Cross-Linking of Cold-insoluble Globulin by Fibrin-stabilizing Factor*

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Cold-insoluble globulin (CI globulin) was purified from human plasma and identified on the basis of its sedimentation coefficient, electrophoretic mobility, and concentration in normal plasma. CI globulin was distinguished from antihemophilic factor (AHF) by amino acid analysis, position of elution from 4% agarose, and electrophoretic migration in polyacrylamide gels in the presence of sodium dodecyl sulfate without prior reduction. CI globulin and AHF could not be distinguished by polyacrylamide gel electrophoresis in sodium dodecyl sulfate after reduction and probably have very similar subunit molecular weights. CI globulin apparently consists of two polypeptide chains, each of molecular weight 2.0 x 10^5, held together by disulfide bonds.

CI globulin was a substrate for activated fibrin-stabilizing factor (FSF, blood coagulation factor XIII). FSF catalyzed the incorporation of a fluorescent primary amine, N-(5-aminopentyl)-5-dimethylaminonaphthalene-1-sulfonamide, into CI globulin and also catalyzed the cross-linking of CI globulin into multimers, as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate after reduction. In the presence of fibrin, cross-linking of CI globulin by FSF occurred without the formation of CI globulin multimers. Instead, polypeptides with apparent molecular weights of 2.6 x 10^5 and 3.0 x 10^5 were seen. The formation of these polypeptides coincided with the loss of the α chain of fibrin and CI globulin. The polypeptides were not seen when fibrin alone was cross-linked. The formation of the polypeptides was greater in fine clots than in coarse clots, and greater in clots incubated at 0°C than in clots incubated at 37°C. In clots made from purified fibrinogen, CI globulin, and FSF, the concentration of CI globulin in the clot liquor was greater if either FSF or calcium ion was omitted and cross-linking did not take place. These observations suggest that CI globulin is enzymically cross-linked to one of the chains of fibrin, most likely the α chain, and is thus covalently incorporated into the fibrin clot.

CI globulin is very similar to a protein in the plasma membrane of fibroblasts. The cross-linking of CI globulin to itself and to fibrin may typify reactions also involving the fibroblast membrane protein.
fied from human plasma and shown to be principally CI globulin. Experiments performed subsequently suggest CI globulin is a substrate for FSF and may be incorporated into a fibrin clot by covalent cross-linking.

**Materials and Methods**

**Materials**—Common chemicals were reagent grade. Suppliers of other materials included: Sepharose 4B, Pharmacia Fine Chemicals; dithiothreitol, Calbiochem; microgranular DEAE-cellulose (DE52), H. Reeve Angel and Co.; sodium dodecyl sulfate, Pierce Chemical Co.; cysteine hydrochloride, Sigma Chemical Co.; 2-mercaptoethanol, BioRex 70 (400 mesh), phosphocellulose (Cellex-P), and reagents for polyacrylamide gels. BioRad Laboratories; and dansylcadaverine, Cyclo Chemical Co. Bovine thrombin was purchased from Parke-Davis and Co. and purified further by chromatography on BioRex 70 (18). Human FSF (19) and myosin from rabbit skeletal muscle were generous gifts from Dr. Soo Il Chung, National Institute of Dental Research, and Dr. Eric Fossel, Harvard Medical School, respectively.

**Preparation of Proteins**—Fibrinogen and CI globulin were copurified from fresh-frozen plasma (obtained from donors undergoing repeated phlebotomy for hemochromatosis) by precipitation with ammonium sulfate and ethanol as described previously (17). The preparation was clotted and CI globulin purified from the clot liquor as follows. Protein from the clot was precipitated step, 1 to 2 g in 300 to 600 ml was dialyzed against 0.1 M sodium chloride, 0.01 M Tris-chloride, and 1 mm EDTA, pH 6.8, and clotted at 22° by addition of thrombin to give a final concentration of a unit/ml. After 3 hours, clot synarization was induced with a stir rod and the clot removed. To remove thrombin, the clot liquor was passed through a bed of phosphocellulose (1.5 x 10 cm) equilibrated with the clotting buffer. Protein in the effluent was dialyzed against by adding 1/2 volume of saturated ammonium sulfate. The protein was dissolved in a small volume of 0.3 M sodium chloride and 0.01 M Tris-chloride, pH 7.3, and applied to a freshly poured column of Sepharose 4B equilibrated and developed at 22° with the same buffer. Protein eluted from the column as three peaks (Fig. 1). The protein in Peak I was probably the large molecular weight protein associated with AHF activity, since its amino acid composition (Table I) and migration in polyacrylamide gels in the presence of sodium dodecyl sulfate with and without prior reduction were similar to those reported previously (20, 21). CI globulin eluted in Peak II and could not be distinguished from AIH by gel electrophoresis in sodium dodecyl sulfate after prior reduction, suggesting that the subunit molecular weights of CI globulin and AHF are similar; 2.0 x 105 (20) (Fig. 2). The amino acid composition of CI globulin was identical to that of AHF (Table I), and CI globulin migrated with a smaller apparent molecular weight (3.9 x 105) than AHF (greater than 1.0 x 105) in polyacrylamide gels in sodium dodecyl sulfate without prior reduction (Fig. 2).

CI globulin in Peak II was precipitated by adding 1/2 volume of saturated ammonium sulfate. The precipitate was dissolved in and dialyzed against 0.05 M sodium chloride and 0.01 m Tris-chloride, pH 8.1. The protein was chromatographed on microgranular DEAE-cellulose at 22° in a column (2.5 x 15 cm) developed with a linear 0.05 to 0.3 M sodium chloride gradient (total volume of 1000 ml) at pH 8.1 with 0.01 M Tris-chloride. Fractions from the main protein peak, eluting at pH concentrations of 0.16 to 0.19 M, were pooled, concentrated by precipitation with an equal volume of saturated ammonium sulfate, and dissolved in and dialyzed against the desired buffer. Portions were then frozen in Dry Ice-methanol and stored at -70° until used.

The yield of CI globulin isolated from plasma in this manner was 9%, significant losses being sustained during aluminum hydroxide adsorption, the precipitation steps, clotting, gel filtration, and chromatography on DEAE-cellulose (Table II). The final product migrated as a single band upon polyacrylamide gel electrophoresis of the native protein (30 µg) at alkaline pH, of the reduced protein (20 µg) in sodium dodecyl sulfate, and of the reduced and carboxymethylated protein (20 µg) in 8 M urea at alkaline pH. CI globulin, 1.8 mg/ml, sedimented with a sedimentation coefficient (s20, w) of 13.8 S. Antiserum to CI globulin was produced in rabbits and reacted with a fast β-globulin upon immunoelectrophoresis. The concentration of CI globulin in the plasma of 20 young adults was 279 ± 79 µg/ml (mean ± S.D.), as assayed by Laurell's electroimmunoassay (26). No differences were found when CI globulin prepared without exposure to thrombin (3) and CI globulin prepared by the present method were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by immunoelectrophoresis.

Fibrinogen free of CI globulin was prepared by chromatography at 22° of 1 to 2 g of the protein obtained after ethanol precipitation on a column (5 x 35 cm) of microgranular DEAE-cellulose, developed with a 0.04 to 0.30 M linear sodium chloride gradient (total volume of 3800 ml) buffered at pH 6.1 with 0.01 M Tris-chloride. Flow rates of 350 to 400 ml/hour were achieved by gravity flow, and 12 to 18 ml fractions were collected. In this system, fibrinogen was eluted as two peaks (27).

![Fig. 1. Chromatography of the concentrated clot liquor on Sepharose 4B. The sample, 14.4 ml containing 108 mg of protein, was chromatographed on a column (5 x 51 cm) of Sepharose 4B developed with upward flow. The flow rate was 60 ml/hour, 10-ml fractions were collected. Fractions containing protein were pooled as indicated, and the protein was concentrated by ammonium sulfate precipitation. Of the total protein eluted from the column, estimated by absorbance, 3% was in I, 74% in II, and 22% in III. The void column for the column, determined using blue dextran, is indicated by the arrow.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>CI globulin</th>
<th>Peak I</th>
<th>AHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>3.6</td>
<td>4.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.6</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.4</td>
<td>4.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.5</td>
<td>9.5</td>
<td>9.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>10.8</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Serine</td>
<td>8.1</td>
<td>6.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.5</td>
<td>11.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Proline</td>
<td>6.2</td>
<td>6.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.6</td>
<td>7.5</td>
<td>7.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.2</td>
<td>5.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.5</td>
<td>7.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Valine</td>
<td>8.2</td>
<td>9.8</td>
<td>8.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.6</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.5</td>
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<td>3.5</td>
</tr>
<tr>
<td>Leucine</td>
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<td>7.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.3</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.4</td>
<td>2.8</td>
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</tr>
</tbody>
</table>

* Analyzed as half-cystine.

† Analyzed as cysteic acid.

6615
Purification of CI globulin from 1580 ml of citrated plasma

Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction of CI globulin from 1580 ml of citrated plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Plasma</td>
<td>7300</td>
</tr>
<tr>
<td>Adsorbed plasma</td>
<td>601</td>
</tr>
<tr>
<td>First 25% ammonium sulfate</td>
<td>491</td>
</tr>
<tr>
<td>precipitation</td>
<td></td>
</tr>
<tr>
<td>Second 25% ammonium sulfate</td>
<td>383</td>
</tr>
<tr>
<td>precipitation</td>
<td></td>
</tr>
<tr>
<td>7% ethanol precipitation</td>
<td>274</td>
</tr>
<tr>
<td>Clot liquor</td>
<td>193</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>193</td>
</tr>
<tr>
<td>Sepharose 4B, Peak II</td>
<td>82</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>71</td>
</tr>
</tbody>
</table>

8 In the presence of other proteins, the concentration of CI globulin was estimated by electroimmunoassay. Absorbance was used when CI globulin was greater than 95% pure.

9 For the initial five steps, CI globulin concentration was divided by the concentration of total protein. The amount of contamination in the latter steps was estimated by gel electrophoresis in sodium dodecyl sulfate.

CI globulin eluted as a third peak at chloride concentrations of 0.16 to 0.18 M. Fibrinogen free of FSF was prepared by treating the protein from the first fibrinogen peak with 3.3 M urea (28).

Cross-linking reactions—Solutions containing fibrinogen and CI globulin were dialyzed against 0.14 M sodium chloride buffered at pH 7.4 with 0.01 M Tris-chloride. FSF was added. Cross-linking was initiated by adding a solution containing calcium chloride and thrombin; in some experiments dithiothreitol or cysteine was added as well (when fibrinogen was present, cross-linking was accompanied by clotting). Approximate final concentrations were: calcium ion, 2 mM; dithiothreitol, 10 mM; cysteine, 17 mM; and thrombin, 1 unit/ml. The cross-linking reactions were stopped either by adding EDTA in 5-fold excess of calcium ion or by adding an equal volume of 2% sodium dodecyl sulfate, 8 M urea, and 2% 2-mercaptoethanol.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.0 was performed using the method of Weber and Osborn (29). Prior to electrophoresis, samples were incubated for 10 min at 100° in 4 M urea, 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol. For analysis of unreduced proteins, samples were incubated for 2 hours at 37° in 4 M urea and 1% sodium dodecyl sulfate. Discontinuous gel electrophoresis in 8 M urea was performed according to the method of Davis (30), using a 3.5% running gel and 2.0% stacking gel. Electrophoresis in 8 M urea at pH 9.5 was done with multiphasic buffer system A of Rodbard and Chrambach (31), with the upper and lower gels being made 8 M in urea; proteins to be subjected to electrophoresis were reduced and carbamylated in 8.4 M guanidine hydrochloride (32). Gels were stained with Coomasie brilliant blue and destained by diffusion (29). Densitometry was performed with a Gilford gel scanning apparatus. Photographs of gels containing danylated proteins were taken with Polaroid type 105 positive-negative film and a Kodak Wratten No. 22 filter (Eastman Kodak Co.); a long wavelength ultraviolet box (Ultra-Violet Product, Inc.) was the light source.

Immunological Techniques—Antisera were prepared in rabbits by inoculating animals intramuscularly with 300 to 500 mg of CI globulin in complete Freund's adjuvant (Difco Laboratories) followed by repeated subcutaneous inoculations with antigen, 100 to 200 μg, at 2-week intervals. Immunoelectrophoresis was carried out at room temperature in 1% agarose in pH 8.6 barbital buffer (I = 0.05) on glass slides (33). Laurell's electroimmunoassay (26) was performed in 1% agarose in the same barbital buffer on glass slides placed on a plate incubator at 10°. The electrophoresis was run at 300 volts for 3 to 4 hours. Purified CI globulin served as a primary standard. Pooled plasma was stored in portions at -70° and served as a secondary standard. Indirect immunofluorescence of WI-38 human fibroblasts, obtained from American Type Culture Collection and grown in basal Eagle's medium (diploid) (Grand Island Biological Co.) supplemented with 10% fetal bovine calf serum, was performed as described by Ruoslahti et al. (34).

Analytical Techniques—The concentrations of purified fibrinogen and CI globulin were determined spectrophotometrically using absorption coefficients (μ = A280 cm⁻¹) at 280 nm of 1.528 (35) and 1.28 (3), respectively. In the presence of other proteins, CI globulin was quantitated using Laurell's electroimmunoassay (26). Protein concentration was determined by the method of Lowry et al. (36). Hydrogen ion concentration was measured with a glass electrode at 22°; pH values reported for the various buffers have not been corrected to the temperature at which the buffer was used.

Amino acid analyses were carried out on a Beckman model 121 amino acid analyzer. The sample (100 to 300 μg) was placed in 1 ml of 6 N hydrochloric acid, de-aerated, sealed in glass tubes, and hydrolyzed for 24 hours at 110°.

Sedimentation coefficients were determined at 20° in a Spinco model E analytical ultracentrifuge equipped with schlieren optics.

RESULTS

CI globulin was purified from human plasma as described under "Materials and Methods" and identified on the basis of its sedimentation coefficient (2), electrophoretic mobility (2), and concentration in normal plasma (3). The purified protein migrated on polyacrylamide gels as a homogeneous band under three different electrophoretic conditions. CI globulin could be distinguished from AHF by position of elution from 4% agarose, amino acid analysis, and electrophoretic migration in polyacrylamide gels in the presence of sodium dodecyl sulfate without prior reduction. Cooper et al. (37) and Weinstein et al. (38) also concluded that CI globulin and AHF were distinct proteins. The apparent molecular weight of CI globulin, estimated by polyacrylamide gel electrophoresis in sodium dodecyl sul-
fate, was $2.0 \times 10^5$ when analyzed after reduction and $3.9 \times 10^4$ when analyzed without prior reduction (Fig. 2). These observations suggest that CI globulin consists of two polypeptide chains held together by disulfide bonds. Molecular weights obtained by sedimentation equilibrium for bovine CI globulin ($5.4 \times 10^4$ (38)) and human CI globulin ($4.5 \times 10^4$ (39)) are consistent with a dimeric structure.

To show that CI globulin was a substrate for FSF, CI globulin and FSF were incubated with 1.4 mM dansylcadaverine, a fluorescent primary amine, in the presence of 17 mM cysteine. Gel electrophoresis in sodium dodecyl sulfate of the reaction mixture indicated that the fluorescent amine was being incorporated into CI globulin (Fig. 3A). Similar incorporation was seen when no sulfhydryl reagents were present. Incorporation was not seen in the absence of enzyme or calcium ion. These findings indicate that CI globulin contains 1 or more glutaminy1 residues susceptible to the action of FSF.

Attempts were made to cross-link CI globulin with FSF in the presence and absence of sulfhydryl reagents, which are known to enhance FSF activity (40). FSF cross-linked CI globulin when 10 mM dithiothreitol was present; as monitored by polyacrylamide gel electrophoresis in sodium dodecyl sulfate after reduction, progressively higher multimers appeared with time (Fig. 3B). A nearly linear relationship was found when the logarithms of the molecular weights of the multimers were plotted versus their migrations relative to bromphenol blue, assuming that the progression of cross-linking was monomer to dimer to trimer and so on (open circles in Fig. 2). The trimers were a doublet, suggesting that there may be several modes of cross-linking resulting in complexes with different apparent molecular weights. The rate and extent of cross-linking in the presence of dithiothreitol were variable from experiment to experiment and on several occasions were greater than shown in Fig. 3B; the reasons for the variability are not understood. No cross-linking was noted in the absence of enzyme, and the reaction could be stopped by addition of EDTA. The reaction proceeded less well if 17 mM cysteine was substituted for 10 mM dithiothreitol and did not proceed at all without added sulfhydryl reagents, even when the concentration of FSF was doubled or tripled.

A faint band migrating slightly farther than CI globulin monomer developed with time when CI globulin was incubated with FSF and thrombin, 1.6 units/ml (Fig. 3B). In other experiments, CI globulin was incubated for 2 hours at 37°C with thrombin, 16 units/ml, and then denatured, reduced, and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Partial degradation of the $2.0 \times 10^4$ molecular weight polypeptide to fragments with molecular weights of 1.7 to $1.8 \times 10^4$ and 2 to $3 \times 10^4$ was noted. Thus, CI globulin appears to be susceptible to proteolysis at high concentrations of thrombin. However, the reaction was slow at low thrombin concentrations, and the disappearance of the CI globulin band from polyacrylamide gels of fibrin clots must be mediated by FSF. To study this phenomenon, a series of experiments was performed comparing the cross-linking of CI globulin and fibrin, as analyzed by gel electrophoresis in sodium dodecyl sulfate after reduction, to cross-linking of CI globulin alone and of fibrin alone.

These experiments indicate that the cross-linking of CI globulin by FSF in the presence of fibrin differs from the cross-linking of CI globulin alone. First, the reaction took place in the absence of dithiothreitol or cysteine (Fig. 4). Under conditions in which coarse clots formed, the amount of the CI globulin monomer diminished with time, and material appeared which barely entered the polyacrylamide gel. Second, at no time were CI globulin polymers seen. Instead, at intermediate times polypeptides were present with molecular weights estimated to be $6 \times 10^4$ and $1.0 \times 10^5$ greater than the CI globulin monomer (c and b in Fig. 2). These polypeptides were not seen in gels of fibrin alone, suggesting that they must arise from cross-linking between CI globulin and fibrin. At a constant CI globulin concentration of 750 µg/ml, the polypeptides were seen at CI globulin to fibrin (w/w) ratios ranging from 1:0.1:10 to 1:0.1:2.

The rates of loss of bands corresponding to CI globulin and the three chains of fibrin were estimated by performing densitometry of the gels (Fig. 5). Monomeric CI globulin and the α chain of fibrin disappeared at similar rates. A similar correlation between the amounts of remaining monomeric CI globulin and α chain was found when the FSF concentration was varied, resulting in variable amounts of cross-linking. When fine clots were formed using 0.18 M sodium chloride, the rate and extent of α chain cross-linking were less (Fig. 4) as has been reported by Doolittle (42). Under these conditions, the polypeptides of molecular weight $2.6 \times 10^4$ and $3.0 \times 10^4$ were more prominent and persisted longer when compared to gels of coarse clots formed in 0.12 M sodium chloride (Fig. 4). The cross-linking of CI globulin and fibrin was also studied at 0°C (Fig. 6). At this temperature, clots formed in 0.12 M and 0.18 M sodium chloride were both coarse. The time of formation of a visible clot was 1 to 2 min compared to 10 to 20 s at 37°C, and 7 dimer formation took 1 hour to go to completion as compared to 2 min at 37°C. The CI globulin monomer was converted almost completely to the $M_c = 2.6 \times 10^4$ polypeptide over a 1-hour period. The $M_c = 3.0 \times 10^4$ polypeptide appeared at the same time that α dimers and trimers were noted in clots formed of fibrin alone. A small amount of protein appeared at the position of the CI globulin dimer, although CI globulin could not be cross-linked in the absence of fibrin under otherwise identical conditions.

Experiments were performed in which each of the components of the clotting mixture was left out, and the amount of CI globulin left in the clot liquor after syneresis was quantitated (Table III). After clotting in the presence of FSF and calcium ion, the amount of CI globulin in the clot liquor was diminished compared to clots in which cross-linking did not occur, corroborating the evidence from gel electrophoresis that CI globulin was enzymically cross-linked to fibrin. In addition, there appeared to be less CI globulin in liquids of non-cross-linked clots than in mixtures lacking fibrin, suggesting that fibrin occluded CI globulin during syneresis.

**DISCUSSION**

The present studies indicate that CI globulin is a substrate for FSF. CI globulin was enzymically labeled by the exogenous amine, dansylcadaverine, and cross-linked in physiologic buffers containing dithiothreitol. From studies of guinea pig liver transglutaminase, it seems likely that there is specificity in the reaction between transglutaminases and their substrates;

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6 Ferry and Morrison (41) distinguished between two types of fibrin clots: a fine clot, which is transparent, friable, and non-synerizing, and a coarse clot, which is opaque, plastic and nonfriable, synerizes readily, and forms at lower pH and ionic strength.
Fig. 3. (upper left). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of CI globulin (CIG) incubated with FSF. A, CI globulin, 360 µg/ml, was incubated at 37° for 2 hours in a solution containing FSF, 12 µg/ml; thrombin, 1 unit/ml; 2 mM calcium ion; 1.4 mM dansylcadaverine; and 20 mM cysteine. EDTA was added to stop the reaction, the protein was denatured and reduced, and 4 µg were subjected to electrophoresis on 4% gels. The fluorescence at the bottom of the gel represents free dansylcadaverine. B, CI globulin, 600 µg/ml, was incubated at 37° for the indicated time in a solution containing FSF, 5 µg/ml; thrombin, 1.8 units/ml; 2 mM calcium ion; and 10 mM dithiothreitol. EDTA was added to stop the reaction, the proteins were denatured and reduced, and 6 µg were subjected to electrophoresis on 3.5% gels.

Fig. 4 (lower left). Comparison of the time courses of cross-linking at 37° of fibrin alone and of fibrin and CI globulin (CIG), analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Fibrinogen, 3.1 mg/ml, was clotted by thrombin, 1 unit/ml, in the presence of FSF, 14 µg/ml, and 3.3 mM calcium ion; CI globulin, 450 µg/ml, was present (A) or omitted (B). Two sodium chloride concentrations were used: 0.12 M resulting in coarse clots; and 0.18 M resulting in fine clots. At the designated time, the proteins were denatured and reduced, and 20-µl samples, containing approximately 31 µg of fibrin and 4.5 µg of CI globulin, were subjected to electrophoresis on 4% gels. The anode was toward the bottom. Band designation is discussed in the text.

Fig. 5 (upper right). Time course of disappearance of the monomeric polypeptide chains of fibrin and CI globulin from polyacrylam-
TABLE III

Amounts of CI globulin in clot liquors of cross-linked and noncross-linked clots

<table>
<thead>
<tr>
<th>Missing reagent</th>
<th>CI globulin in clot liquor (mean ± S.D.)</th>
<th>Percentage of CI globulin lost into the clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;15</td>
<td>&gt;97</td>
</tr>
<tr>
<td>Calcium ion</td>
<td>412 ± 62</td>
<td>28</td>
</tr>
<tr>
<td>Cysteine</td>
<td>146 ± 4</td>
<td>75</td>
</tr>
<tr>
<td>FSF</td>
<td>390 ± 76</td>
<td>32</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>668 ± 116</td>
<td>0</td>
</tr>
</tbody>
</table>

Fibrinogen, 3.3 mg/ml, was clotted at 37°C for 2 hours in a solution which, when complete, contained CI globulin, 573 µg/ml, FSF, 18 µg/ml, 16.6 mM cysteine, 3.3 mM calcium ion, and thrombin, 1 unit/ml. At the end of the incubation, EDTA was added and syneresis induced. CI globulin in the clot liquor was quantitated by electrophoresis. When all reagents were present, polycrylamide gel electrophoresis in sodium dodecyl sulfate revealed that nearly all of the α chains and CI globulin had been converted to higher molecular weight species. Gels of clots from which cysteine was omitted revealed partial polymerization of the α chains and CI globulin. Gels of clots from which calcium ion or FSF were omitted revealed no cross-linking. Incubations were performed in triplicate and analyses in duplicate.

The experiments were most consistent with cross-linking of CI globulin to the α chain of fibrin. First, CI globulin monomer and the α chain were lost at similar rates as estimated by polycrylamide gel electrophoresis in sodium dodecyl sulfate (Fig. 5). Second, the bands of molecular weight 2.6 × 10⁶ and 3.0 × 10⁶ did not progress to bands of higher molecular weight in fine clots with inhibited α chain polymerization. Finally, at 0°C the M, = 2.6 × 10⁶ band, which formed at a rate comparable with γ dimerization (presumably reflecting the increased affinity of CI globulin for fibrinogen (2, 5) and fibrin (7) at low temperatures), was not converted to higher molecular weight species until α dimer and trimer formation occurred. It should be emphasized that these arguments are based solely on interpretations of polycrylamide gels. Sodium dodecyl sulfate-protein complexes containing cysteine and ε-(γ-glutamyl)lysine may be expected to migrate anomalously (52), and thus the molecular weights assigned these complexes (Fig. 2) are probably lower than the actual molecular weights. Experiments are in progress to isolate the presumptive CI globulin-α complex from cross-linked clots formed at 0°C and prove the identity of the complex chemically and immunologically. As discussed by Doull et al. (42), the COOH-terminal half of the αa chain of fibrinogen (and α chain of fibrin) is especially sensitive to chemical modification and proteolytic cleavage. It may be that this part of the fibrin molecule, which is thought to contain reactive glutaminyl residues (50), is the site of cross-linking to CI globulin. Such a proposal can be tested: plasmin-degraded derivatives missing COOH-terminal portions of the α chain (53) should not cross-link to CI globulin.

We can only speculate on the biological significance of CI globulin-CI globulin and CI globulin-fibrin cross-linking and should like to consider several possibilities. Such reactions may explain why there is less CI globulin in serum than in plasma (3). However, the differences between plasma and serum CI globulin concentrations reported by Mosesson and Ulfmann (3) were only 19 to 52% and are similar to the 28% and 32% losses of CI globulin into fibrin we found in the absence of FSF activity (Table III). CI globulin has been identified complexed to fibrinogen in protein precipitates forming in chilled plasma from patients with cryofibrinogenemia (4, 5). Studies of Rhesus monkeys experimentally infected with Rocky Mountain spotted fever suggest that plasma levels of the CI globulin may fall with the formation and lysis of fibrin. Thus, only certain proteins (43) and glutaminyl residues (44, 45) were susceptible to the enzyme. After oxidation (44, 45) or succinyl-Font (46), more glutaminyl residues became susceptible, suggesting that tertiary protein structure is an important determinant of specificity. Of the plasma fractions and proteins studied by Clarke et al. (43), radioactive cadaverine was enzymically incorporated by guinea pig liver transglutaminase into fibrinogen, ceruloplasmin, and α globulin whereas little or no incorporation into albumin or γ-globulins was seen. FSF has a different and more restricted specificity than the guinea pig liver enzyme (47-49). In addition to catalyzing the ordered cross-linking of the γ and α chains of fibrin, as described in the introduction, FSF catalyzed the rapid incorporation of dansylcadaverine into the γ chain of fibrin (48), presumably by transamidation with the glutaminyl residue in the COOH-terminal region known to incorporate radioactive glycine ethyl ester (13), and the slower incorporation of dansylcadaverine into the α chain (48). Two fluorocent peptides were identified upon plasmin digestion of the α chains, indicating that 2 glutaminyl residues probably specifically incorporate the fluorescent amine (50, 51). Schwartz et al. attempted to cross-link serum proteins with guinea pig liver transglutaminase and FSF (49); cross-linking was noted when the proteins incubated with the liver enzyme were analyzed on 7.5% polycrylamide gels in sodium dodecyl sulfate but none when the proteins incubated with FSF were analyzed. CI globulin may not have been detectable in their gels.

Several observations indicate that CI globulin may be cross-linked to one of the chains of fibrin. First, when fibrin was present, the cross-linking of CI globulin did not require di-thiothreitol or cysteine, whereas CI globulin could be demon-
the cross-linking reaction may take place in vivo. There are remarkable similarities between the hydrodynamic behavior, subunit molecular weight, and subunit composition of mammalian CI globulin and the clottable protein of lobster blood (34, 50), suggesting a possible homology between these proteins. The lobster protein is cross-linked directly by a transglutaminase without prior proteolysis (56), and perhaps the property of lobster fibrinogen to be cross-linked by transglutaminases has been preserved and put to new uses in mammals.

It may be that CI globulin, which apparently is synthesized by fibroblasts (6, 34), functions on the surface of these cells. Given the similarities between the fibroblast membrane protein and CI globulin, it seems likely that the membrane protein has an affinity for fibrin and is a substrate for FSB. Niewiarowski et al. (58) found fibroblasts adhered to polyelectrolyte/PAAm and induced FSB retraction. Patients with FSB deficiency have been reported to have problems with wound healing (59–61), and Beck et al. (62) found that the presence of FSB in clots from patients with a defect in CI globulin was quantitatively and qualitatively inferior to that found in clots from normal plasma. Addition of 1% normal plasma, which corrected the defect in clot solubility, did not correct the defect in fibroblast growth. Partial correction of growth was achieved by adding 10% normal plasma and full correction by adding 50%. It has been shown that fibrin γ dimer formation correlated well with clot insolubility and required only 1% of the normal concentration of FSB, while α polymer formation required 10 to 100% of the normal concentration (49, 63, 64). Beck et al. hypothesized that FSB promoted fibroblast growth by rendering the fibrin resistant to proteolysis by cellular proteolytic enzymes (62), and indeed, Schwartz et al. (49) found that α polymerization made fibrin resistant to proteolysis by plasmin. The present work, however, suggests an alternative hypothesis: FSB is required for the covalent association of the fibroblast membrane protein with fibrin.

Vaheri and Ruoslahti found less of the membrane protein similar to CI globulin on the surface of transformed fibroblasts than on the surface of normal fibroblasts and suggested that the protein may be involved in the intercellular control of growth and movement (8). Evidence for the existence of r-(γ-glutamyl)lysine cross-links in the membrane proteins of L cells derived from mouse fibroblasts has been reported by Birck et al. (65). Thus, the fibroblast membrane proteins similar to CI globulin may interact with one another in a manner typified by the cross-linking of CI globulin molecules in solution.

Acknowledgments—I am deeply indebted to Drs. Leonard Spero, Sue Il Chang, Michael Mosesson, and Erkki Ruoslahti for discussions about this work.

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