QUANTITATIVE DETERMINATION OF AMINO-TERMINAL AMINO ACIDS IN CROSSLINKED AND NON-CROSSLINKED FIBRIN

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In a recent communication (Fuller and Doolittle, in press) we presented evidence for the involvement of the ε-amino groups of lysyl residues in the crosslinking of mammalian fibrin by the plasma transamidating enzyme known variously as Factor XIII, fibrin stabilizing factor, fibrinase and/or Laki-Lorand factor. This finding stood in direct contrast to the widely held assumption that fibrin crosslinking involves the α-amino groups of the amino-terminal glycines exposed during the conversion of fibrinogen to fibrin by thrombin (Lorand, Konishi and Jacobsen, 1962; Loewy, Dahlberg, Dorwart, Weber and Eisele, 1964; Loewy, Matačić and Darnell, 1966). An obvious corollary of our finding should be that the amino-endgroup patterns of crosslinked and non-crosslinked fibrins should be the same. In this regard a previous report (Lorand, Konishi and Jacobsen, 1962) had noted a great reduction in the number of endgroups in crosslinked fibrin. Quantitative amino-terminal determinations on a urea-insoluble protein are often less efficient than determinations on a more readily dispersed preparation, however, and it seemed possible that the penetration of crosslinked fibrin by amino-group reagents was sufficiently hindered that lower values resulted artifactually.

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In this communication we report the measurement of crosslinked fibrin amino-terminal amino acids under conditions where hindrance is apparently not a factor. Our data unequivocally show that the amounts of amino-endgroups in crosslinked and non-crosslinked fibrins are the same within the limits of experimental error, thereby eliminating any notions that any of the α-amino groups of fibrin are involved in the crosslinking reaction.

MATERIALS

Bovine fibrinogen (Pentex) was further purified by the method of Laki (1951). These preparations, although having a high clottability, are sufficiently contaminated with the crosslinking enzyme that they can be fully crosslinked by the addition of thrombin, calcium ions and a sulfhydryl activator. The $E^{1\,cm}_{1\%}$ for fibrinogen at neutral pH was taken to be 15.0 at 280 mμ (cf. Blombäck, 1958). Optical density measurements were made on a Zeiss PMQ-II spectrophotometer. Bovine thrombin containing about 250 N.I.H. units/mg. was the generous gift of Dr. Staffan Magnusson. The phenylisothiocyanate (Eastman) used in the endgroup determination was redistilled under reduced pressure before use. All other reagents used in the determination were of analytical grade.

EXPERIMENTAL

Clotting and Crosslinking. Equal volumes (2.5 mls) of a stock fibrinogen solution (7.8 mg/ml. saline) were pipetted into a number of separate tubes. To some of these were added 1.0 ml. of a thrombin solution (final = 2 N.I.H. units/ml) containing calcium chloride (final concentration = 25 mM) and cysteine (final concentration = 12.5 mM) to activate the latent crosslinking enzyme. A thrombin-saline solution of the same potency was added to the tubes destined to be clotted but not crosslinked. Paired mixtures of identical composition were set up simultaneously in order to quantitate the degree of crosslinking.

Degree of Crosslinking. Insolubility of a fibrin preparation in 1% monochloroacetic acid was taken to be a measure of crosslinking. After clotting for one hour at room temperature, an equal volume of 2% MCA[2] was added to the paired duplicates to ascertain the degree of crosslinking. The preparations deprived of calcium ions and cysteine were readily dispersed; the other clots remained intact. After standing for an additional hour, the tubes were centrifuged and the OD_{280} of the supernatant fluids determined. In the case of the preparations designed to be crosslinked very little of the original protein was rendered soluble, whereas in the non-crosslinked controls virtually all of the original protein was recovered as MCA-soluble material (Table 1).

Determination of Amino-terminal Residues. The mixtures for endgroup analysis were also let clot for one hour, after which the fibrin clots were wound out on glass rods and transferred to glass-stoppered tubes containing 2.5 mls of a solution containing 6.7 M urea, 0.03 M tris, and 0.2 M mercaptoethanol at pH 9. The amount of original protein transformed into the fibrin clots was determined by reading the OD_{280} of the residual clot liquors (Table 1). The non-crosslinked preparations dispersed readily, but the crosslinked material did not. After some minutes, however, the crosslinked fibrin became exceedingly transparant and open-structured, although it did not dissolve.

The preparations were mixed with an equal volume of pyridine followed by the addition of 0.2 mls phenylisothiocyanate. Coupling was allowed to proceed for 90 min. at 40° to insure full penetration of the reagent into the crosslinked fibrin. Thereafter the preparations were carried through the Edman procedure used by Blombäck and Yamashina (1958). The final extracts containing the PTH derivatives were evaporated to dryness and the residues dissolved in 50 microliters of dichloroethane. Duplicate applications (20 microliters each) were made on separate chromato-

[2] Abbreviations used: MCA = monochloroacetic acid; OD_{280} = optical density at 280 mμ; PTH = phenylthiohydantoin.

Table 1

Clottable Protein and Degrees of Crosslinking*

		OD_{280} Original Mixture	OD_{280} Clot Liquor	Percent Clottable Protein	OD_{280} of MCA Super. x 2	Percent Insoluble Protein
Crosslinked	1	8.4	0.59	93	0.48	95
	2	8.4	0.60	93	0.40	95
Non-crosslinked	1	8.4	0.49	94	7.8	7
	2	8.4	0.50	94	7.9	6

* The degree of crosslinking was determined on separate paired tubes of identical compositions to those on which the clottability and the amino-terminal amino acids were measured.

grams; chromatography and elution were conducted according to the method of Sjöquist (1960). PTH-glycine and PTH-tyrosine were both determined on Sjöquist's Solvent III. The molar extinction coefficients for PTH-glycine (14,900) and PTH-tyrosine (15,600) were taken from Sjöquist (1957). Complete spectral determinations were made in the range λ = 245-320 mμ to verify the authenticity of the eluted derivatives.

RESULTS AND DISCUSSION

The results of the endgroup analyses are listed in Table 2. Under the conditions used, no diminution of endgroups in the crosslinked fibrin could be detected. In fact, the uncorrected values in all cases are in accord with an 80% overall recovery based on a molecule with four moles of glycine and two moles of tyrosine for every 330,000 molecular weight. The ratio PTH-glycine/PTH-tyrosine was 2.0 $\pm$ 0.1 in six separate determinations (four crosslinked and two non-crosslinked).

Table 2

PTH-Amino Acids Recovered from Crosslinked and Non-crosslinked Bovine Fibrin

Moles/330,000 grams*

			PTH Glycine	PTH Tyrosine	Ratio Gly/Tyr
Crosslinked Fibrin	Batch I	1	3.47	1.77	1.97
		2	3.13	1.64	1.91
	Batch II	1	3.28	1.75	1.88
		2	3.21	1.55	2.07
Non-crosslinked Fibrin	Batch II	1	3.10	1.48	2.09
		2	3.14	1.62	1.94

* Uncorrected for procedural losses. Calculations based on protein concentration of starting fibrinogen solutions (corrected for 93-94% clottability) using $E_{1\%} = 15.0$. Each value is the average determined from eluates of duplicate chromatography runs. Batches I and II refer to two different starting fibrinogen preparations.

The first suggestion that the amino-endgroups of various clots might differ was offered by Middlebrook (1955) who found a 25% reduction in the amount of amino-terminal glycine in "plasma clots" relative to regular (non-crosslinked) fibrin. The difficulties inherent in working with the crude plasma system precluded a reliable comparison, however. Furthermore, the accepted molecular weight for fibrin at that time (450,000) gave the appearance that the yields were one-third better than they actually were. More recently Lorand *et al.* (1962) reported that crosslinked fibrin had only one-quarter of the glycine endgroups and one-half the tyrosine endgroups found in non-crosslinked preparations. Coupled with their finding that the crosslinking process was inhibited by various glycyl

esters and peptides, this gave rise to the generally accepted scheme whereby the α-amino groups of the terminal glycines were thought to be the donors in a transpeptidating or transamidating process leading to crosslinking. In both of these studies (Middlebrook, 1955; Lorand et al., 1962) it was noted that the number of lysine ϵ-amino groups was indistinguishable for the two kinds of fibrin. There are more than 200 moles of lysine per mole of fibrin (330,000 MW), however, but only 2-4 crosslinks per mole have been implicated on the basis of ammonia release and radioactivity incorporation studies (Loewy, Dahlberg, Dorwart, Weber and Eisle, 1964; Matačić and Loewy, 1966). It would seem unlikely that the lysine ϵ-amino measurements would have been able to detect a change of this magnitude (1-2%).

In summary, there is no basis for the implication of the glycine or tyrosine α-amino groups of mammalian fibrin in the crosslinking process. It remains to be explained, however, why glycine derivatives are such powerful inhibitors of the crosslinking reaction (Lorand et al., 1962).

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