthalene per liter of dioxane) were added. Counting was performed in a Beckman Liquid Scintillation System, Type CPM-200; no correction was made for quenching. Conductivity of eluant fractions was determined with a Phillips conductivity cell.

Peptide Mapping was performed essentially according to Blombäck *et al.* (10). Samples of each of the purified chains of fibrin, either crosslinked or noncrosslinked, were digested for 17 hr at 37° with TPCCK-treated trypsin (EC 3.4.4.4, Worthington Biochemical Corporation) at pH 8.5 in 0.2 M NH_4HCO_3 buffer. The enzyme to substrate ratio was 1:80. After digestion the samples were freeze-dried and dissolved in buffer to be applied to thin-layer cellulose plates. Electrophoresis was performed at pH 5.5 in pyridine-acetate buffer for 75 min at 300 V. After electrophoresis was completed, the plates were dried and subsequently subjected to ascending chromatography in butanol-pyridine-acetic acid-water 1.5:1.0:0.3:1.2. After the plates were dried, they were developed with ninhydrin.

Amino acid Analysis was performed on a Technicon model TSM Amino Acid Analyzer. Hydrolyses were performed in 5.7 N HCl under reduced pressure at 110° for 22, 48, and 72 hr (11).

Amino-Terminal Amino Acids were determined by the phenylisothiocyanate method described by Edman (12). The phenylthiohydantoin derivatives were identified by thin-layer chromatography on silica-gel plates in solvents I, II, III, D, and E as described by Brenner *et al.* (13).

Fig. 1. Chromatographic profile of 135 mg of tritiated *S*-carboxymethyl noncrosslinked fibrin on a column of CM-cellulose, $5 \text{ cm}^2 \times 16.5 \text{ cm}$. Flow rate was 13 ml/hr, and the yield was 88%. Absorbance at 280 nm (—); radioactivity (●—●); conductivity (O—O). Horizontal bars indicate pooled fractions. Polyacrylamide electropherograms at pH 2.5 in 7% gels are shown above each pooled fraction.

Polyacrylamide Gel Electrophoresis was performed in 7% gels at pH 2.5 essentially according to Brummel and Montgomery (14). Electrophoresis was run in 75-mm tubes for 120–150 min at 120 V. After electrophoresis, the gels were stained with Coomassie brilliant blue and destained with 12% trichloroacetic acid. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate at pH 7.0 was performed as described (15). Samples were first incubated in phosphate buffer containing 1% sodium dodecyl sulfate. Electrophoresis was run in 3.5 or 7.0% gels at a constant current of 7 mA per tube. Protein standards were reduced and alkylated and treated in the same manner as the test samples. Molecular weights of the samples were estimated by comparison of their relative mobilities to the relative mobilities of the protein standards of known molecular weight.

RESULTS

Chromatographic separation of *S*-carboxymethyl fibrin chains

Representative chromatograms of the reduced, *S*-carboxymethyl derivatives of crosslinked and noncrosslinked fibrins on CM-cellulose are shown in Figs. 1 and 2. Polyacrylamide gel electropherograms of the pooled fractions at pH 2.5 are shown above each of the major fractions. Also shown on the chromatograms are the radioactivity and conductivity profiles. It can be seen that with this chromatographic procedure the *S*-carboxymethyl derivatives of both crosslinked and noncrosslinked fibrins were separated into three major components. Electrophoretic analysis on polyacrylamide gels confirmed that the pooled fractions I, III, and V from both chromatograms each contained one relatively pure component. These pooled fractions were dialyzed, freeze-dried, and used for further analysis.

Fig. 2. Chromatographic profile of 175 mg of tritiated *S*-carboxymethyl crosslinked fibrin on a column of CM-cellulose, 5 cm² × 20 cm. Flow rate was 16 ml/hr, and the yield was 86%. See Fig. 1 for details.

By aminoacid analysis, amino-terminal analysis, molecular weight estimation, and peptide mapping (see below), it was determined that fractions I, III, and V of crosslinked fibrin were composed of the *S*-carboxymethyl derivatives of γ - γ -dimer, free β -chain, and α -polymer, respectively. Similarly, the same fractions of noncrosslinked fibrin contained the derivatives of free γ -chain, β -chain, and α -chain.

Aminoacid analysis

The aminoacid compositions of the separated chains of crosslinked and noncrosslinked fibrin are shown in Table 1. Cysteine and cystine are reported as carboxymethylcysteine (CM-Cys), since all half-cystines were converted to the carboxymethyl derivative. The values of threonine and serine were calculated by extrapolation to zero hydrolysis time. Tryptophan was not measured. It can be seen that free α -chain and α -polymer have essentially the same aminoacid composition. Likewise the β -chains of crosslinked and noncrosslinked fibrins have the same composition, as do also the γ -chain and the γ - γ -dimer. These aminoacid analyses are in agreement with those reported previously for the *S*-carboxymethyl derivatives of fibrinogen (9, 16) and the *S*-sulfo derivatives of crosslinked fibrin (6).

Amino-terminal analysis

Amino-terminal aminoacid analysis by the phenylisothiocyanate method showed that the γ -chain and the γ - γ -dimer had only tyrosine as the amino-terminal amino acid, while the β -chains, the α -chains, and the α -polymer had glycine. These results agree with the established amino-terminal amino acid of the respective human fibrin chains. No purified component was found that had both tyrosine and glycine as amino-terminal residues.

Molecular weight determination

The molecular weights of each of the separated chains were estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (15). The α -chain of noncrosslinked fibrin consistently appeared as a doublet with average molecular weights of 61,000 and 63,000; β -chain was 54,000; γ -chain was 47,000. The molecular weight for crosslinked fibrin β -chain was 55,000, and for γ - γ -dimer, 80,000. Most of the α -polymer material was of too great a molecular weight to enter gels at acrylamide concentrations as low as 3.5%. However, about 10% of the protein did enter the gel as a broad, diffuse band and had a relative mobility indicating that it was composed of 6–8 crosslinked α -chains. However caution should be taken in assigning a minimum molecular weight for this material because of the lack of appropriate standards in this molecular weight range.

Peptide mapping

Data obtained by peptide mapping or fingerprinting provide further characterization and identification of the separated chains of the two types of fibrin. In Fig. 3 are shown the tryptic peptide maps of each of the *S*-carboxymethyl derivatives, with the chains of noncrosslinked fibrin in the upper half of the figure and the corresponding chains of crosslinked fibrin beneath them. Spots that stained yellow with ninhydrin are represented by the shaded areas. It is evident that the α -chain and the α -polymer have similar peptide maps. The majority of the ninhydrin-staining peptides appeared in the same relative positions, and the positions of the four characteristic yellow spots were nearly identical. Likewise, the peptide maps of the β -chains from crosslinked and noncrosslinked fibrins are also similar, both in general appearance and with regard to the two yellow-staining spots. The similarities in the peptide maps of the γ -chain and the γ - γ -dimer are

TABLE 1. Aminoacid composition of *S*-carboxymethyl crosslinked and noncrosslinked fibrin chains

Amino acid	Non-crosslinked fibrin			Crosslinked fibrin		
	α -chain	β -chain	γ -chain	α -polymer	β -chain	γ - γ -dimer
CM-Cys	0.064	0.123	0.126	0.075	0.146	0.147
Asp	0.788	0.909	0.977	0.844	1.106	1.055
Thr*	0.530	0.340	0.472	0.518	0.450	0.504
Ser*	1.005	0.472	0.392	0.995	0.595	0.505
Glu	0.814	0.907	0.862	0.854	1.090	0.975
Pro	0.474	0.399	0.231	0.508	0.451	0.280
Gly	0.735	0.637	0.595	0.755	0.708	0.647
Ala	0.246	0.349	0.459	0.265	0.405	0.496
Cys/2	0	0	0	0	0	0
Val	0.355	0.384	0.273	0.370	0.427	0.294
Met	0.132	0.217	0.152	0.122	0.238	0.153
Ile	0.234	0.261	0.446	0.238	0.311	0.475
Leu	0.383	0.439	0.465	0.394	0.503	0.501
Tyr	0.101	0.279	0.313	0.113	0.320	0.343
Phe	0.205	0.143	0.286	0.220	0.191	0.324
His	0.156	0.109	0.162	0.156	0.124	0.163
Lys	0.477	0.513	0.590	0.485	0.562	0.617
Arg	0.450	0.390	0.175	0.472	0.435	0.186

Values are given in $\mu\text{mol/mg}$ and represent the average of 6–12 determinations of 2–4 preparations.

* Extrapolated to 0 hydrolysis time from 22, 48, and 72 hr.

FIG. 3. Peptide maps of tryptic digests of the *S*-carboxymethyl derivatives of crosslinked, insoluble fibrin and noncrosslinked, soluble fibrin. X indicates point of application of 2–5 μ l of sample on thin-layer cellulose plates. Electrophoresis was performed in the horizontal dimension and was followed by ascending chromatography. [Typical peptide maps of α -chain and α -polymer have appeared in a preliminary report (5) and are included here for comparison with the peptide maps of the other fibrin components].

FIG. 4. Tryptic peptide map of equal amounts of *S*-carboxymethyl α -chain and γ -chain, which were mixed together before digestion with trypsin. See Fig. 3 for details.

also apparent. The differences in the three types of peptide maps were sufficiently great to allow positive identification of each of the chains.

Experiments, designed further to characterize the types of chains involved in fibrin crosslinking, are shown in Fig. 4. If an α - γ type of crosslink existed in fibrin, one would expect to find evidence of it by peptide mapping. To test this idea, tryptic hydrolysates of α -chain and γ -chain from noncrosslinked fibrin were mixed together in equal proportions, and a peptide map was made of this mixture. Comparison of this peptide map with those of α -polymer and γ - γ -dimer clearly showed that neither of these components could be mistakenly identified as an α - γ crosslink.

DISCUSSION

The mammalian fibrinogen molecule is composed of three pairs of polypeptide chains, which are designated as α (A), β (B), and γ . These chains are held together by disulfide bonds, and the fibrinogen molecule consists of a dimeric structure, so that the formula of the molecule may be expressed as $[\alpha(A)\beta(B)\gamma]_2$. The primary structure of the amino-terminal portion of each chain has been established (17, 18).

When thrombin converts fibrinogen to fibrin by a limited proteolysis, two fibrinopeptides, A and B, are released (19). The structure of the resultant fibrin may then be expressed as $(\alpha\beta\gamma)_2$. The final step in fibrin formation is the enzymatic introduction of *N*⁶- γ -glutamyllysyl bonds between the polypeptide chains of fibrin, which is catalyzed by activated factor XIII.

The data presented in this paper provide further charac-

terization of the polypeptide chains of fibrin and evidence for the way in which crosslinking occurs. The chromatographically purified, *S*-carboxymethyl derivatives of the fibrin chains could be positively identified by aminoacid analysis, amino-terminal analysis, molecular weight determination, and peptide mapping. Comparison of these analyses for noncrosslinked fibrin with those for fibrinogen showed the expected similarities. Thus we have conclusive evidence that the *S*-carboxymethyl derivative chains of noncrosslinked human fibrin eluted from CM-cellulose in the order: γ -chain, β -chain, and α -chain (Fig. 1).

When crosslinked human fibrin was reduced and alkylated, three major components were again obtained by chromatography on CM-cellulose (Fig. 2). The first component had amino-terminal tyrosine and an aminoacid composition and peptide map that identified it as being composed of γ -chain. By polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the molecular weight of this component was estimated to be 80,000. From these data we could conclude that this component was the *S*-carboxymethyl derivative of two covalently crosslinked γ -chains. Although the estimated molecular weight was lower than the sum of two γ -chains, this was to be expected since the introduction of covalent bonds between polypeptide chains increases the relative mobility and, thus, yields molecular weight values that are lower than the expected value (15).

The second component of crosslinked fibrin to elute from CM-cellulose had amino-terminal glycine and a molecular weight of 55,000. The aminoacid composition and peptide map were that of the β -chain. The identity of this component

of crosslinked fibrin as free β -chain provides further evidence that the β -chain of fibrin is not a substrate for activated factor XIII and is not covalently crosslinked.

The third component of crosslinked fibrin had the amino-acid composition and peptide map of α -chain. It had amino-terminal glycine, as does α -chain. A molecular weight could not be obtained by polyacrylamide electrophoresis in sodium dodecyl sulfate since most of this material did not penetrate the gels. About 10% of total α -polymer, which did enter the gels, appeared as a diffuse band with a molecular weight range consistent with polymers composed of 6–8 crosslinked α -chains. Attempts to obtain a molecular weight by sedimentation equilibrium were also unsuccessful, since the material was polydisperse (McDonagh, R.P. & Blombäck, B., unpublished observations). From these data this material was identified as the *S*-carboxymethyl derivative of covalently crosslinked α -chains. The high molecular weight and the disperse nature of the material suggest that a large and variable number of α -chains are crosslinked by activated factor XIII. This hypothesis is supported by gel filtration experiments on Sepharose. Thus, the crosslinking of α -chains is in contrast to that of γ -chains, which stops when only two γ -chains have been linked together.

The formation of α - γ crosslinks in fibrin is also theoretically possible, since both chains contain the necessary donor and acceptor sites. However, no evidence could be obtained in these experiments for this type of crosslinking reaction in human fibrin. If activated factor XIII does catalyze the formation of these bonds, it must do so only to a very slight degree. In these experiments it was not possible to isolate a purified derivative with both glycine and tyrosine as the amino-terminal amino acids, as would be expected for α - γ -crosslinked chains. Confirmation of absence of a significant amount of α - γ crosslinking was obtained from peptide maps of mixtures of α - and γ -chains. This peptide map (Fig. 4) clearly could not be matched to the maps of any of the purified components of crosslinked fibrin. While these experiments eliminate the possibility of any significant formation of α - γ crosslinks occurring in a one to one proportion, they cannot completely rule out the further possibility that γ -chains are incorporated to a small extent into polymers consisting predominantly of α -chains. However, kinetic experiments to determine the rate of formation of crosslinked fibrin components have shown that quantitative crosslinking of γ -chains occurs before α -polymer formation begins (20).

From the results obtained in these experiments it is apparent that the *S*-carboxymethyl derivatives of the chains of fibrin can be isolated by CM-cellulose chromatography, and these isolated components are sufficiently pure to allow their positive identification and characterization. With this procedure it has been possible not only to characterize the chains of crosslinked and noncrosslinked fibrins, but also to show conclusively that γ - γ -dimer and α -polymer formation are the pathways by which activated factor XIII catalyzes the covalent crosslinking of fibrin.

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