*IDENTIFICATION OF THE POLYPEPTIDE CHAINS INVOLVED IN THE CROSS-LINKING OF FIBRIN**

By Renné Chen and Russell F. Doolittle

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA (SAN DIEGO)

Communicated by Bruno H. Zimm, April 7, 1969

Abstract.—The stabilization of fibrin clots by activated factor XIII involves two different sets of cross-linked chains. In one case (type I) two γ-chains are linked to each other, indicating that γ-chains have both donor (suitable lysyl-) and acceptor (suitable glutaminyl-) functions. A second system (type II) consists of a γ-chain linked to an α-chain. Experiments with a substitute donor (glycine ethylester) indicate that only γ-chains have enzyme-accessible acceptor sites, suggesting that α-chain participation is limited to lysyl side chains. A model molecular arrangement for fibrin has been suggested which accommodates all the data.

The vertebrate fibrinogen molecule in its native form is generally presumed to be a dimer, each half molecule of which is made up of three nonidentical polypeptide chains.[1] For every 330,000 mol wt, the formula $\alpha_2\beta_2\gamma_2$ applies. The transformation of these molecules into fibrin gels is a self-assembly process resulting directly from the thrombin-mediated removal of the fibrinopeptides A and B from the amino-terminals of the α and β chains, respectively. Under appropriate conditions, and most assuredly *in vivo*, the gel is reinforced by the introduction of covalent cross-links; this process is catalyzed by the thrombin-activated transamidase known as factor XIII.[2] The formation of these cross-links has been laid to a donor (lysine ϵ-amino group) and an acceptor (presumably a glutaminyl side chain) forming ϵ-(γ-glutamyl) lysine isopeptide links.[3–5] Until now, nothing was known as to which of the three classes of constituent polypeptide chains were involved in these cross-links. The subject is of considerable interest since there is apparently little or no rearrangement of the individual molecules in the conversion of non-cross-linked to cross-linked fibrin, and therefore any information about which chains are in contact with which chains in the cross-linked fibrin should also hold for the fibrin formed by self-assembly. This information in turn should tell us something about the arrangement of polypeptide chains in the original parent fibrinogen molecules.

In this article we present evidence for two kinds of interchain involvement. In the first kind, γ-chains are hooked to other γ-chains, each subunit apparently containing both a donor and an acceptor site. In the second category, γ-chains are hooked to α-chains. We also present evidence that only γ-chains possess acceptor sites; presumably the α-chains are participating only through lysyl ϵ-amino groups.

Ordinarily, cross-linked fibrin is distinguished from non-cross-linked fibrin experimentally by the insolubility of the cross-linked material in dispersing agents such as concentrated urea or guanidine solutions or dilute acid. After complete disruption of the disulfide linkages by sulfitolysis, however, even

cross-linked fibrin can be dispersed by 6 *M* urea. The experiments that we report here depended on the separation of the larger cross-linked subunits from their unlinked neighbors by gel filtration on Sephadex G-200 in 6 *M* urea and on the separation of the two classes of linked types on carboxymethyl cellulose in 8 *M* urea.

Methods.—Bovine fibrinogen (Pentex) was further purified by the method of Laki.[6] These preparations are 93–96% clottable and contain factor XIII in such amounts that they can be fully cross-linked by the addition of thrombin, cysteine, and calcium ion. Bovine thrombin was obtained from Parke-Davis; in some experiments we used a preparation given us by Dr. S. Magnusson. In order to obtain fully cross-linked fibrin, we prepared a mixture which contained fibrinogen (5 mg/ml), thrombin (2 U.S. units/ml), cysteine (0.0125 *M*), and calcium ion (0.025 *M*). In the case of non-cross-linked fibrin, the cysteine-calcium ion contribution was replaced by an equivalent amount of sodium chloride solution of appropriate ionic strength. In all cases the degree of cross-linking was checked by measuring the solubility in 8 *M* urea and 2% monochloroacetic acid. Fibrin clots, either cross-linked or non-cross-linked, were washed well with water, snipped into small pieces, dispersed in 8 *M* urea, and subjected to sulfitolysis.[7–9] The S-sulfo-fibrins were dialyzed extensively[7] and freeze-dried. Carboxymethyl cellulose (CMC) (Whatman CMC-52) was soaked in 0.1 *M* sodium acetate, pH 5.2, for at least 2 hr and then washed with water and starting buffer (0.005 *M* sodium acetate, pH 5.2, in 8 *M* urea). Columns (2 × 11 cm) were packed by gravity and equilibrated with at least 300 ml of starting buffer; the pH and conductivity of the effluent buffer were checked before use. About 40 mg of the S-sulfo-proteins was dissolved in 2.0 ml of starting buffer and dialyzed against the same buffer before application. After application of the sample, the column was developed with 50 ml of starting buffer followed by a linear concentration gradient made with equal volumes (300 ml each) of starting and limit buffer (0.1 *M* sodium acetate, pH 5.2, in 8 *M* urea). After elution, the peaks (absorbance at 280 mμ) were pooled, dialyzed extensively against distilled water, and freeze-dried. Sephadex G-200 columns (2.5 × 40 cm) were packed from 50% slurries made with 6 *M* urea. Samples were usually dissolved in 8 *M* urea and diluted to 6 *M* before application to the G-200 columns. Both the G-200 and CMC columns were developed at room temperature. Aliquots from the various pools were hydrolyzed *in vacuo* with constant boiling hydrochloric acid at 110° for 24 hr. Amino acid analyses were performed on a modified (long light path) Spinco 120B amino acid analyzer. Amino-terminal amino acids were determined by the phenylthiohydantoin method of Edman.[10]

^{14}C-glycine ethylester was obtained from New England Nuclear; 2.2 mg (50 μc) was dissolved in 4.0 ml of 0.15 *M* ^{12}C-glycine ethylester solution, pH 7.5. The specific activity of this solution was 0.081 μc/μM. The diluted radiolabeled material was mixed with a fibrinogen (100 mg) solution; and cysteine, calcium ion, and thrombin were added in the usual manner for producing cross-linked fibrin. The final concentration of glycine ethylester was 0.03 *M*. After 40 min, the clot was washed with water and subjected to sulfitolysis and CMC chromatography. Aliquots (1.0 ml) of the effluent were counted in 10 ml of scintillation fluid (Triton-X-100:Liquifluor:Toluene = 20:3:37)[11] on a Beckman LS-233 scintillation counter.

Results.—Carboxymethyl cellulose: Sulfitolyzed non-cross-linked fibrin can be resolved into its constituent γ-, β-, and α-polypeptide chains by chromatography on carboxymethyl cellulose conducted in 8 *M* urea (Fig. 1, *bottom*). Amino acid composition studies and end group analysis on the various pooled peaks yielded values consistent with those previously reported by Henschen.[12] The elution pattern of sulfitolyzed cross-linked fibrin was markedly different, however, and not readily interpretable (Fig. 1, *top*). About one third of the "γ-chain peak" disappeared in all runs, and the β- and α-chains, which are difficult to separate

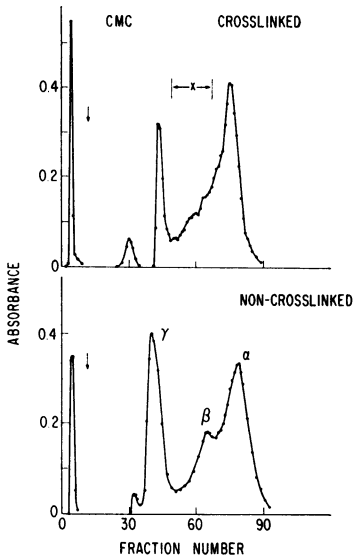

FIG. 1.—Carboxymethylcellulose chromatography of sulfitolyzed cross-linked (*top*) and non-cross-linked (*bottom*) fibrins. Absorbance read at 280 mμ. Fraction size = 5.9 ml. Arrow designates start of linear concentration gradient of sodium acetate buffer, pH 5.2.

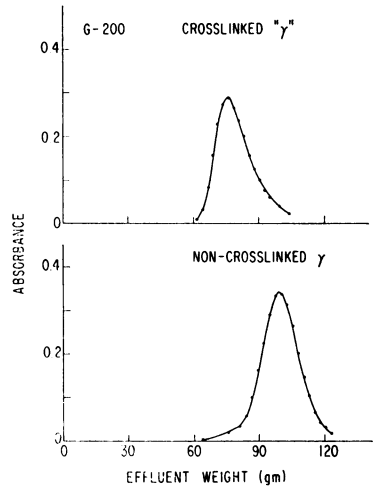

FIG. 2.—Gel filtration on Sephadex G-200, conducted in 6 *M* urea, of γ-chain peaks from CMC. *Top:* γ-chain peak from cross-linked fibrin (2.5 × 41-cm column); *bottom:* γ-chain peak from non-cross-linked fibrin (2.5 × 41-cm column).

under any conditions, became more indistinguishable. An additional shoulder in this region (designated *X* in Fig. 1) obscured the normal profile of the non-cross-linked type. Apparently the material disappearing from the γ-chain peak was reappearing in the *X* region.

Sephadex G-200: When cross-linked and non-cross-linked sulfitolyzed fibrins were compared on Sephadex G-200 in the presence of 6 *M* urea, the cross-linked material clearly contained more large molecular weight material than the non-cross-linked did. The presence of a large molecular weight contaminant (presumably incompletely sulfitolyzed fibrinogen) which eluted early during chromatography on CMC in the case of cross-linked and non-cross-linked fibrins alike, made the results difficult to quantitate and the assignment of chain identities impossible. Consequently, each of the pools from CMC chromatography (from both the cross-linked and non-cross-linked preparations) was subjected to the gel filtration procedure.

Two different pools from the CMC-chromatographed cross-linked fibrin yielded large molecular weight components upon G-200 gel filtration: the γ-peak (Fig. 2) and the *X* peak (Fig. 3). We have designated these two situations as type I and type II cross-linked systems, respectively. The type I material, which elutes at the same position as non-cross-linked γ-chains on CMC, is in fact comprised of cross-linked γ-chains, probably two in number. This conclusion is based on its amino acid composition (Table 1) and the presence of

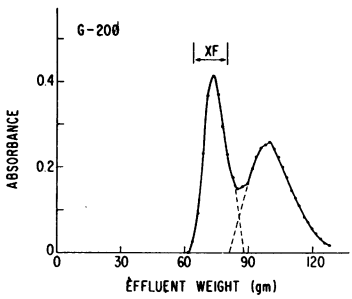

FIG. 3.—Gel filtration on Sephadex G-200 (2.5 × 41 cm), conducted in 6 *M* urea, of *X* peak from CMC chromatography of cross-linked fibrin (Fig. 1). The region designated *XF* was subjected to amino acid analysis.

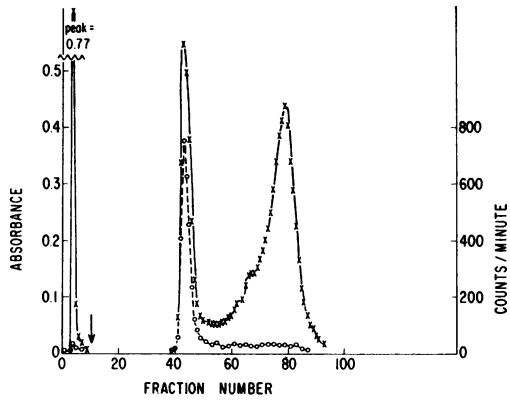

FIG. 4.—CMC chromatography of sulfitolyzed fibrin which had been clotted in the presence of ^{14}C-glycine ethylester. *Solid line*, absorbance at 280 mμ; *broken line*, radioactivity.

tyrosine as its only amino-terminal amino acid. That there are only two chains in each unit is a surmise based on the observation that the material is slightly included on the G-200 columns and elutes a little behind unsulfitolyzed fibrinogen. It is important to note that no free γ-chains were present in cross-linked fibrin (Fig. 2). The type I (γ- γ) material corresponds to two thirds of the constituent γ-chains (Table 2). The type II system accounts for the one third of the γ-chains which disappeared on CMC chromatography. This latter cross-linked system is apparently made up of a γ-chain linked to an α-chain, its amino acid composition being intermediate between those of the two individual chains (Table 1). The constituent chains are most readily distinguished by

TABLE 1. *Basic amino acid compositions of chains and chain systems from cross-linked and non-cross-linked fibrins.**

	Chain from non-cross-linked fibrin			Observed for cross-linked chain system	
	γ	β	α	Type I	Type II
Lysine	54.6 ± 0.3‡ (58.4)§	48.3 ± 0.3 (50.4)	46.8 ± 0.5 (44.4)	55.3 ± 0.9	50.4 ± 0.9
Histidine	15.4 ± 0 (13.3)	9.6 ± 0.3 (10.4)	11.9 ± 0.8 (10.5)	15.4 ± 0.2	14.4 ± 0.6
Arginine	30.3 ± 0.3 (28.3)	42.1 ± 0.4 (39.1)	41.3 ± 0.9 (45.2)	29.3 ± 1.0	35.2 ± 0.9

	Calculated for various combinations†		
	γ − α	γ − β	α − β
Lysine	50.7	51.5	47.6
Histidine	13.7	12.3	10.8
Arginine	35.7	33.7	41.7

* Values are based on three to five separate determinations including at least two different preparations of each and are given in mole % basic amino acids.

† Calculated values are simple means based on our values for individual chain compositions.

‡ Standard deviation.

§ Values in parentheses have been calculated from data of Henschen[12] who has previously reported amino acid compositions of isolated chains from bovine fibrin.

TABLE 2. *Percentage of total protein* included at γ position on CMC.*

Run no.	Fibrinogen	Non-cross-linked fibrin	Cross-linked fibrin
1	31.3	33.4	24.0
2	28.4	29.8	18.8
3	30.1	31.0	20.7
4	—	30.4	19.7
5	—	29.7	23.5
Mean	29.9 ± 1.5†	30.9 ± 1.5	21.3‡ ± 2.3

* Not including the material which passed through before start of gradient.
† Standard deviation.
‡ The mean amount of γ position material in cross-linked fibrin = 68.9% of the mean amount from non-cross-linked fibrin, or approximately two thirds.

their basic amino acid compositions. Since one third of the γ-chains are hooked to an equal number of α-chains, it is apparent that there are equal numbers of the two kinds of cross-linked chain systems.

Identification of the acceptor chains: Although in the case of type I systems it is obvious that the γ-chains must possess both donor and acceptor functions for cross-linking, type II systems could conceivably interact in a number of different combinations, depending on which potential functions the α-chain has. The cross-linking of fibrin can be inhibited by a number of amines, including various glycine derivatives.[13] Suitably activated factor XIII has been shown to incorporate these inhibitors into various natural and unnatural acceptors, including fibrin itself.[14, 15] Accordingly, we clotted some fibrin in the presence of ^{14}C-glycine ethylester with the object of identifying which chains possessed suitable acceptor sites. The concentration of the radioactive inhibitor was chosen at a level just sufficient to produce inhibition of the cross-linking process in order to eliminate as much indiscriminate incorporation as possible. The resulting fibrin was sulfitolyzed and subjected to CMC chromatography. All the incorporated radioactivity, amounting to somewhat more than 1 mole per 330,000 mol wt (calculated for 50% counting efficiency) eluted with the γ-chain peak (Fig. 4). These results indicate that the γ-chains are the only chains which have acceptor functions (presumably suitably disposed glutaminyl side chains) in fibrin.

Discussion.—The intermolecular array in cross-linked fibrin cannot be very much different from that of non-cross-linked fibrin. Apart from their identical appearance in the electron microscope,[16] it should be expected *a priori* that the molecules in the original fibrin gel are more or less fixed in specific positions by noncovalent interactions. The cross-linking enzyme (activated factor XIII) presumably catalyzes the formation of intermolecular peptide bonds between side chains which are already in the appropriate juxtaposition. If this line of reasoning is correct, then any finding with regard to which portions of the molecule are joined by the cross-linking process should also pertain to discussion of which regions are brought into contact during the initial fibrin polymerization. It is clear from the experiments reported here that one major contact is established between γ-chains, presumably belonging to neighboring molecules. In fact *all* γ-chains are involved in cross-linking situations, one system (type I)

being comprised of (two) γ-chains, and a second system (type II) being made up of a γ-chain and an α-chain.

It is obvious that γ-chains must have both donor and acceptor sites. Furthermore, since both the participating γ-chains in the type I system should be chemically identical, one can postulate an antiparallel type of contact in which each pair of chains is linked twice (Fig. 5). The fact that only two chains seem to be linked together (as opposed to 3,4,..n) seems to eliminate the possibility that cross-linking occurs in a staggered overlap fashion. Type II should have only one isopeptide cross bridge since the α-chains did not incorporate any radioactivity when ^{14}C-glycine ethylester was used as an inhibitor of cross-linking. The acceptor functions are apparently the exclusive domain of the γ-chains, and α-chains must be participating by way of the ϵ-amino group of lysyl side chains. If this interpretation is correct, then the maximum number of ϵ-(γ-glutamyl)-lysine bridge peptides per fibrin monomer (330,000 mol wt) will be 2.0. Previous measurements on the number of these isopeptides per mol (330,000 mol wt) have ranged from 1.2 to 2.8 (refs. 3–5).

The regularity of the distribution of types I and II linkage systems from one

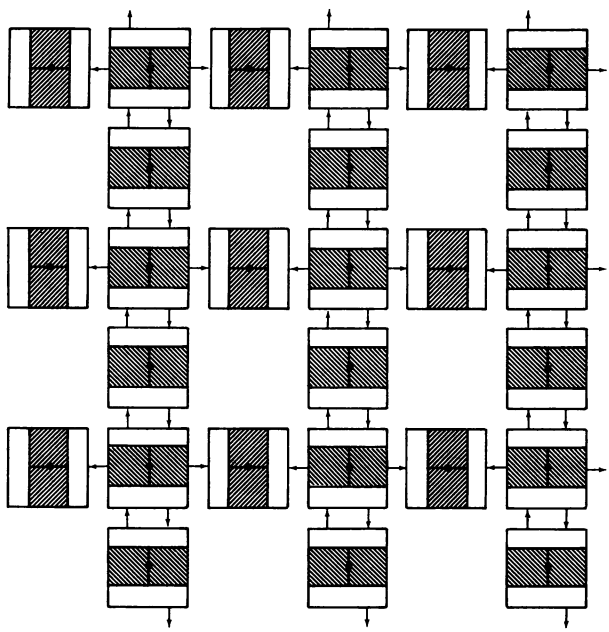

FIG. 5.—Schematic representation of a molecular arrangement for fibrin which is consistent with all available cross-linking data, including the mean number of isopeptide bonds per 330,000 (2.0) and the distribution of $\gamma\gamma$- and $\gamma\alpha$-linkage systems. The square, shaded regions represent α-chains, whereas the oblong, unshaded areas indicate γ-chains. Note that *all* γ-chains are involved in cross-linking situations; β-chains, which are not involved in the cross-linking process, are not included in the diagram. Arrows depict the directional aspects of the ϵ-(γ-glutamyl) lysine cross-links and point from the donor (lysine) to the acceptor (glutamine). The diagram of each molecular unit is drawn transverse to a dyad axis of symmetry.

batch of cross-linked fibrin to another (Table 2) is suggestive of a packing restriction in the gel. Any proposed model arrangement must be consistent with the following restrictions: (*a*) all γ-chains must be involved in intermolecular cross-linking; (*b*) one third of the γ-chains should be linked to α-chains; (*c*) the remaining γ-chains should be hooked to other γ-chains; (*d*) the directional aspect of the isopeptide bonds should be such that all the acceptor sites are on γ-chains; (*e*) the number of isopeptide bonds should be 2.0 per fibrin monomer; and (*f*) the molecular array should be sufficiently branched to be consistent with space polymer formation. The molecular arrangement depicted in Figure 5 satisfies all these conditions. On the average, each molecule (fibrin monomer) is hooked to 2.67 other molecules, although in actuality one third are hooked to four partners and two thirds to two partners. The number of isopeptide links is exactly 2.0 per fibrin monomer, and the donor-acceptor directionality is consistent with the fact that only γ-chains have acceptor sites. The ratio of type I (γ-γ) linkage systems to type II is exactly 1:1, in complete accord with the data, two thirds of the γ-chains being involved in type I and the remaining one third being paired with an equal number of α-chains in type II.

The significance of these observations with regard to the initial events in fibrin formation should not be overlooked. The logical suggestion has been made that the cross-linking sites are masked in the parent fibrinogen molecule and that unmasking is concomitant with the removal of the fibrinopeptides by thrombin.[2] In fact, it is necessary to remove only the fibrinopeptides A for gelation to occur.[17–19] This limit fibrin, lacking only its A fibrinopeptides, can be cross-linked by activated factor XIII.[20] In theory, only one kind of site function (i.e., donor *or* acceptor) needs to be masked to prevent the incipient polymerization of fibrinogen. Our experiments suggest that the unique sites made available by the removal of fibrinopeptide A are the acceptor side chains on the γ-chains. Amino acid sequence studies on the isopeptide regions of the type I and type II systems should tell us whether or not the same glutaminyl acceptor is involved in both systems.

Note added in proof: L. Lorand, responding to a preprint of this article, has drawn our attention to the fact that when the fluorescent probe dansyl-cadaverine is enzymatically incorporated into fibrin, most of the fluorescence is in the tyrosine (γ-) chain (Lorand and Tokura, unpublished data). This work (as yet unpublished) has been referred to previously by Lorand *et al.* (in *Biochem. Biophys. Res. Commun.*, **25**, 629 (1966)).

We are pleased to acknowledge the technical assistance of Mrs. Louise Schmidt. We are also grateful to Joseph Prentice for valuable discussions about the use of G-200 in concentrated urea solutions. We thank S. J. Singer, M. Weinstein, and B. Zimm for significant suggestions about this manuscript.

* This work was supported by NIH grant AI-07781, NSF grant GB-4619, and NIH Program Project grant GM-12027.

[1] Blombäck, B., and I. Yamashina, *Arkiv Kemi*, **12**, 299 (1958).

[2] Lorand, L., *Federation Proc.*, **24**, 784 (1965).

[3] Matačić, S., and A. G. Loewy, *Biochem. Biophys. Res. Commun.*, **30**, 356 (1968).

[4] Pisano, J., J. S. Finlayson, and M. P. Peyton, *Science*, **160**, 892 (1968).

[5] Lorand, L., J. Downey, T. Gotoh, A. Jacobsen, and S. Tokura, *Biochem. Biophys. Res. Commun.*, **31**, 222 (1968).

[6] Laki, K., *Arch. Biochem.*, **32**, 317 (1951).
[7] Pechére, J. F., G. H. Dixon, R. H. Maybary, and H. Neurath, *J. Biol. Chem.*, **233**, 1364 (1958).
[8] Henschen, A., *Arkiv Kemi*, **32**, 317 (1951).
[9] McKee, P. A., L. A. Rogers, E. Marler, and R. L. Hill, *Arch. Biochem. Biophys.*, **116**, 271 (1966).
[10] Edman, P., *Ann. N.Y. Acad. Sci.*, **88**, 602 (1960).
[11] Benson, R., *Anal. Chem.*, **38**, 1353 (1966).
[12] Henschen, A., *Arkiv Kemi*, **22**, 375 (1964).
[13] Lorand, L., K. Konishi, and A. Jacobsen, *Nature*, **194**, 1148 (1962).
[14] Lorand, L., and A. Jacobsen, *Biochemistry*, **3**, 1939 (1964).
[15] Matačić, S., and A. G. Loewy, *Biochem. Biophys. Res. Commun.*, **24**, 858 (1966).
[16] Kay, D., and B. J. Cuddigan, *Brit. J. Haematol.*, **13**, 341 (1967).
[17] Blombäck, B., and T. C. Laurent, *Arkiv Kemi*, **12**, 137 (1958).
[18] Doolittle, R. F., J. L. Oncley, and D. M. Surgenor, *J. Biol. Chem.*, **237**, 3123 (1962).
[19] Doolittle, R. F., *Biochem. J.*, **94**, 735 (1965).
[20] Sasaki, T., I. H. Page, and J. R. Shainoff, *Science*, **152**, 1069 (1966).
[21] Lorand, L., H. H. Ong, B. Lipinski, N. G. Rule, J. Downey, and A. Jacobsen, *Biochem. Biophys. Res. Commun.*, **25**, 629 (1966).