THE IDENTIFICATION OF ISOPEPTIDE CROSSLINKS IN INSOLUBLE FIBRIN*

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It has been an open question for a number of years whether isopeptide crosslinks between peptide chains can occur within or between protein molecules. In this communication we shall demonstrate by direct means that a number of isopeptide crosslinks per molecule are formed when plasma transglutaminase** converts soluble fibrin into insoluble fibrin.

The role of plasma transglutaminase in the formation of insoluble fibrin was discovered and studied by Barkan and Gaspar (1923), Robbins (1944), Laki and Lorand (1948), Lorand (1948, 1950), Loewy and Edsall (1954), and Loewy et al. (1957). The transamidase properties of the enzyme were demonstrated by Loewy et al. (1964). That glutamine was the acceptor in the transamidation reaction was demonstrated by Loewy et al. (1966) and by Matačić and Loewy (1966). The involvement of the ε-amino groups of lysine as the donors in the transglutamination re-

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** This enzyme has in the past been given a number of names such as serum factor, fibrin stabilizing factor (FSF), Laki-Lorand factor, and fibrinase. We shall use "Factor XIII" for the inactive precursor purified from plasma and "plasma transglutaminase" for the thrombin-activated enzyme responsible for the formation of insoluble fibrin.

action was demonstrated by Fuller and Doolittle (1966), Lorand et al. (1966), and Doolittle and Fuller (1967).

Though the work cited above has demonstrated the involvement of glutamine residues and of ε-amino groups of lysyl residues in the formation of insoluble fibrin, it remained to be shown by direct means that isopeptide crosslinks do actually occur in insoluble fibrin. We have done this by synthesizing the isodipeptide ^{14}C-γ-glutamyl-ε-lysine and adding it to enzyme hydrolysates of insoluble fibrin. By purifying the isodipeptide and showing a decrease in specific activity of the isodipeptide it was possible to demonstrate the presence of unlabelled γ-glutamyl-ε-lysine in the insoluble fibrin hydrolysate.

The isodipeptide ^{14}C-γ-glutamyl-ε-lysine was synthesized enzymatically by incorporating ^{14}C-lysine into casein with plasma transglutaminase. It turned out that this incorporation involved specifically the ε-amino group of lysine since the α-amino group cannot act as an amine donor when the neighboring α-carboxyl group is free. The incorporation reaction of free lysine is very slow when compared with that of an esterified amino acid and therefore high levels of enzyme and long periods of incubation had to be used. The incubation mixture was as follows: 20 mg of casein, 4 mg of Factor XIII, fraction 5 (Loewy et al. 1961), 50 μcuries ^{14}C-L-lysine (237 mc/μmole), 15 μmoles $CaCl_2$, 0.1 mg human thrombin (200 Iowa units per mg N), 15 μmoles mercaptoethanol, all in 3 ml of 0.3 M tris buffer pH 7.5. This mixture was incubated for 24 hours at 30°C after which 2 mg of additional Factor XIII were added and the incubation continued for another 24 hours. The labelled casein was then purified by precipitation with 10% TCA for 20 minutes at 4°C, the precipitate being freed of unincorporated ^{14}C-lysine by repeated washing and centrifugation with a total of 100 ml of 5% TCA. The precipitate was then washed repeatedly with a total of 30 ml ethanol-ether (1:1) followed by 10 ml of ether, and finally dried

under vacuum over P_2O_5. Activities of 50-150,000 cpm/mg of casein were obtained. The ^{14}C-lysyl derivative of casein was digested with pronase (Calbiochem) and leucine aminopeptidase (Worthington) by a method described by Haley, et al. (1966) and modified by Matačić and Loewy (1966). This method hydrolyzes 95% or more of the peptide bonds of casein but leaves the isopeptide bond intact as judged by the absence of radioactive lysine in the hydrolysate. It was found that a culture of gram positive bacilli, originally isolated from a contaminated casein digest, absorbed most of the amino acids, leaving the isodipeptide in the supernatant after the bacteria were removed by centrifugation. We therefore made it a practice to utilize these bacilli to absorb the amino acids released by the enzyme digestion, which allowed us to chromatograph on our 0.6 x 130 cm resin column the hydrolysate of as much as 100 mg of casein in one single run. The chromatography, scintillation counting, and other allied methods used have been described by Matačić and Loewy (1966). The radioactive peak was found in a position just preceding that of leucine, that is, after elution with 280 ml of sodium citrate buffer which matches exactly the elution properties of synthetic γ-glutamyl-ε-lysine (Cyclo Chem. Corp.). The elution properties of synthetic α-glutamyl-ε-lysine are entirely different, the peak appearing after 436 ml of sodium citrate buffer. The radioactive fraction was desalted on a Dowex 50X-8 column according to the method described by Haley, et al. (1966), freeze-dried and dissolved in 0.01 M HCl. Upon acid hydrolysis this material could be shown to consist of equal proportions of glutamic acid and ^{14}C-lysine only. The amount of lysine was measured by comparing the area of the ninhydrin positive peak with that of a norleucine standard. The radioactivity was measured by scintillation spectrometry using a ^{14}C-toluene standard to estimate quenching.

The ^{14}C-isodipeptide marker prepared in the manner described above

was utilized to detect the presence of isopeptide crosslinks in insoluble fibrin by using the following procedure. Bovine fibrinogen (fraction 3a) and Factor XIII were prepared by the procedure of Loewy, et al. (1961). The fibrinogen was stored as frozen solutions and the Factor XIII as a freeze-dried powder at -15°C. The incubation mixture for the formation of insoluble fibrin involved 100 mg of bovine fibrinogen, 10 mg of Factor XIII, 15 µmoles of $CaCl_2$, 15 µmoles of mercaptoethanol, 0.5 mg of bovine thrombin, all in 3 ml of 0.3 M tris buffer pH 7.5 and incubated for 24 hours at 30°C. Soluble fibrin was prepared in the same manner but 15 µmoles of ethylene dinitrilotetraacetate (EDTA) was substituted for the calcium. The clot was cut into small pieces and homogenized in a Servall Omnimixer in 0.3 M NaCl after which the proteins were precipitated with 10% TCA. The washing procedure utilized was the same as the one described for the ^{14}C-lysyl derivative of casein. The insoluble and soluble fibrin was digested with pronase and leucine aminopeptidase according to the procedure of Haley, et al. (1966) and modified by Matačić and Loewy (1966). We found that in the case of the fibrin digest the bacilli, used to such advantage with casein, did not absorb the amino acids specifically. In this instance considerable quantities of amino acids were not absorbed and some losses of the isodipeptide marker were experienced. Since we had found that, in order to obtain reliable results, we had to scale up our methods and utilize as much as 100 mg of fibrin, we developed the following modification of our chromatographic procedure. To a digestion mixture of 100 mg of fibrin we added 30,000 cpm of the ^{14}C-γ-glutamyl-ε-lysine marker. After digestion a preliminary purification was carried out on a large (2 x 130 cm) Dowex 50X-8 column kept at room temperature utilizing the same gradient buffer elution schedule as described by Matačić and Loewy (1966). The additional column purifications were then carried out on the "Technicon A" column and by the procedure

TABLE I

NUMBER OF ISOPEPTIDE CROSSLINKS FOUND IN INSOLUBLE FIBRIN

Amounts and specific activities of lysine obtained from HCl hydrolysates of purified γ-glutamyl-ε-lysine

	from "Marker" added to Hydrolysate		from Soluble Fibrin Hydrolysates			from Insoluble Fibrin Hydrolysates		
μmoles Fibrin Monomer (MW 320,000)	μmoles	Specific Activity cpm/μmole	μmoles	Specific Activity cpm/μmole	No. Crosslinks / Fibrin Monomer	μmoles	Specific Activity cpm/μmole	No. Crosslinks / Fibrin Monomer
3.13×10^{-2}	5.19×10^{-2}	2.70×10^{5}	1.4×10^{-2}	2.48×10^{5}	0.15	2.8×10^{-2}	1.22×10^{5}	2.0
3.13×10^{-2}	2.85×10^{-2}	5.35×10^{5}	5.4×10^{-3}	4.8×10^{5}	0.11	4.0×10^{-2}	1.50×10^{5}	2.3
3.13×10^{-1}	3.23×10^{-1}	1.86×10^{5}				2.51×10^{-1}	5.04×10^{4}	2.8

previously described (Matačić and Loewy, 1966). Column temperature was 45°C for 90 minutes and 60°C for the remaining time. The next purification was carried out at 30°C. This procedure of chromatography at two different temperatures proved to be very effective in separating the γ-glutamyl-ε-lysine from leucine and other minor impurities. After three such chromatographic purifications it was possible to demonstrate that an HCl hydrolysate of the radioactive material consisted of equal proportions of glutamic acid and lysine with no impurities being detected.

The analytical procedure employed consisted of measuring the amino acid quantity and the radioactivity of the lysine obtained from an HCl hydrolysate of purified γ-glutamyl-ε-lysine from soluble and insoluble fibrin. Table 1 shows some typical examples of the data we have obtained.

In no instance did the specific activity of the lysine from the digest of the isodipeptide purified from soluble fibrin differ significantly from the specific activity of the lysine from the purified marker. We can conclude that soluble fibrin does not contain significant quantities of the isopeptide crosslinks. In the case of insoluble fibrin, however, significant differences in specific activity could be determined amounting to values of 2-3 crosslinks per fibrin monomer of 320,000.*

The procedure we have employed also lends itself to the identification of isopeptide crosslinks in other proteins. We are now en-

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* Before scaling the method up to the use of 100 mg of fibrin for a measurement, we obtained some higher values but since our amino acid levels were very low (2×10^{-2} μmoles of lysine) we do not consider these values to be reliable. The value of 3 crosslinks per fibrin monomer obtained from a hydrolysate of 100 mg insoluble fibrin we consider to be reliable since it involved the measurement of 2.5×10^{-1} μmoles of lysine.

gaged in studies to determine whether isopeptide crosslinks are a more widely utilized principle of protein structure especially in relation to processes of biological importance.

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ADDENDUM

After this manuscript was accepted we heard from Drs. J. J. Pisano, J. S. Finlayson and M. P. Peyton that they have submitted a manuscript to Science which contains substantially identical results.