MICROHETEROGENEITY OF CROSS-LINKED γ DIMERS ISOLATED FROM BOVINE STABILIZED FIBRIN

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SUMMARY: Bovine stabilized fibrin was reduced, carboxymethylated and separated by chromatography on a Sepharose 4B column. The fraction containing cross-linked γ dimers was then subjected to linear gradient chromatography on a CM-52 column. On this column, the γ dimers were separated into an adsorbed and unadsorbed fraction. The components in these fractions were designated as the γ-1 and γ-2 dimers. They each gave a single band on SDS-polyacrylamide gel electrophoresis and both had a molecular weight of 90,000 (±2,000). The identities of the γ-1 and γ-2 dimers were also shown by their amino acid compositions, terminal residues and tryptic and plasmic maps. However, they differed in electrophoretic mobilities on gels at pH 8.3 and pH 3.6 and in carbohydrate composition. The γ-1 dimer was slightly acidic and contained more hexoses and glucosamine than the γ-2 dimer. These results indicate that the characteristics of the bovine monomeric γ chains, γ-1 and γ-2, previously reported by Gerbeck et al., are transferred to their corresponding cross-linked γ dimers, formed in the stabilization of fibrin.

Recently, many workers have reported that the three pairs of subunit polypeptides, $A\alpha_2$, $B\beta_2$ and γ_2, which build up the fibrinogen molecule of 340,000 unit weight, are heterogeneous chromatographically and electrophoretically (1-6). The existence of two chromatographically different γ chains in bovine fibrinogen was first reported by Gerbeck et al. (2). A similar heterogeneity of human γ chains was reported by Henschen

and Edman (4). Mosesson et al., on the other hand, reported that the two types of human fibrinogen, which could be separated by chromatography on DEAE-cellulose, resulted from the presence of a γ chain variant in one of the protein molecules (5, 6). These data suggest that stabilized fibrin probably may contain two γ dimers, which are distinguishable chromatographically and electrophoretically. The present results demonstrate their existence.

MATERIALS AND METHODS

Fibrinogen was prepared from bovine plasma containing citrate by the method of Blombäck and Blombäck (7). The coagulability of the preparation, as determined spectrophotometrically, was about 98 %. Bovine thrombin (Topical, Park Davis Co.) was purified by the method of Lundblad (8).

Factor XIII was prepared from bovine plasma by the method of Loewy et al. (9) and after heat treatment at 56°C for 3 min was used as the enzyme preparation. Sepharose 4B (Lot, 3765) and Whatman CM-52 were products of Pharmacia, Uppsala, Sweden and W.R. Balston, Ltd., England, respectively.

Stabilized fibrin was prepared as follow: 900 ml of 2.7 % fibrinogen in 0.05 M veronal buffer, pH 7.6, 500 NIH units of thrombin, 1 ml of Factor XIII (0.9 mg/ml), 14 mmoles of L-cysteine and 14 mmoles of $CaCl_2$, in a total volume of 1,182 ml, were incubated at room temperature. After incubation for 6 hrs, the resulting stabilized fibrin was collected, washed twice with 1 liter of 0.05 M veronal buffer, pH 7.6, and with 2 liters of distilled water and lyophilized. One gram of lyophilized material was suspended in 8.4 M guanidine-HCl at pH 8.5, and reduced and carboxymethylated by the method of McDonagh et al.(10). The resulting alkylated sample was

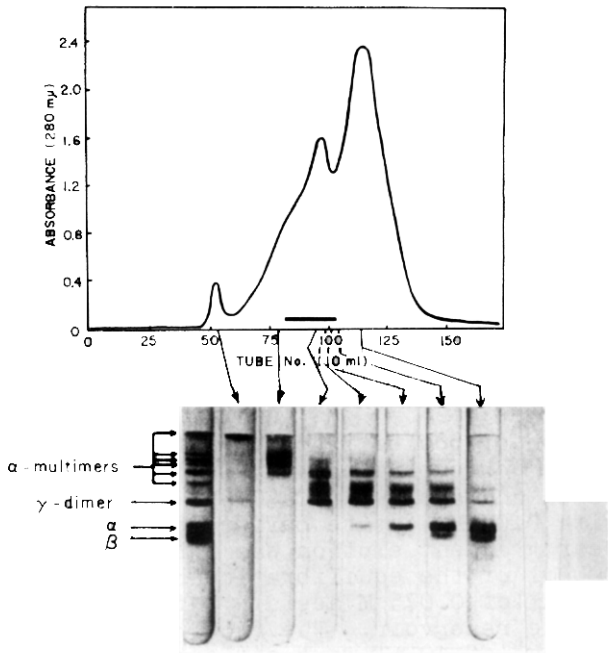

Fig. 1. Separation of cross-linked chains of bovine stabilized fibrin on a Sepharose 4B column. A carboxymethylated sample (800 mg) dissolved in 8 M urea was applied to a column (5.5 x 60 cm) of Sepharose 4B, equilibrated with 0.05 M acetate buffer, pH 5.2, containing 8 M urea. Elution was performed with the equilibration buffer at a flow rate of 60 ml per hr and 10 ml fractions were collected at room temperature. Absorbancy at 280 mμ was measured with a Hitachi, model 124, spectrophotometer. SDS-polyacrylamide gel (5 %) electrophoresis was done essentially by the method of Weber and Osborn (14). Electrophoresis was performed at room temperature at a constant current of 7.0 mA per tube for 4 hrs. During this time the marker dye, bromphenol blue migrated to within 50 mm from the bottom of the 60 mm long gel. The gels were stained with Coomassie brilliant blue and destained with 10 % trichloroacetic acid. Migration was from top (cathode) to bottom (anode). The pattern of reduced cross-linked fibrin showing all the γ chains as γ dimers and most of the α chains as a variety of α multimers, was shown on the left.

used as the preparation of cross-linked γ dimers.

RESULTS

Separation of Cross-linked γ Dimers

The elution pattern of alkylated cross-linked fibrin on agarose chromatography is shown in Fig. 1, with the electrophoretic patterns of various fractions of elute on SDS-poly-

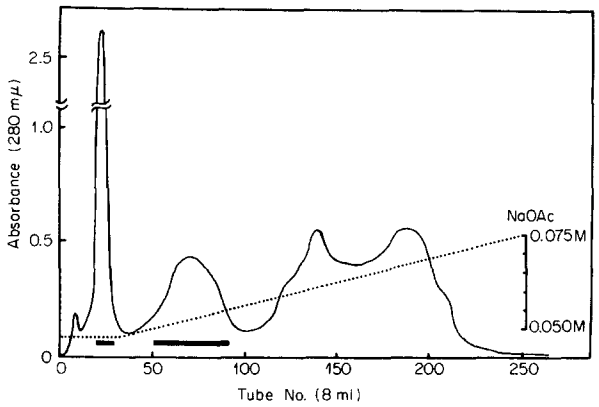

Fig. 2. Separation of pooled fractions of γ dimers on a CM-52 column. Fractions corresponding to tube numbers 81 to 103 in Fig. 1 were pooled and applied to a CM-52 column (2.7 x 22 cm), equilibrated with 0.05 M acetate buffer, pH 4.8, containing 8 M urea. Linear gradient elution was performed at room temperature with 1,000 ml of the equilibration buffer in the mixing vessel and 1,000 ml of 0.075 M acetate buffer, pH 6.0, containing 8 M urea in the reservoir. The flow rate was about 80 ml per hr and fractions of 10 ml were collected at room temperature.

acrylamide gel. On electrophoresis, all the α multimers were separated into the first peak and the front shoulder of the second peak. The main component of the second peak consisted of γ dimer, with small amounts of α multimers and monomeric β chain. The last peak contained mainly β chain, which is not cross-linked in the formation of stabilized fibrin. The fractions shown as a solid bar in Fig. 1 were combined, dialyzed for two days against distilled water, and lyophilized. The material thus obtained was dissolved in 0.05 M acetate buffer containing 8 M urea and subjected to chromatography on a CM-52 column. The elution pattern is shown in Fig. 2. The first large peak contained only the γ dimer, as judged by its mobility on SDS-gel. The second peak also showed the presence of a γ dimer-like component. The two fractions had almost the same total extinction at 280 mμ, indicating that they contained equivalent amounts of protein. These components were desig-

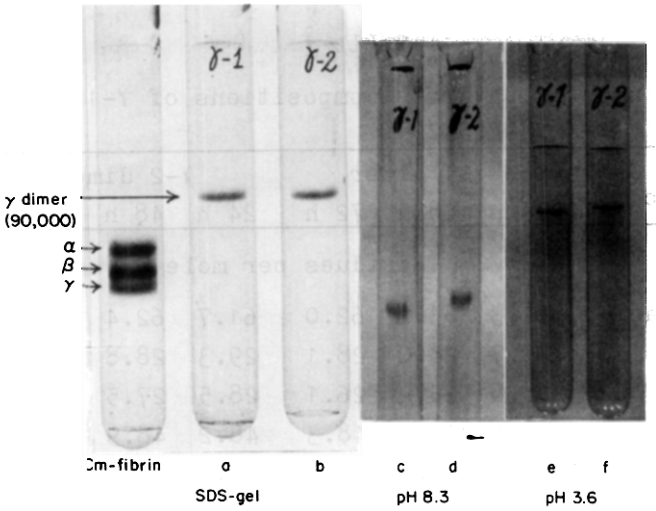

Fig. 3. Disc polyacrylamide gel electrophoresis of isolated γ dimers under various conditions. Electrophoreses (a) and (b) were carried out under the same conditions as those described in Fig. 1. Electrophoreses (7 % gel, 8 M urea) (c) to (f) were carried out at 4°C at 3 mA per tube for 8 hrs as in previous work (15).

nated γ-1 and γ-2, since their chromatographic behaviors were the same as those of monomeric γ-1 and γ-2 found in bovine fibrinogen by Gerbeck et al. (2). The two fractions shown as solid bars in Fig. 2 were collected, dialyzed extensively against distilled water and lyophilized.

Fig. 3 shows the patterns of dimeric γ-1 and γ-2 on electrophoresis under different conditions. They showed identical mobilities on SDS-gels, indicating that both their molecular weights were 90,000 (±2,000). However, on disc gel electrophoresis at pH 8.3, they showed slightly different mobilities, dimeric γ-1 moving further to the anode than dimeric γ-2 (Fig. 3c and d). Their electrophoretic mobilities at pH 3.6 were also different (Fig. 3e and f).

Characterizations of γ-1 and γ-2 Dimers

The terminal residues, amino acid and carbohydrate compositions of the γ-1 and γ-2 dimers were determined. On Edman

Table I

Amino acid and carbohydrate compositions of γ-1 and γ-2 dimers

Amino acid	γ-1 dimer			γ-2 dimer		
	24 h	48 h	72 h	24 h	48 h	72 h
	Residues per mole					
Aspartic acid	61.3	63.2	62.0	61.7	62.4	61.7
Threonine	29.7	28.0	28.1	29.3	28.8	28.5
Serine	28.8	26.9	26.1	28.5	27.5	26.6
Glutamic acid	48.6	47.5	48.3	48.2	48.9	48.3
Proline	11.4	11.9	11.7	11.5	11.9	12.2
Glycine	37.6	36.8	37.3	38.2	38.2	32.8
Alanine	20.9	20.6	21.5	21.3	21.1	21.2
1/2 Cystine	10.6	10.3	10.7	10.5	10.2	10.3
Valine	17.8	19.5	20.7	17.5	17.5	19.7
Methionine	6.8	6.7	6.9	7.0	6.8	7.3
Isoleucine	23.7	24.6	24.9	23.5	24.4	24.6
Leucine	26.7	26.7	26.6	26.2	26.5	26.3
Tyrosine	22.4	22.3	22.2	22.7	22.2	22.7
Phenylalanine	16.6	16.6	16.7	16.7	16.5	16.3
Histidine	8.4	8.5	8.7	8.5	8.6	8.7
Lysine	30.4	30.0	31.5	30.0	30.7	31.2
Arginine	13.8	14.9	12.2	14.8	14.3	14.4
Hexoses	Residues per mole					
Orcinol-H_2SO_4	11.4			8.8		
Phenol-H_2SO_4	10.7			9.0		
Glucosamine	9.4			8.0		

The material (1-1.2 mg) was hydrolyzed for the indicated times in 1.0 ml of 5.7 N HCl at 110°C in an evacuated tube. Excess HCl was removed over KOH pellets *in vacuo*, and the residue was dissolved in 3 ml of 0.2 M sodium citrate buffer, pH 2.20. The analysis was performed with an amino acid analyzer, model JLC-5AH, Japan Electron Optics Lab. Ltd., by the method of Spackman *et al.* (16). Hexoses were determined by the methods of Winzler (17) and Dubois *et al.* (18), and hexosamine was determined by the method of Gardell (19).

degradation (11), both dimers were found to have amino-terminal tyrosine, and they both liberated carboxyl-terminal valine on hydrazinolysis (12). The amino acid compositions of the two γ dimers were also indistinguishable (Table 1). However, they differed in carbohydrate composition. On analysis of duplicate samples using two different methods, dimeric γ-1 was consistently found to contain more hexoses than γ-2 (Table 1). It also contained more hexosamine. Peptide maps of tryptic and plasmic digests of the γ-1 and γ-2 dimers did not show any difference in the locations of ninhydrin positive peptides. Thus, the amino acid sequences of the γ-1 and γ-2 dimers seem to be identical.

DISCUSSION

As reported by Gerbeck et al. (2), bovine fibrinogen contains two types of monomeric γ chain, γ-1 and γ-2. These chains are distinguishable by column chromatography on CM-cellulose and by disc electrophoresis on polyacrylamide gel. It is of interest to know which types of monomeric γ chains are cross-linked with each other in formation of stabilized fibrin. Three cross-linkages are possible: γ-1-γ-1, γ-2-γ-2 and γ-1-γ-2. The results obtained here indicate that the third type does not occur. The evidence for this is as follows:

1. The γ dimers isolated from alkylated cross-linked fibrin were separated into adsorbed and unadsorbed fractions on a CM-cellulose column, and their chromatographic behaviors were the same as those of the two monomeric γ chains reported by Gerbeck et al..

2. The isolated γ-1 and γ-2 dimers differed in electrophoretic mobilities on gels at pH 8.3 and 3.6. The γ-1 dimer was somewhat more acidic than the γ-2 dimer. These properties are the same as those of monomeric γ-1 and γ-2.

3. The characteristics of monomeric γ-1 of containing relatively more hexoses and glucosamine than monomeric γ-2, are the same as those of the corresponding γ-1 dimer. These results may be related to the chromatographic heterogeneity of cross-linked bovine fibrin observed by Chen and Doolittle (13).

Differing from the findings of Gerbeck et al., we could not detect any difference in the amino acid compositions of the γ-1 and γ-2 dimers. Moreover, peptide maps of tryptic and plasmic digests from the two dimers were indistinguishable. Thus the amino acid sequences of dimeric γ-1 and γ-2 were probably identical.

It should be noted that the characteristics of monomeric γ-1 and γ-2 were transferred to their respective dimers formed on stabilization of bovine fibrin. This means that in the cross-linking of monomeric γ chains pairs of γ-1 or pairs of γ-2 must be linked covalently with each other. Assuming the existence of a pseudo-identical γ pair in a single molecular species, it also seems likely that the cross-linking between monomeric γ chains must be formed intermolecularly in fibrin molecules.

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