THE FORMATION OF CROSSLINKED FIBRINS: EVIDENCE FOR THE INVOLVEMENT OF LYSINE ε-AMINO GROUPS

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In vertebrates the final step of the blood coagulation process is the crosslinking of fibrin. There is now good evidence that the crosslinking reaction is a transamidation (Lorand, Konishi and Jacobsen, 1962; Loewy, Dahlberg, Dorwart, Weber and Eisele, 1964; Loewy, Matacić and Darnell, 1966; Matacić and Loewy, 1966; Lorand-Bruner, Urayama and Lorand, 1966). The transamidase that mediates the reaction is the calcium dependent enzyme which has previously been known as Factor XIII, fibrin stabilizing factor (FSF), fibrinase, or the Laki-Lorand factor. The amide acceptor has now been shown to be a glutaminyl residue (Matacić and Loewy, 1966).

The amine donors have been thought to be the amino-terminal glycine residues of the fibrin produced by the action of thrombin (Lorand et al., 1962; Loewy et al., 1964; Loewy et al., 1966). The rationale for involving the amino-terminal glycine residues depended on (a) the effectiveness of the various glycine derivatives to inhibit crosslinking, and (b) reports that crosslinked fibrin has fewer glycine end groups than the non-crosslinked fibrin (Lorand et al., 1962). In this report, we present evidence indicating that the amino-terminal glycines of fibrin are not involved in the crosslinking process at all. Rather, our experiments point to the ε-amino groups of lysine as the primary amine donors in the

crosslinking process.

Our experimental plan was first to chemically block the amino groups (α and ϵ) of fibrinogen, clot the fibrinogen with thrombin, thereby exposing the α-amino groups of the fibrin amino-terminal glycines, and then to establish whether or not such a fibrin deprived of ϵ-amino lysines could be crosslinked by the transglutaminating enzyme (Factor XIII).

MATERIALS AND METHODS

Bovine fibrinogen (Calbiochem, Cohn Fraction I) was purified according to the method of Laki (1951). Fibrinogen preparations (85-95% clottable) were partially amidinated with ethyl acetimidate (Hunter and Ludwig, 1962) according to the procedure described by Wofsy and Singer (1963). The reaction was conducted in sodium borate buffer, pH 8.5, for two hours at 0°, conditions known to block 65% of the amino groups of bovine gamma globulin (Wofsy and Singer, 1963). Crude Factor XIII, the crosslinking enzyme, was prepared according to the method of Loewy, Donathan, Kriel and Wolfinger (1961). The preparation was determined to have about 200 units/ml. In order to assay the enzyme and to unequivocally demonstrate its activity, it was also necessary to prepare fibrinogen free of Factor XIII (Loewy et al., 1961). Not enough of this preparation was available for the amidination experiments, however; the amidinated fibrinogen and its non-amidinated controls referred to above were not previously freed of any contaminating enzyme. Indeed, under suitable conditions the non-amidinated fibrinogen could be crosslinked without the addition of the Factor XIII preparation. Fibrinogen preparations were clotted with semi-purified bovine thrombin (ca. 250 N.I.H. units/mg). The terminal amino groups of this non-crosslinked fibrin produced from amidinated and non-amidinated fibrinogen were determined by the Edman technique as described by Blombäck and Yamashina (1958).

Those fibrin preparations which were to be examined for crosslinking ability were clotted in the presence and absence of Factor XIII preparation.

Calcium ions and cysteine were present in all systems. In a typical experiment, an equal volume of a solution containing thrombin (ca. 3 N.I.H. units/ml), 0.04 M $CaCl_2$, 0.2 M ammonium acetate, 0.02 M cysteine, and Factor XIII (ca. 12 units/ml) was added to a 1% solution of fibrinogen (amidinated or non-amidinated) dissolved in 0.2 M ammonium acetate. Control experiments were run in which the Factor XIII was absent. All determinations were made in duplicate.

The degree of crosslinking was determined by finding how much of the various fibrins were rendered soluble by treatment with 1% monochloroacetic acid (MCA) at room temperature for one hour. To this end, an equal volume of 2% MCA was added to each of the reaction mixtures after incubation for one hour at room temperature.

RESULTS

Fibrin produced from amidinated fibrinogen cannot be crosslinked under conditions in which non-amidinated controls are rendered completely insoluble (Table I). This is true even though the clots formed from amidinated fibrinogen are indistinguishable from the controls by other means. Hence, the overall clottability of amidinated fibrinogen is not impaired, the clotting time is unchanged, and the number of amino-terminal glycines appearing in the fibrin is not reduced (Table II).

Since the glycine amino groups are fully available, whereas the most readily accessible ε-amino lysine groups are blocked, one must conclude that it is the amidination of the latter which has prevented crosslinking.

DISCUSSION

The crosslinking of mammalian fibrin is remarkably similar to the overall coagulation process observed in lobsters (Doolittle and Lorand, 1962). In particular lobster coagulation is inhibited by the same glycine derivatives which prevent crosslinking in mammalian systems. The striking

TABLE I

Comparison of MCA Solubility of Fibrin Clots Formed from Amidinated and Control Fibrinogens

	Fibrinogen Preparation	OD_{280} Fibrinogen Solution	OD_{280} Additive * Solution	OD_{280} Clot Liquor	OD_{280} after Dispersion with 1% MCA: Theoretical if all Dissolved	Theoretical if none Dissolved	Actual Measurement	Insoluble Clot Visible
CONTROL	Amidinated	4.6	0.005	0.506	2.3	0.253	2.7	-
Factor		4.6	0.005	0.462	2.3	0.231	2.4	-
XIII	Control	4.8	0.005	0.528	2.4	0.264	0.44	+
Not		4.8	0.005	0.473	2.4	0.237	0.48	+
Added	Enzyme-free	2.0	0.005	0.484	1.0	0.242	1.0	-
		2.0	0.005	0.550	1.0	0.275	1.0	-
EXPERIMENTAL	Amidinated	4.6	0.502	0.846	2.55	0.423	2.55	-
		4.6	0.502	0.844	2.55	0.422	2.36	-
Factor	Control	4.8	0.502	0.848	2.65	0.424	0.462	+
XIII		4.8	0.502	0.942	2.65	0.472	0.638	+
Added	Enzyme-free	1.65	0.502	0.844	1.05	0.422	0.550	+
		1.65	0.502	0.726	1.05	0.363	0.308	+

* The additive solution contained thrombin, $CaCl_2$, cysteine and, in the experimental mixtures, Factor XIII.

TABLE 2

PTH-Amino Acids Recovered from Fibrin Prepared from Amidinated and Control Fibrinogens

	Moles / 340,000 grams *	
	PTH-Glycine	PTH-Tyrosine
Amidinated	3.4	0
	3.1	0
Control	2.4	1.0
	2.9	1.3

* Uncorrected for procedural losses. Values based on protein concentration of urea-dispersed fibrin as determined by OD_{280} using $E_{1\%} = 15.9$. These preparations were not clotted under conditions which would allow crosslinking.

parallel has been further developed by the more recent studies of Lorand-Bruner et al. (1966). In essence, the lobster clotting process proceeds directly from a soluble "fibrinogen" to a crosslinked "fibrin." In view of the similarities of inhibition, we were surprised to find that glycine end groups were not present in lobster fibrinogen (Fuller and Doolittle, 1966). Our subsequent finding that mildly amidinated lobster fibrinogen was completely nonclottable led us to the experiment described in this paper.

It is important to emphasize the mildness and specificity of the reagent used to block amino groups in these experiments. This is attested to by the initial stages of fibrin formation due to the action of thrombin. Furthermore, amidination does not affect the overall charge on the molecule, nor is the lysine derivative formed vastly different in its bulkiness from the native group (Wofsy and Singer, 1963). Under the conditions used, the reagent is specific for amino groups.

The maintenance of biological integrity of the amidinated fibrinogen was absolutely necessary to the conduct of the experiment, since advantage

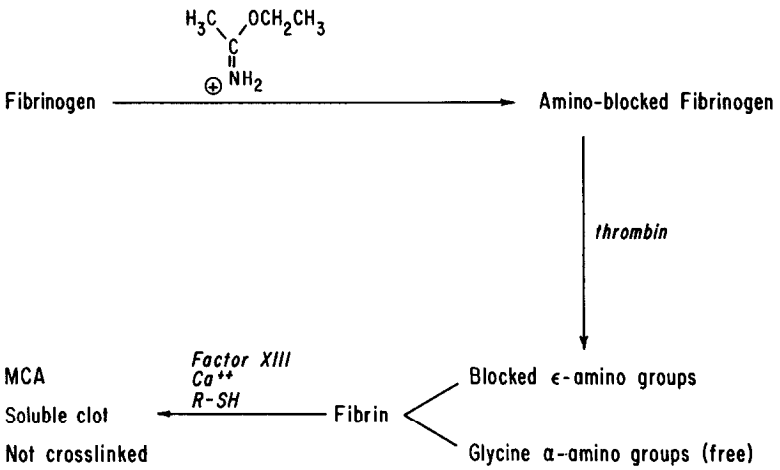

Fig. 1. The rationale of the experiment. When fibrinogen controls were handled similarly except for the omission of the ethyl acetimidate the clot produced by the action of Factor XIII was completely insoluble in 1% monochloroacetic acid and presumed to be fully crosslinked.

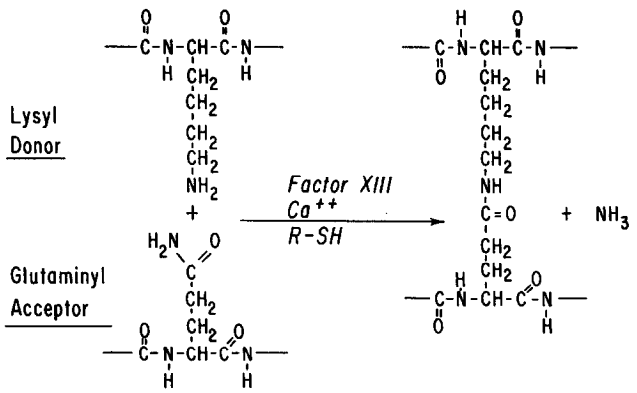

Fig. 2. Postulated mode of crosslinking when mammalian fibrin is acted upon by Factor XIII.

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had to be taken of the exposure of the glycine end groups by thrombin in order to distinguish these from amidinated amino groups. In this regard we must mention that in addition to lysine amino groups, one other amino group has been modified in the amidinated fibrin: the tyrosine amino-terminal of the C chain of fibrinogen. We would presently minimize the influence of this residue relative to the involvement of the ε-amino groups of lysine on the same basis which led us to the present experiments. Namely,

tyrosine does not appear to be an amino-terminal in lobster fibrinogen (Fuller and Doolittle, 1966) or even in some primitive vertebrate fibrinogens and fibrins (Doolittle, 1965), nor are tyrosine derivatives inhibitors of the crosslinking process. Lysine derivatives, on the other hand, are second only to glycine derivatives in their inhibitory power in the lobster system (Doolittle and Lorand, 1962).

In summary, blocking of the amino groups of bovine fibrinogen does not change its clottability by thrombin. The ensuing fibrin cannot be crosslinked by the transglutaminating enzyme (Factor XIII), however, despite the fact that its terminal glycine amino groups are exposed. The prevention of crosslinking is evidently effected by the amidination of the ε-amino groups of lysine.

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