Chain Pairs in the Crosslinking of Fibrin

L. Lorand, D. Chenoweth and R. A. Domanik

Biochemistry Division, Department of Chemistry
Northwestern University, Evanston, Illinois

Received August 11, 1969

Summary: After sultifolysis, enzymatically crosslinked fibrin yields fragments which are different from those obtainable from non-crosslinked material. The α and γ chains of the latter disappear almost entirely and crosslinking bands appear which move slower in electrophoresis. Prominent among these is one with the mobility calculated for an α-γ hybrid chain dimeric combination. The β chain of fibrin appears to be unaffected by crosslinking.

Crosslinking of fibrin is the last enzymatically controlled reaction in the events of normal blood coagulation. Mixing of pure fibrin with the fibrinoligase enzyme[1], which acts as a transpeptidase, results in the formation of unique γ-glutamyl-ε-lysine bonds between fibrin molecules (Lorand et al., 1968 a and b; Matacic and Loewy, 1968). Though few in number (ca. 2 moles per mole of fibrin[2]), the newly formed peptide bonds appear to be responsible for a significant rise in the elastic modulus of the gel (Roberts et al., 1969) and for a very great increase in its resistance to digestion by fibrinolytic enzymes (Lorand and Jacobsen, 1962; Bruner-Lorand et al., 1966; Lorand, 1969).

Inherent in the proposal that crosslinking occurred through a transpeptidating mechanism (Lorand et al., 1962) was the prediction that, after sulfitolysis, crosslinked fibrin would give rise to fragments differing from those of the parent molecule. This concept is illustrated in Fig. 1, depicting a protein with three subunits.

---

[1] Obtained through the limited proteolytic activation by thrombin of the fibrin stabilizing factor isolated from plasma (Lorand and Konishi, 1964; Konishi and Lorand, 1966; Lorand et al., 1968 b)

[2] This figure varies somewhat with reaction conditions.

Both endgroup studies (Lorand and Middlebrook, 1952; Blomback and Yamashina, 1958) and direct isolation of the S-sulfo-chains obtained by sulfitolysis (Clegg and Bailey, 1962; Henschen, 1963) indicate an ($\alpha\beta\gamma$) type of three-chain structure for fibrin. The α and β chains possess N-terminals of glycine, while γ ends in tyrosine (Lorand and Middlebrook, 1952). If the molecular weight of fibrin is taken to be about 330,000 [i.e., 340,000 for fibrinogen (Shulman, 1953) minus a 3% allowance for the release of fibrinopeptide (Lorand, 1951)] , the endgroup data fit an $(\alpha\beta\gamma)_2$ structure containing four chains ending in glycine and two in tyrosine (Lorand and Middlebrook, 1952)[3]. It has recently been proposed (Gerbeck et al., 1967; Brummel and Montgomery, 1969) that the six polypeptide chains are not really three identical pairs; in disc gel electrophoresis α resolves into α-1 and α-2, γ into γ-1 and γ-2.

Because of the ambiguity in molecular weight estimates and the microheterogeneity of the component chains, we prefer not to allude at this stage to any of the possible topographical models of fibrin, but simply choose to illustrate the general concept as presented in Fig. 1. For the sake of simplicity, only a single inter-molecular contact leading to a dimerically crosslinked pair is shown. The donor amino group is on subunit 1, whereas the acceptor carbonyl function is located on subunit 2. In this simple situation the crosslinked protein would give rise to different and fewer fragments than the parent one.

It should be recalled, of course, that in terms of actual chemistry both the α and γ chains in fibrin contain acceptor crosslinking sites (Lorand et al., 1966; Lorand and Chenoweth, 1969), as demonstrated by titration with fluorescent N-(5-aminopentyl)-5-dimethylamino-1-naphtalenesulfonamide and with 1-^{14}C-glycine ethyl ester. The acceptors in the γ chains are more reactive.

The recently developed acrylamide electrophoretic method of Brummel and Montgomery (1969) lends itself particularly well for investigating the problem

[3] If the molecular weight estimates have to be revised, as suggested by the work of Capet-Antonini and Guinand (1967), only a simple ($\alpha\beta\gamma$) pattern would have to be reckoned with.

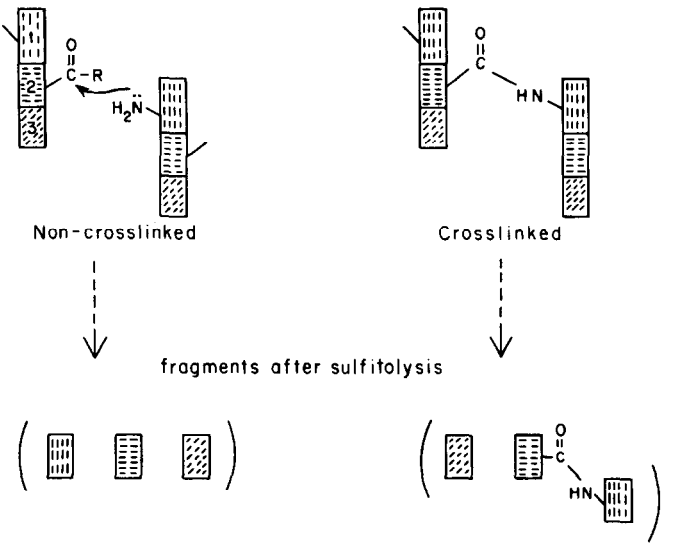

Fig. 1. Fragmentation pattern of a protein by sulfitolysis, before and after the introduction of inter-molecular peptide crosslinks.

outlined. Analysis of the S-sulfo-fragments is facilitated both by the rapidity and the high resolving power of this technique.

Fibrin and its enzymatically crosslinked derivative (Lorand and Ong, 1966; Lorand et al., 1966) were sulfitolyzed by the method of Henschen (1963). The modified proteins were stored in freeze-dried form and were dissolved just prior to electrophoretic application.

Fig. 2 shows the pattern obtained with human fibrin. However, very similar observations were made also with bovine material. Whereas the β chain remains, the α and γ chains disappear almost entirely on crosslinking. At the same time a prominent band (marked X-1 in Fig. 2) appears with a measured mobility corresponding to that calculated for a hybrid α-γ dimeric combination (i.e., taking an average mobility for α and γ and dividing by two).

Since both the α and γ chains carry acceptor sites (Lorand et al., 1966; Lorand and Chenoweth, 1969), it may be assumed that -- when fully crosslinked -- the dimeric α-γ pair would contain two γ-glutamyl-ε-lysines and that the direction of the crosslinking $\overrightarrow{CO}$-NH peptide would have to be represented as $\alpha \not\geq \gamma$

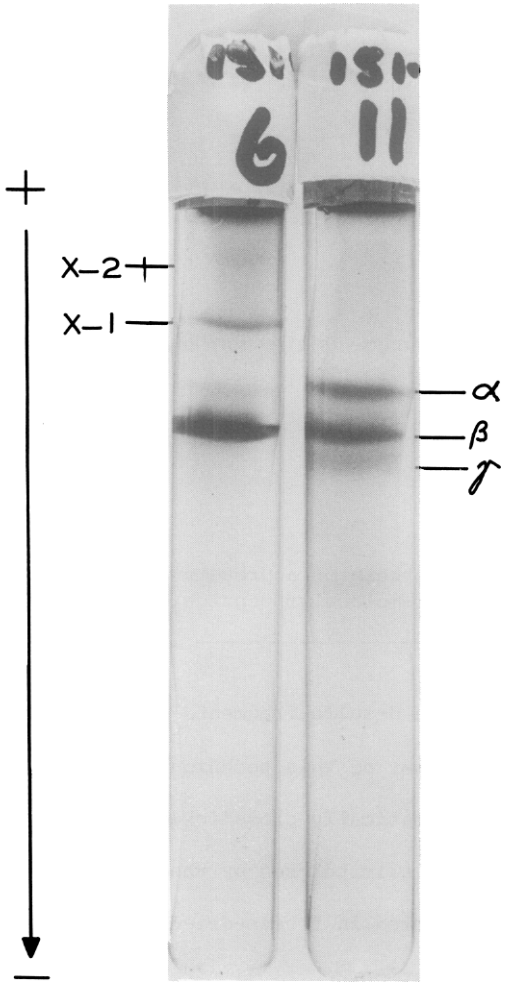

Fig. 2. Acrylamide gel electrophoretic patterns of the S-sulfo-derivatives of crosslinked (left) and non-crosslinked fibrin (right). Experimental procedure according to Brummel and Montgomery (1969).

(with the apostrophy indicating that the chain is derived from another fibrin molecule).

Though not readily seen on the photograph, visual inspection of the gels relating to the crosslinked material reveals weak bands immediately ahead and behind X-1. Mobilities of these are compatible with those calculated for homologous γ-γ' and α-α' dimers. In addition, there are some quite slowly moving bands, one of which is marked as X-2 in Fig. 2. It is likely that it repre-

sents a chain combination of a higher order (e.g., tetrameric). Altogether, the electrophoretic pattern of these fragments is expected to vary with the degree of crosslinking of the fibrin gel.

In sulfitolyzed non-crosslinked human fibrin[4], we observe the same type of microheterogeneity within the α and γ chains which has been reported for bovine material, (Gerbeck et al., 1967; Brummel and Montgomery, 1969), particularly when Coomassie blue is employed for staining[5]. The disappearance of almost the entire α and γ region after crosslinking indicates that α-1, γ-1 as well as α-2 and γ-2 participate in the transpeptidation reaction.

Demonstration of differences between the S-sulfo-fragments of non-crosslinked and crosslinked fibrin clearly eliminates the possibility that disulfide bridges play a primary role in crosslinking (Loewy and Edsall, 1954). If crosslinking depended on inter-chain disulfides, the pattern of fragments after sulfitolysis would be identical for fibrin and for its crosslinked counterpart.

Acknowledgements

Special thanks are due to Professor Rex Montgomery of the State University of Iowa, Iowa City, for sending us the manuscript of Brummel and Montgomery (1969) prior to publication.

This work was aided by a U. S. Public Health Service Research Career Award and by grants from the National Heart Institute (HE-02212) and from the American Heart Association. It was also supported by a training grant (TI GM-626) from the Division of General Medical Sciences.

REFERENCES

Blomback, B. and Yamashina, I., Arkiv f. Kemi, 12, 299, 1958.
Brummel, M. C. and Montgomery, R., Anal.Biochem., In Press, 1969.
Bruner-Lorand, J. A., Pilkington, T. R. E. and Lorand, L. Nature, 210, 1273, 1966.
Capet-Antonini, F. and Guinand, S., CR Acad. Sci. D (Paris) 265, 2093, 1967.
Clegg, J. B. and Bailey, K., Biochim. Biophys. Acta, 63, 525, 1962.
Gerbeck, C. M., Yoshikawa, T. and Montgomery, R., Fed. Proc., 26, 537, 1967.

---

[4] The starting fibrinogen (fraction I-4) was obtained from the Karolinska Institute in Stockholm.

[5] Also a doublet is seen in the β-chain derived from crosslinked fibrin.

Henschen, A., Arkiv f. Kemi, 22, 1, 1963.
Konishi, K. and Lorand, L., Biochim. Biophys. Acta, 121, 177, 1966.
Loewy, A. G. and Edsall, J. T., J. Biol. Chem, 211, 829, 1954.
Lorand, L. Nature, 167, 992, 1951.
Lorand, L. and Middlebrook, W. R., Biochem. J., 52, 196, 1952.
Lorand, L. and Jacobsen, A., Nature, 195, 911, 1962.
Lorand, L., Konishi, K. and Jacobsen, A., Nature, 194, 1148, 1962.
Lorand, L. and Konishi, K., Arch. Biochem, Biophys., 105, 58, 1964.
Lorand, L. and Ong, H. H., Biochem. Biophys. Res. Comm., 23, 188, 1966.
Lorand, L., Ong, H. H., Lipinski, B., Rule, N. G., Downey, J. and Jacobsen, A., Biochem. Biophys. Res. Comm., 25, 629, 1966.
Lorand, L., Rule, N. G., Ong, H. H., Furlanetto, R., Jacobsen, A., Downey, J., Oner, H. and (Bruner)Lorand, J. A., Biochemistry, 7, 1214, 1968 a.
Lorand, L. Downey, J., Gotoh, T., Jacobsen, A. and Tokura, S., Biochem. Biophys Res. Comm., 31, 222, 1968 b.
Lorand, L., in "Dynamics of Thrombus Formation and Dissolution", ed. S. A. Johnson and M. M. Guest, J. B. Lippincott Co., Philadelphia, pp. 212-244, 1969.
Lorand, L. and Chenoweth, D., Proc. Natl. Acad. Sci., In Press, August, 1969.
Matacic, S. and Loewy, A. G., Biochem. Biophys. Res. Comm., 30, 356, 1968.
Roberts, W., Mockros, L. and Lorand, L., Unpublished data, 1969.
Shulman, S., J. Am. Chem. Soc., 75, 5846, 1953.