STUDIES ON FIBRIN CROSSLINKING. NATURE OF THE ACCEPTOR GROUPS IN TRANSPEPTIDATION

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The physiological crosslinking of fibrin (F) units, thought to occur by a transpeptidation reaction (Lorand, et al., 1962), can be inhibited by low concentrations of hydroxylamine (Lorand and Jacobsen, 1964; Lorand, 1965). This compound was further shown (Lorand, et al., 1966; Lorand and Ong, 1966) to terminate crosslinking by virtue of actually becoming incorporated into fibrin: $H_2N.F.CO.Y + H_2N.OH \longrightarrow H_2N.F.CO.NH.OH + HY$. Such a specific tracer, introduced by the crosslinking enzyme itself [i.e. by the thrombin activated fibrin stabilizing factor (Lorand and Konishi, 1964; Konishi and Lorand, 1966)], may be assumed to label the same acceptor functions (CO.Y) of fibrin which -- in the absence of hydroxylamine -- would participate in forming crosslinks: $H_2N.F.CO.Y + H_2N.F'.CO.Y \longrightarrow H_2N.F.CO.NH.F'.CO.Y + HY$, as shown for dimerization. Therefore, fibrin-hydroxamate seemed well-suited for exploring the acceptor crosslinking sites of fibrin.

The enzymatically derivatized hydroxamate of bovine fibrin was prepared as described elsewhere (Lorand and Ong, 1966). If the molecular weight of the protein is taken as 330,000 [i.e. the molecular weight of fibrinogen (Shulman, 1953) minus 3%

allowance for the release of fibrinopeptide material (Lorand, 1951) in the fibrinogen-fibrin conversion], the starting material contained 3.2 moles of hydroxamate per mole of fibrin. By contrast, the control fibrin which was mixed with hydroxylamine in the absence of the crosslinking enzyme, contained less than 0.3 moles of hydroxamate per mole of protein. The two protein preparations were treated throughout all consecutive steps in a parallel manner. They were first solubilized by treatment with trypsin (Lorand and Ong, 1966); this was followed by reaction with 1-fluoro-2,4-dinitrobenzene and a Lossen-type of rearrangement in base (Blumenfeld and Gallop, 1962). As a result, the hydroxamates of glutamic acid residues of the protein would be converted to aminoethyl, while those of aspartic acid to aminomethyl side chains. When hydrolyzed in acid (5.7N HCl), the former would yield diaminobutyric acid and the latter would give rise to diaminopropionic acid.

Separation and chromatographic identification of the basic amino acids, including diaminobutyric and diaminopropionic acids, was performed with an accelerated physiological fluid column (PA-35; size 0.9 x 22 cm) on a Beckman Spinco Model 120B amino acid analyzer (Spackman et al., 1958). Flow rate was adjusted to 50 ml per hour at 33°, using 0.38N citrate buffer of pH 3.5. Under these conditions phenylalanine, tyrosine and ammonia emerge together at 120 ml effluent volume, diaminobutyric acid appears at 141 ml, diaminopropionic acid at 150 ml, ornithine at 199 ml and histidine at 254 ml. The top curve in Fig. 1 (curve 4) shows the elution pattern obtained when L-diaminobutyric acid (Calbiochem), DL-diaminopropionic acid (Nutritional Biochemicals) and L-ornithine (Mann Laboratories) were added to a standard (Beckman) mixture of amino acids.

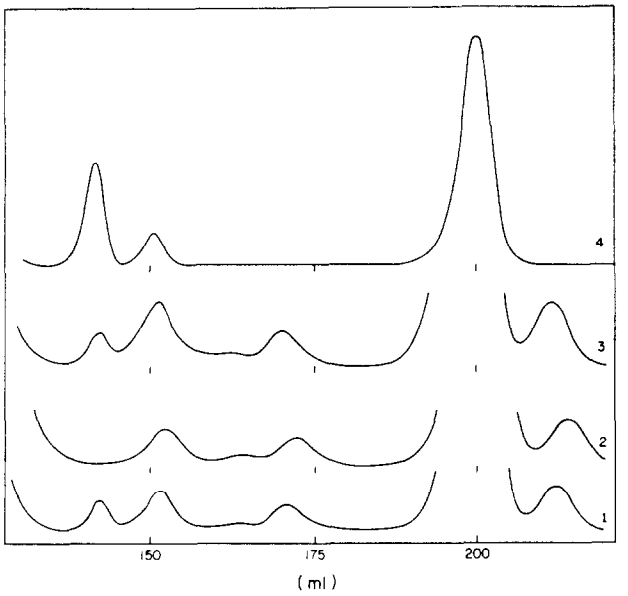

Fig. 1. Curve 1: Hydrolysate of Lossen-rearranged control fibrin, enriched with L-diaminobutyric acid. Curve 2: Hydrolysate of Lossen-rearranged control fibrin. Curve 3: Hydrolysate of Lossen-rearranged, enzymatically derivatized fibrin-hydroxamate. Curve 4: Standard amino acids; (from left to right) L-diaminobutyric acid, DL-diaminopropionic acid and L-ornithine.

The most striking difference between the hydrolysate (24 hr.) of the enzymatically derivatized protein (curve 3) and that of the control protein (curve 2) pertains to the area where authentic L-diaminobutyric acid would emerge. It will be noticed that the control has quite a flat baseline here. However, when the hydroly sate of the control protein is enriched by adding 0.0167 μmoles of L-diaminobutyric acid to it (curve 1), a peak very much like that observed in curve 3 is seen.

In order to evaluate the amount of presumed diaminobutyric acid recovered in curve 3 in terms of the quantity of the protein used, the acidic and neutral amino acid contents of the hydrolysate were measured (Spackman et al., 1958). Based on a number of

reference amino acids (Table 1) and using the known amino acid composition of fibrinogen (Mihalyi et al., 1964) and of fibrin (Henschen, 1963) as standard[1], the amount of diaminobutyric acid (curve 3) would correspond to approximately 0.8 moles per mole of fibrin. This value accounts for about 25% of the original hydroxamate as presumed diaminobutyric acid. Another independent experiment gave identical results.

TABLE I

Reference Amino Acid		Moles of Reference Amino Acid per 10^5g of Protein A: Mihalyi et al.,1964 B: Henschen, 1963	Moles of Presumed Diaminobutyric Acid in 10^5g of Protein
Asp	A	103.2	0.237
	B	104	0.239
Glu	A	93	0.245
	B	93	0.245
Pro	A	46.1	0.255
	B	33	0.183
Gly	A	83.1	0.230
	B	85	0.235
Ala	A	36.6	0.250
	B	40	0.274
Val	A	44.2	0.293
	B	43	0.286
Met	A	14.8	0.266
Leu	A	49.7	0.258
	B	53	0.276
Ileu	A	36.5	0.258
	B	39	0.276
Phe	A	26.1	0.246
	B	26	0.245

[1] Disregarding losses to possible dinitrophenylation.

In addition to diaminobutyric acid, a comparison between curves 2 and 3 in Fig. 1 reveals a difference in peak areas in

the region where diaminopropionic acid is expected. The latter has such a low color yield with ninhydrin (Blumenfeld and Gallop, 1962) that the difference, if indeed due to diaminopropionic acid, could be highly significant. This problem is now being investigated.

While it is, of course, desirable that the chromatographic identification of presumed diaminobutyric acid be supported by further characterization of the product, the evidence on hand seems to implicate, at least in part, the participation of γ-carbonyl groups of glutamyl residues as acceptor functions in the transpeptidation reaction which leads to crosslinking of fibrin. This in turn would emphasize the similarities between the mode of action of the crosslinking enzyme and that of guinea pig liver transglutaminase (Clarke et al., 1959). It should also be recalled that acetyl and benzyloxycarbonyl derivatives of diesters and diamides of both aspartic and glutamic acids can serve as inhibitors of fibrin crosslinking (Lorand and Jacobsen, 1964; Lorand, 1965). Benzyloxycarbonyl-L-glutaminylglycine, (Cyclo Corp.) a known transglutaminase substrate (Folk and Cole, 1965), is also somewhat inhibitory. Thus, judging from the pattern of inhibition, crosslinking could conceivably utilize either glutamic or aspartic acid type residues.

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