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Intramolecular crosslinking of monomeric fibrinogen by tissue transglutaminase

(hemolysis/atherosclerosis/tumor cells)

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ABSTRACT **In addition to generating polymeric products from human fibrinogen, human erythrocyte transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) was shown to catalyze the intramolecular reaction of crosslinking two of the constituent chains within monomeric fibrinogen itself. This internally fused protein derivative contains appreciable amounts of the N^{ϵ}-(γ-glutamyl)lysine bridge peptide and displays the Aα·γ hybrid chain pattern of crosslinking, characteristic for the actions of tissue transglutaminases on fibrinogen. Diagnostic analysis in pathological situations, where such enzymes might have escaped from cells into the plasma environment, should include a search for the internally crosslinked soluble fibrinogen monomer.**

It is known that human fibrinogen, upon reaction with a tissue transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) from human erythrocytes or from guinea pig liver, forms high molecular weight multimeric products by a program of polymerization different from that seen with coagulation factor $XIII_a$ (1, 2). Factor $XIII_a$ promotes first γ-to-γ and then Aα-to-Aα chain homologous crosslinking (3), whereas Aα·γ type of hybrid crosslinking is the unique feature of the action of transglutaminase on fibrinogen. The question arises (1) whether these unusual crosslinks, apart from participating in the intermolecular process of polymerization, might also be introduced intramolecularly between the constituent Aα and γ chains of monomeric fibrinogen itself. This issue became even more pertinent when we found that, even after extended exposure of fibrinogen to transglutaminase, a sizeable portion of the protein remained in the unpolymerized $M_r \approx 340{,}000$ form. The present report shows that the monomeric species of fibrinogen, reisolated from the mixture after reaction with erythrocyte transglutaminase, contains significant amounts of the N^{ϵ}-(γ-glutamyl)lysine bridge peptide and shows the characteristic Aα·γ hybrid chain pattern of crosslinking (1).

MATERIALS AND METHODS

The contents of one vial of human fibrinogen (40 mg, IMCO, American Diagnostica, Greenwich, CT) were dissolved in 3 ml of buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA) and were dialyzed against 4 liters of the same buffer for 16 hr at 4°C. The concentration of fibrinogen (11.6 mg/ml) was estimated using $A_{280}^{1\%} = 15.1$ (4). Human erythrocyte transglutaminase was purified by David Schilling to apparent SDS/PAGE homogeneity based on a published procedure (5). Concentration of the erythrocyte enzyme [1.7 mg/ml of 20 mM imidazole·HCl, pH 6/1 mM EDTA/1 mM dithiothreitol/0.35 M KCl/10 units of Trasylol per ml/5% (vol/vol) glycerol] was estimated by using the BCA assay kit (Pierce) according to the manufacturer's instructions. Erythrocyte transglutaminase was stored at −80°C.

Crosslinking of fibrinogen was typically carried out at 37°C for 1 hr in reaction mixtures of about 24 ml containing approximately 2.3 μM fibrinogen, 0.1 M NaCl, 50 mM Tris·HCl (pH 7.5), and *ca.* 0.3 μM erythrocyte transglutaminase, with 5 mM $CaCl_2$ added last. The crosslinking reaction was stopped by the addition of 1.5 ml of 0.1 M EDTA/50 mM Tris·HCl, pH 7.5.

The reaction mixture of transglutaminase-treated fibrinogen was concentrated by lyophilization to about 9 ml and fractionated on Sepharose 4B gel filtration column (1.6 × 96 cm) in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA. Elution was carried out with the same buffer at a flow rate of 12 ml/hr, and ≈2-ml fractions were collected. Absorbancy was measured at 280 nm.

Agarose (2%)/SDS (0.1%) electrophoresis under nonreducing conditions (6) was carried out (*ca.* 24°C) on protein samples (*ca.* 10 μg) following treatment at 37°C with 6 M urea/2% SDS for 30 min. SDS (0.1%)/PAGE (5% acrylamide) analysis (*ca.* 24°C) was performed as described by Weber and Osborn (7) on samples of *ca.* 10 μg after a 30-min (37°C) treatment of the proteins with 40 mM dithiothreitol/6 M urea/2% SDS. Two-dimensional electrophoresis was by the procedure of O'Farrell (8) with Ampholine (LKB) of pH 3.5–10 in the first dimension. Separation in the second dimension was by SDS/PAGE as above. Electroblotting to nitrocellulose (0.2 μm, Schleicher & Schuell) was carried out with LKB model 2005 electroblotting unit for 2 hr at 4°C by the method of Towbin *et al.* (9) in 25 mM Tris/192 mM glycine, pH 8.3/20% methanol. Protein staining of the gels was with 0.025% Coomassie brilliant blue R (in 10% acetic acid/25% methanol), and staining of the nitrocellulose blots was with 1% amido black (in 10% acetic acid/50% methanol).

For purposes of immunostaining, a rabbit antiserum against Aα chains (residues 241–476) and a mouse monoclonal antibody against the γ chains (residues 392–406) of fibrinogen were provided by Joan Sobel of the College of Physicians and Surgeons, Columbia University and by Bohdan J. Kudryk of The New York Blood Center, respectively. The antiserum against Aα (code H 51-8) was diluted 1:120,000, and the antibody against γ (code 6/20/5) was diluted 1:420,000 into 0.05% (vol/vol) TWEEN 20 (Sigma) phosphate-buffered saline. Vectastain ABC Kits (Vector Labs) were used for the peroxidase-based staining of immunoblots as described (10).

For crosslink analysis, ≈2 ml of the monomeric fibrinogen eluted from the Sepharose 4B column, containing about 0.2 mg of protein, was mixed with 1 ml of 30% (wt/vol) trichloroacetic acid (Sigma). The precipitated protein (4°C overnight) was collected by centrifugation and was washed successively with 1 ml of 5% trichloroacetic acid (three times), 1 ml of 50% ethanol/50% acetone (vol/vol) (three times), and 1 ml of

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acetone (twice). To the dried precipitate, 0.5 ml of 0.1 M ammonium bicarbonate and a small crystal of thymol were added. Enzymatic digestion was carried out by the procedure of Cariello *et al.* (11) with the sequential addition of subtilisin (10 μg and 4 μg), pronase (10 μg and 4 μg), carboxypeptidase Y (5 μg), leucine aminopeptidase and prolidase (2 μg each), and, again, leucine aminopeptidase (2 μg). The samples were dried and dissolved in 0.2 ml of water, and amino acid analysis was performed according to the *o*-phthalaldehyde derivatization procedure of Griffin *et al.* (12). Three aliquots (10 μl each, containing about 3.6 μg of digested protein), one of which had 10 pmol of authentic N^{ϵ}-(γ-glutamyl)lysine added, were mixed with 50 μl of 20% (vol/vol) triethylamine in ethanol and dried. They were redissolved in 10 μl of water/50 μl of the triethylamine, and dried again. This procedure was repeated three more times. A control containing all of the enzymes, but no fibrinogen, was treated in the same manner. The samples were dissolved in 20 μl of 50 mM sodium phosphate (pH 7.5). Five microliters of 5 mM sodium phosphate (pH 7.5) was added to one of the original aliquots and to the aliquot to which N^{ϵ}-(γ-glutamyl)lysine was added (see above). To the third sample, 5 μl of partially purified γ-glutamylamine cyclotransferase was added (13). The samples were incubated at 37°C for 90 min and then dried. Determination of N^{ϵ}-(γ-glutamyl)lysine peptide by HPLC was carried out as described by Cariello *et al.* (11) with a Waters model 600E solvent delivery system. Data were collected by a Waters maxima 825 workstation.

RESULTS

Gel filtration chromatography (Fig. 1, *Top*) coupled with nonreducing electrophoretic analysis of the effluent on agarose in SDS (Fig. 1 *Middle*) showed that, even after prolonged exposure (1 hr at 37°C with 5 mM Ca^{2+}; ionic strength $\mu \approx$ 0.15 mol/kg of solvent) of human fibrinogen (*ca.* 2.3 μM) to human erythrocyte transglutaminase (*ca.* 0.3 μM), a significant portion of the protein still remained in the monomeric form ($M_r \approx 340{,}000$). As seen in Fig. 1 *Middle*, Fraction 42 contained the high molecular weight polymeric products that were discussed in a previous publication (1); fraction 60 consisted mostly of dimers and trimers; fractions 65 and 66 consisted of monomers and dimers, whereas the later emerging fractions 67–72 consisted of the monomers with only faint and diminishing traces of the dimers. This latter pool was the focus of our attention, and it was of special interest to find with reducing SDS/PAGE analysis (Fig. 1 *Bottom*) that these monomeric protein fractions represented an unusual, internally crosslinked form of fibrinogen. Just as in the polymeric products, the characteristic Aα·γ band ($M_r \approx 115{,}000$) was present in the monomeric species, but the higher $A\alpha_p{\cdot}\gamma_q$ structures (lanes 1–3 in Fig. 1 *Bottom*) were absent.

Two-dimensional electrophoresis offers perhaps the best means for assessing the hybrid Aα·γ crosslinked chain pattern generated in the reaction of fibrinogen with tissue transglutaminase (1). This technique was used to compare the starting fibrinogen with its monomeric equivalent reisolated from the reaction mixture after treatment with transglutaminase. The reference fibrinogen (Fig. 2 *Upper*, labeled "I") revealed the standard profile with a compact spot for the γ chain, the isoelectrically heterogeneous Aα and Bβ chains, and some partially degraded Aα chain remnants. These breakdown products, marked $\overline{A\alpha}$, were well revealed by immunoblotting with an Aα chain-specific antibody (Fig. 2 *Upper Right*). The reference protein contained a small amount of crosslinked γ-γ′ chain dimers, seen both by amido black staining (Fig. 2 *Upper Left*) and better by immunoblotting with a γ chain-specific antibody (Fig. 2 *Upper Center*); the presence of these γ-γ′ dimers was perhaps due to a previous exposure of the fibrinogen (during blood collection or before) to the action of factor $XIII_a$.

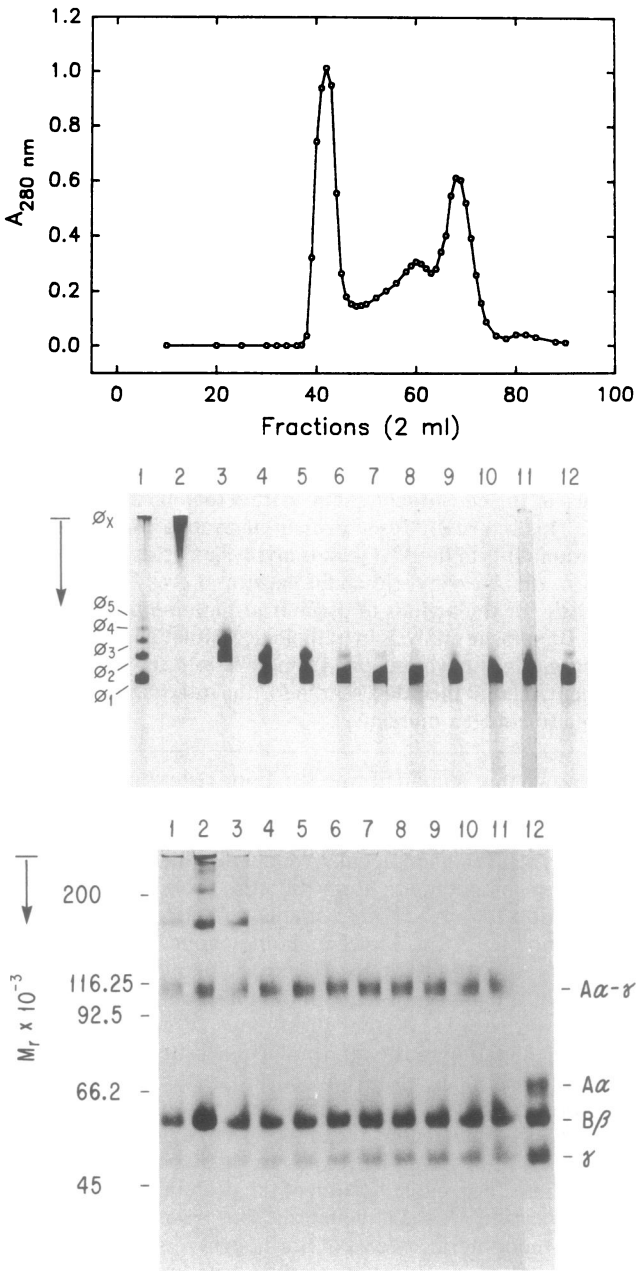

FIG. 1. Isolation of the monomeric form of human fibrinogen after reaction with human erythrocyte transglutaminase. (*Top*) Elution profile of the reaction mixture from Sepharose 4B. The abscissa shows the numbers of the 2-ml fractions collected; the ordinate presents absorbancies of the effluent at 280 nm. (*Middle*) Nonreducing electrophoresis of designated fractions from *Top* in SDS (0.1%) on 2% agarose. Lanes: 1, fibrinogen incubated with transglutaminase; 2, isolated fraction 42 from *Top*; 3–11, fractions 60, 65, 66, 67, 68, 69, 70, 71, and 72, respectively; 12, fibrinogen before treatment with transglutaminase (control). Positions of monomeric "fibrinogen" (ϕ_1) and those of oligomeric species (ϕ_2, ϕ_3, ϕ_4, ϕ_5, and ϕ_x) are marked. (*Bottom*) Fractions analyzed in *Middle* were examined by SDS/PAGE under reducing conditions. Positions of the bands corresponding to the three constituent chains of fibrinogen (Aα, Bβ, and γ) and that of the crosslinked Aα·γ hybrid chain combination are marked.

In sharp contrast to the above profile, the monomeric product of fibrinogen isolated after the reaction with erythrocyte transglutaminase (Fig. 2, *Lower*, labeled "II") showed essentially a total depletion of Aα chains. Protein

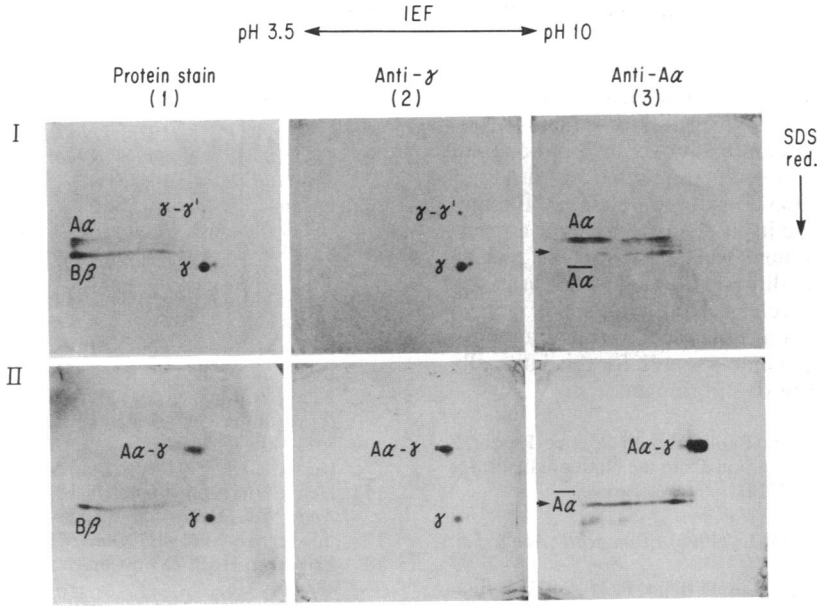

FIG. 2. Two-dimensional electrophoresis, coupled by immunoblotting with anti-Aα and anti-γ chain antibodies, demonstrates that the monomeric pool of protein reisolated after reaction with transglutaminase is, in fact, an internally crosslinked form of the fibrinogen. A buffer range from pH 3.5 (left) to pH 10 (right) was used for isoelectric focusing (IEF) in the first dimension. Separation vertically in the second dimension was carried out in SDS under reducing conditions. (*Upper*) Set I shows the pattern for fibrinogen before treatment with transglutaminase (control). (*Lower*) Set II presents the profiles for the monomeric pool of "fibrinogen" (i.e., fractions 67–74, from Fig. 1 *Top*) reisolated after reaction with transglutaminase. The nitrocellulose transblots were stained with amido black (*Left*) or were developed by immunostaining (*Center* and *Right*) by using antibodies either to the γ chains (*Center*) or to the Aα chains (*Right*) of fibrinogen. Chain assignments (see ref. 1) on the map are indicated, including the small amounts of γ-γ' dimers and the degraded Aα chain remnants (marked as $\overline{A\alpha}$ with an arrowhead) found as contaminants in the commercial fibrinogen preparation. Characteristically, the hybrid Aα·γ chain combination is present only in transglutaminase-treated fibrinogen (*Lower*).

staining (Fig. 2 *Lower Left*) and immunoblotting (Fig. 2 *Lower Center* and *Right*) confirmed that all Aα chains—with the exception of the broken $\overline{A\alpha}$ remnants—became crosslinked to γ chains, moving on the two-dimensional map to a position characteristic for a one-to-one crosslinked combination of Aα and γ chains (1). No higher orders of $A\alpha_p{\cdot}\gamma_q$ hybrids were present, which would have appeared as a ladder directly above the Aα·γ spot. A residual amount of γ chains remained presumably because heterologous crosslinking to intact Aα chain partners could not be fully accommodated because of the prior breakdown of Aα chains. As expected, the Bβ chains did not participate in the crosslinking event.

HPLC analysis of N^{ϵ}-(γ-glutamyl)lysine was then performed on the protein pool of interest after sequential digestion by proteases (subtilisin, pronase, carboxypeptidase Y, leucine aminopeptidase, prolidase, and again leucine aminopeptidase) according to published procedures (11). A minimum estimate of about 1 mol of bridge peptide per mol of fibrinogen was obtained for the transglutaminase-treated monomeric product, whereas no measurable amount of N^{ϵ}-(γ-glutamyl)lysine was recovered from the starting fibrinogen control. Apart from the report relating to the short single chain β-endorphin molecule (14), our finding may be an initial example for the intramolecular introduction of N^{ϵ}-(γ-glutamyl)lysine bridges into a protein of considerable size.

DISCUSSION

Human fibrinogen ($M_r \approx 340{,}000$) is comprised of three different constituent chains (Aα, Bβ, and γ) linked together by disulfide bonds into a doublet ensemble of $(A\alpha B\beta\gamma)_2$. The six N-terminal regions contain the interchain disulfides and form the central domain on the elongated structure of the protein. The C termini of the two shorter chains (Bβ and γ) are thought to be at the opposite ends of the molecule, whereas the C-terminal portions of the longest Aα chains loop back towards each other, reaching the central domain. In addition to localizing the interchain disulfide bridges in the amino acid sequence of the protein, electron microscopic observations serve as the basis for the above conclusions, (see refs. 15–20).

From the point of view of the physiological role of fibrinogen as a clotting substrate, the relationship of the flexible loop of the Aα chain to the body of the protein seems to be of great interest (21). Because of their exposed location and susceptibility to attack by proteases, the C-terminal loops of the Aα chains are often missing, leaving behind the broken $\overline{A\alpha}$ chain remnants as were seen in our preparation (Fig. 2 *Upper Right*). The question as to precisely which domains of the Aα chains become crosslinked to which sequence of the γ chains by transglutaminase or, for that matter, which chain contributes the reactive γ-glutaminyl and ε-lysyl crosslinking functionalities is yet to be determined. However, from the finding (Fig. 2, *Lower Right*) that the Aα-chain remnants (which were stained with an antiserum to the sequence of residues from 241 to 476) did not participate in the enzymatic crosslinking event, it could be inferred that the relevant intramolecular contact sites are located in the easily digestible portion of the Aα chain, distally from residue 477 towards the C terminus.

Preliminary observations indicate that intramolecular stabilization of fibrinogen through the transglutaminase-generated Aα·γ hybrid crosslinks renders this protein virtually incoagulable by thrombin. However, it still remains to be seen whether this might be due to a refractoriness towards thrombin for releasing fibrinopeptide A from the N terminus of the Aα chain in the central domain of the molecule or to the ensuing immobilization of the C-terminal portion of the Aα chain, which would prevent the swinging out of this flexible loop to approach another fibrin molecule.

When the $A\alpha\cdot\gamma$ and the higher $A\alpha_p\cdot\gamma_q$ hybrid chain combinations were first recognized in the reaction of fibrinogen with tissue transglutaminase (1), their formation was thought to be a special feature of the intermolecular event of the polymerization of fibrinogen. The results presented in this paper now reveal that, to a significant extent, production of the $A\alpha\cdot\gamma$ hybrids (but not of $A\alpha_p\cdot\gamma_q$) arises from the intramolecular crosslinking of two constituent chains of the monomeric fibrinogen molecule itself. Thus, whenever a tissue transglutaminase might escape from cells into the plasma environment where it could directly interact with fibrinogen (as caused by hemolysis or release from endothelial cells in atherosclerotic plaques or from cancer cells around tumors), diagnostic analysis should include a search for this internally crosslinked monomeric form of "fibrinogen."

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