HYBRID FIBRIN: PROOF OF THE INTERMOLECULAR NATURE OF γ-γ CROSSLINKING UNITS

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SUMMARY: Proof that the γ-γ bonding system introduced during the enzymatic crosslinking of mammalian fibrin is intermolecular (as opposed to intramolecular) has been obtained by clotting a mixture of bovine and human fibrinogens under conditions which allow crosslinking and the incorporation of a radioactive substitute donor to occur simultaneously. The formation of a hybrid human-bovine γ-γ crosslinking unit was demonstrated electrophoretically, advantage being taken of the difference in mobilities of the human and bovine tryptic fragments involved.

The covalent crosslinking of vertebrate fibrin involves at least two different combinations of the polypeptide chains which make up the individual fibrin monomers (1). In one situation, anti-parallel oriented γ-chains are reciprocally linked by two ε-(γ-glutamyl)lysine crossbridges near their carboxy-terminals (2). In the second kind, which is formed at a considerably slower rate, α-chains are linked to each other in a multimeric pattern (3). McKee et al. (3) have suggested that the γ-γ dimers might be the result of internal crosslinks between γ-chains of the same fibrin monomer (structural formula = $\alpha_2\beta_2\gamma_2$), especially since the introduction of γ-γ crosslinks has only a slight influence on the urea-solubility of fibrin gels. We have argued (4), on the other hand, that the γ-γ crosslinking segments are not only intermolecular, but that they may also be the contact regions involved in the initial fibrin polymerization process.

A way of proving the intermolecular nature of γ-γ crosslinking arose when we found that human and bovine γ-chains have slightly different amino acid sequences in the crosslinking segment (2). Thus, the human γ-chain carboxy-terminus heptadecapeptide has a threonine at position 2

where the bovine has an alanine, and a histidine at position 10 where the bovine has a glutamine. The latter change results in the isolated crosslinking peptides from these two species having different electrophoretic mobilities at low pH. We reasoned that if a mixture of human and bovine fibrinogens were clotted and crosslinked, we might be able to isolate a hybrid crosslinked unit of intermediate electrophoretic mobility if γ-γ crosslinking is indeed intermolecular.

Originally, we attempted to identify the hybrid crosslinking unit on the basis of amino acid composition. Although we were successful in isolating a peptide of the expected characteristics from a mixed fibrin clot, the precision of the experiment, involving as it did the measuring of rather small differences in the number of residues, was marginal. Furthermore, it was difficult to distinguish between simple mixtures of the human and bovine crosslinked units and the true hybrid. There was

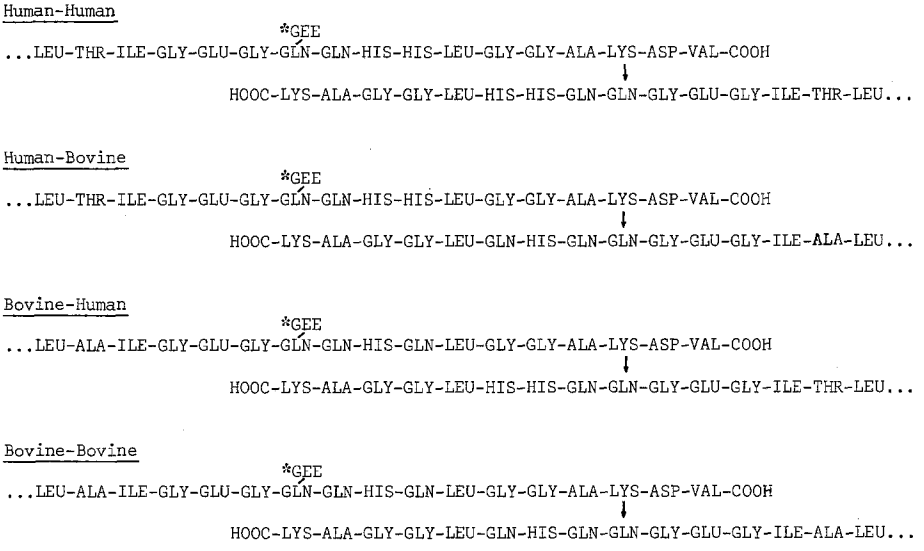

Fig. 1. Structures of anticipated radioactive linked donor-acceptor units. *GEE = radioactive glycine ethyl ester incorporated as a substitute donor. The crosslinking enzyme (activated factor XIII) also introduces some spurious labeling at the glutamine adjacent to the main site of substitution (4). Arrows represent amide bonds between lysine and glutamine sidechains. In systems formed in the absence of substitute donors two reciprocal ε-(γ-glutamyl)lysine crossbridges are formed eight residues apart (4).

also a certain degree of ambiguity introduced by the fact that the cross-linking seldom proceeds to absolute completion and some units are linked by only one ε-(γ-glutamyl)lysine crossbridge instead of two (2), leading to more electrophoretic complications.

Accordingly, we undertook a slightly different approach, taking advantage of the fact that it is possible to isolate crosslinking units comprised of linked chains, but in which one of the acceptor sites has a radioactive substitute donor instead of its natural crosslink (2). The structures of the kinds of linked units which could ensue from such an experiment are shown in Fig. 1. An outline of the experimental strategy is depicted in Fig. 2.

EXPERIMENTAL

Detailed procedures for each of the steps involved have been described in two previous articles (1,2). ^{14}C-glycine ethylester hydrochloride was purchased from New England Nuclear and was cold-diluted with ^{12}C-glycine ethylester for use as a substitute donor (5).

Solutions of purified bovine and human fibrinogen were adjusted to the same concentrations (7.7 mls each at A_{280} = 23.5) and 5.1 mls of 0.15 M glycine ethylester (GEE) solution containing 100 μC of ^{14}C-GEE were added to each. Half of each of these GEE-fibrinogen solutions were then mixed with each other, the remainders being set aside for separate clotting for the control preparation. An equal volume of a thrombin-$CaCl_2$-cysteine solution was added to each of the three GEE-fibrinogen solutions (human, bovine and mixed human-bovine). In each case the final conditions were: fibrinogen, 5 mg/ml; thrombin (Parke-Davis), 0.05 mg/ml; $CaCl_2$, 0.025 M; cysteine, 0.0125 M; glycine ethylester, 0.03 M; KCl, 0.1 M: pH, 7.0. The experimental hybrid mixture contained 60 mg of each kind of fibrinogen. In the control case, 60 mg of each fibrinogen was clotted separately.

The gels were let stand at room temperature for 40 minutes before the clot liquors were squeezed out and the fibrin washed with water. The

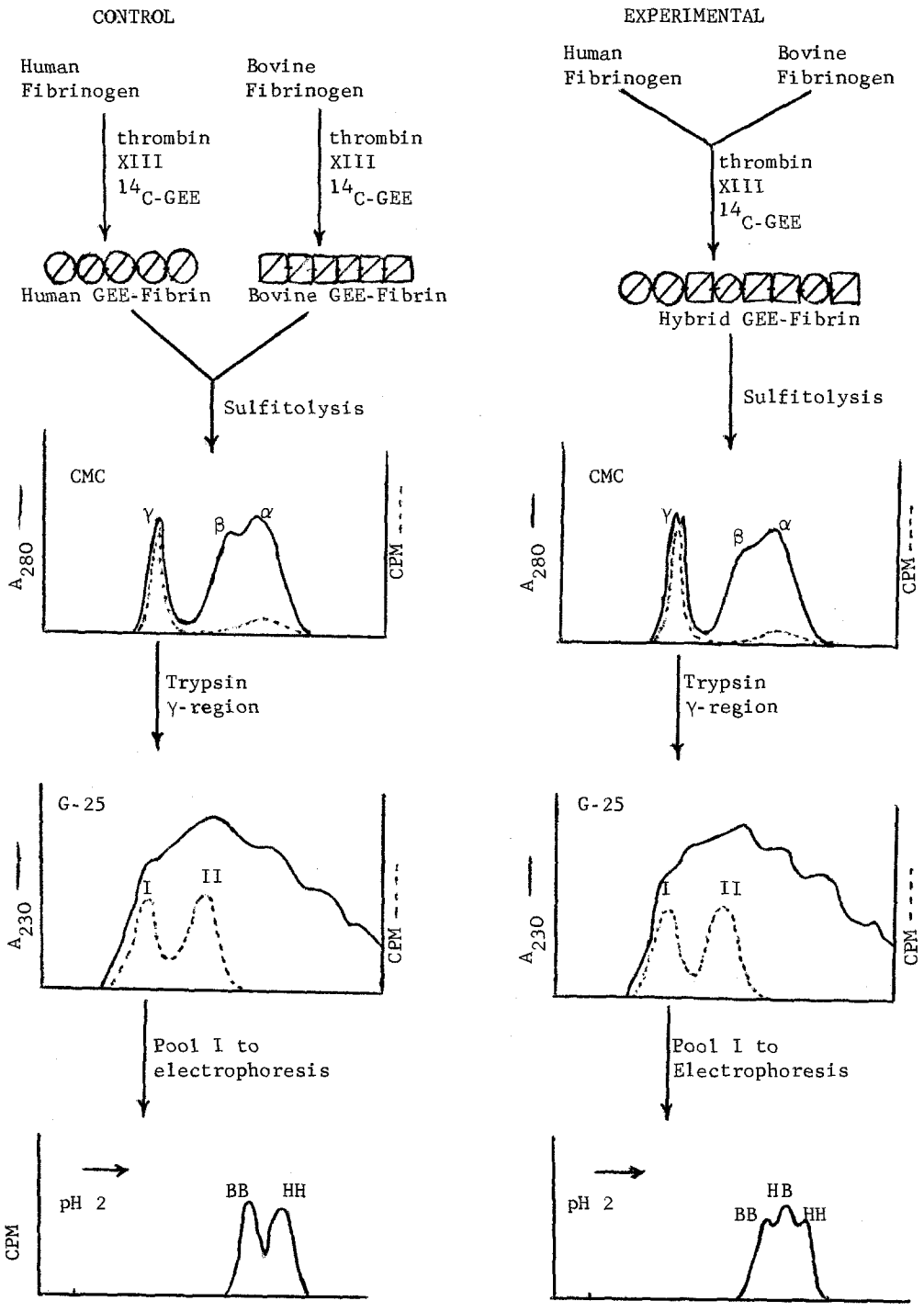

Fig. 2. Experimental strategy for proving intermolecular nature of γ-γ crosslinking.

fibrin was dispersed as well as possible in 8 M urea and then subjected to sulfitolysis, at which point the human and bovine controls were mixed. After thorough dialysis the two preparations (Experimental and Control) were freeze-dried. They were subsequently reconstituted in sodium acetate buffer, pH 5.2, containing 8 M urea, and subjected to carboxymethyl cellulose (CMC) chromatography. Effluent fractions were monitored by their absorbance at λ = 280 mμ and aliquots counted to locate radioactive peaks. In each case the γ-chain regions (containing both linked and unlinked γ-chains) were pooled, dialyzed extensively against distilled water and freeze-dried. The two preparations (Experimental and Control) were then digested with trypsin and the digests applied directly to G-25 columns (2.5 x 60 cms). As in our previous experiments (2,4), two radioactive pools were identified, the first of which corresponded to linked peptides and the second to non-linked acceptor peptides.

The two radioactive peaks from each preparation (referred to hereafter as E-I, E-II, C-I and C-II) were pooled, freeze-dried and re-dissolved in 0.2 mls H_2O each. Aliquots containing about 4000 CMP (40 μL) were applied to paper strips (4 x 41 cms) and electrophoresed at pH 2, 300 V, for 3 or 4 hours. After drying, the strips were scanned in a Packard Model 7201 Strip Counter. An inventory of the radioactivity distribution throughout the experiment is presented in Table I.

RESULTS AND DISCUSSION

In both preparations, electrophoresis of the more retarded peaks from G-25 (C-II and E-II) yielded two sharp radioactive spikes corresponding to the individual 15-residue human and bovine acceptor peptides (Fig. 3). The leading peak from G-25 from the control preparation (C-I) also displayed two peaks upon electrophoresis, although the linked peptide systems do not resolve quite as well as the individual acceptor peptides (Fig. 3). In the case of the leading G-25 peak from the experimental preparation (E-I), however, the valley between the two peaks was occupied by another radio-

TABLE I

INVENTORY OF RADIOACTIVITY RECOVERED THROUGHOUT EXPERIMENT[a]

	Control	Experimental
^{14}GEE incorporated into fibrin (as applied to CMC columns)	196,000 CPM	174,000 CPM
γ-chain pools (digests applied to G-25 columns)	68,000 "	64,000 "
Pool I from G-25	20,000 "	21,000 "
Pool II from G-25	26,000 "	21,000 "
Applied to each electrophoresis strip	~ 4,000 "	~ 4,000 "

[a]The starting fibrinogen solutions contained 100 μC each of ^{14}C-glycine ethylester, but only a small fraction of this material is incorporated into the fibrin. All counting was performed in a Beckman LS-200B liquid scintillation counter.

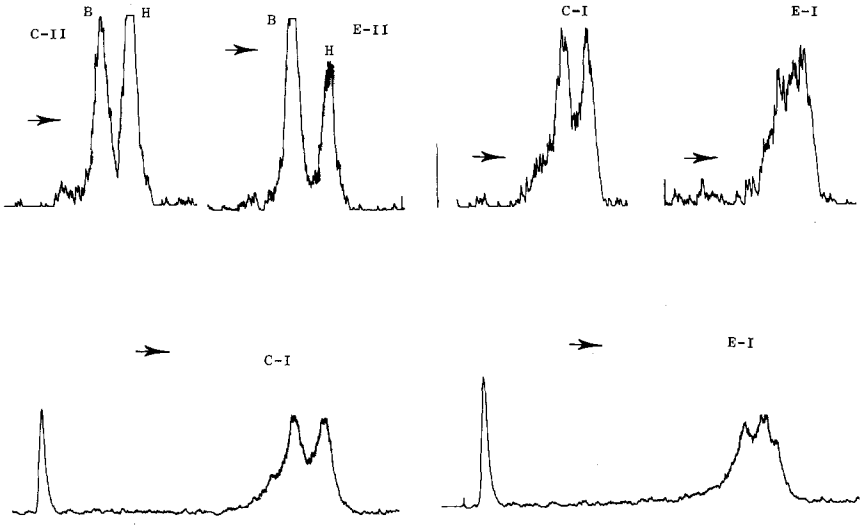

Fig. 3. Radioactivity scans of paper electrophoresis strips. Top series: Three-hour electrophoresis runs of C-II, E-II, C-I and E-I. Scanning slit = 1 mm, scanning speed = 1 cm/min, linear range = 0-300 CPM, time constant = 10 sec. Only the region of the peaks is shown, but no other peaks were present. Bottom: Four-hour electrophoresis runs of C-I and E-I. Scanning slit = 1 mm, scanning speed = 0.1 cm/min, linear range = 0-300 CPM, time constant = 100 sec. ^{3}H-inulin was applied as an origin marker after the electrophoresis and appears at left ends of scans.

active band, its intermediate mobility corresponding to the human-bovine hybrid crosslinked unit (Fig. 3). Identical results were obtained for three separate sets of electrophoretic comparisons.

These observations can only be reconciled with intermolecular cross-linking between γ-chains of adjacent molecules in the fibrin gel. If the arrangement of human and bovine monomers in the gel were completely random, it would be expected that there would be twice as much of the hybrid (HB = BH) as there is of either of the other linked pairs. The data indicate that the three types (human-human, hybrid and bovine-bovine) are all present in approximately the same amounts, however, suggesting some slight preferential specificity favoring homologous pairing at either the assembly or crosslinking steps.

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REFERENCES

1. Chen, R. and Doolittle, R. F., Proc. Natl. Acad. Sci., U.S., 63:420 (1969).
2. Chen, R. and Doolittle, R. F., Proc. Natl. Acad. Sci., U.S., 66:472 (1970).
3. McKee, P. A., Mattock, P. and Hill, R. L., Proc. Natl. Acad. Sci., U.S., 66: 738 (1970).
4. Chen, R. and Doolittle, R. F., manuscript submitted for publication, April,1971.
5. Lorand, L. and Jacobsen, A., Biochemistry, 3:1939 (1964).