TRANSGLUTAMINASE ACTIVITY OF THE FIBRIN CROSSLINKING ENZYME*

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Received August 4, 1966

This communication establishes that the plasma enzyme responsible for the Ca^{++} activated conversion of soluble fibrin into insoluble fibrin incorporates ^{14}C-glycine ethyl ester (GEE) into fibrin by forming γ-glutaminyl but not β-asparaginyl derivatives.

Evidence had accumulated in recent years that the conversion of soluble fibrin into insoluble fibrin occurs via a transmidation reaction between amide donors and amine acceptors with the release of ammonia (Middlebrook, 1955; Lorand et al., 1963; Loewy, 1964; Loewy et al., 1964). The precise identity of the amide donor has not so far been determined unambiguously. Lorand and Jacobsen (1964) reported that carbobenzoxy-L-asparagine amide is an inhibitor of insoluble fibrin formation but these experiments could not distinguish between the alternative mechanisms of 1) competitive inhibition of the enzyme or 2) "chain termination" of polymerization by direct incorporation of the inhibitor into fibrin. The fact that the enzyme, as we shall see, is specific for glutamine side chains and the fact that Lorand and Jacobsen also found carbobenzoxy-glycine β-alanine amide to act as an inhibitor, suggests that these substances are not incorporated into fibrin

* Supported by grant HE-04385 from the National Institutes of Health

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but act as competitive inhibitors of the enzyme. In any case, the absence of data on inhibition by glutamine derivatives in these experiments fails to provide comparisons of the effectiveness of these two amide functions in the inhibition of insoluble fibrin formation. Loewy et al. (1966) incorporated ^{14}C-glycine ethyl ester into glucagon with the plasma enzyme. By hydrolyzing the modified glucagon with leucine amino peptidase and purifying with Dowex-50 column chromatography they were able to demonstrate the presence of γ-glutamyl glycine ethyl ester in the hydrolysate. They showed that two out of three glutaminyl residues were involved in the transamidation reaction, the same two residues which Folk and Cole (1965) identified in their study of guinea pig liver transglutaminase. It is possible that no incorporation into asparaginyl side chains occurred because glucagon has only one asparaginyl group placed one residue away from the C-terminal end. Loewy et al. (1966) also reported preliminary results using fibrin as a substrate which indicated that both glutaminyl and asparaginyl side chains are involved in the transamidation reaction. This result was apparently confirmed by Lorand and Ong (1966) who incorporated hydroxylamine into fibrin and converted the hydroxamates by a Lossen-type rearrangement in base to amino ethyl (for glutaminyl) and amino methyl (for asparaginyl) side chains. After hydrolysis in acid Lorand and Ong were able to demonstrate an increase of diaminobutyric acid suggesting that glutaminyl side chains had reacted with hydroxylamine in the presence of the plasma enzyme. They estimated that some 0.8 moles of hydroxylamine per mole of fibrin had been incorporated-a value, which is at the lower end of the range normally observed. They also observed small differences in diamino propionic acid but, owing to the low ninhydrin color value of this material, they were not able to estimate whether

significant amounts of hydroxylamine had reacted with asparaginyl residues.

We shall present evidence on the specificity of the plasma enzyme for the amide groups of the γ-glutaminyl residues of fibrin. In this respect the plasma enzyme is similar to the mammalian liver transglutaminase system (Neidle et al., 1960; Mycek and Waelsch, 1960; Folk and Cole, 1965). We shall therefore refer to the plasma enzyme (previously called FSF, fibrinase, plasma transamidase) as "plasma transglutaminase" and to its inactive precursor as "factor XIII".

Bovine fibrinogen (fraction 3a) was purified by the method of Loewy et al. (1961). Their "fraction 3" being reprecipitated in 3M NaCl followed by a passage through Sephadex G-50 in 0.3M NaCl to remove traces of NH_4^+, pH adjusted to 7.5 and stored in the frozen state at -15°C. Plasma transglutaminase (factor XIII)* was prepared by the method of Loewy et al. (1961), their "fraction 5" being dissolved in 0.3M NaCl, dialyzed against 0.3M NaCl, pH adjusted to 7.0 and finally freeze dried in 25λ aliquots which contained 0.45 mg of protein and could convert 0.5 g of soluble fibrin into insoluble fibrin.

The procedures employed in this study included the following steps:

(a) The enzymatic incorporation of ^{14}C-glycine ethyl ester (GEE) into fibrin.

(b) The solubilization of insoluble fibrin and the cleavage of disulfide bonds by sulfitolysis.

(c) The enzyme digestion of sulfitolyzed fibrin.

* Unless the precaution is taken to remove prothrombin from plasma with $BaSO_4$ one usually obtains a factor XIII preparation which has become partially activated during fractionation and storage to plasma transglutaminase.

(d) The purification by column chromatography of the radioactive moiety and its identification as γ-glutamyl glycine.

(a) The following incubation mixture was used to incorporate ^{14}C-GEE into fibrin: 16.6 mg of bovine fibrinogen (fraction 3a); 1.8-4.5 mg of plasma transglutaminase; 100 μ moles GEE, specific activity 0.82 millicuries/millimole; 5 μ moles $CaCl_2$; 5 μ moles mercaptoethanol; 0.1 mg of bovine thrombin (200 Iowa units per milligram nitrogen); all in 1 ml of 0.3M tris buffer, pH 7.5. The mixture was incubated at 30° for 24 hours.

(b) The insoluble fibrin was solubilized and its disulfide bonds were broken by sulfitolysis according to the method of Pechère et al. (1958). The sulfitolyzed fibrin, now rendered water soluble, was precipitated and washed 7 times with 10 ml of 5% trichloroacetic acid (TCA). The protein precipitate was then washed with ethanol - ether (1:1), followed by ether and dried under vacuum over P_2O_5.

(c) The dried protein was weighed and digested according to the following procedure. 10 mg of protein were digested by pronase (Calbiochem.) and leucine aminopeptidase (Worthington) according to the method of Haley et al. (in press). We measured the extent of enzyme hydrolysis by comparing the ninhydrin value of the hydrolysate with the value of a 6N HCl (18 hours at 120°C) digest of the hydrolysate and obtained values which were in excess of 90% and thus comparable to those obtained by Haley et al. The radioactivity of the hydrolysate was counted in dioxane scintillation fluid (Bray 1960) in a Model 314 EX Packard scintillation spectrometer. Values ranging from 10 - 30,000 cpm/mg of protein were obtained which represent the incorporation of 1 - 3 moles of GEE per mole of fibrin monomer (molecular weight 320,000).

(d) The radioactive moiety in the hydrolysate was puri-

fied by column chromatography on Dowex 50 X-8 resin according to the following procedures. The apparatus utilized was a modified Technicon amino acid analyzer with a resin column of 130 x 0.6 cm and a Gilford spectrophotometer as a detector and recorder (sensitivity 0.1 - 1.0 absorbancy units per full scale deflection). Three stages of purification by three separate passages through the resin column were employed.

(1) A hydrolysate derived from 5 mg of fibrin was chromatographed (at 45°C for 90 minutes followed by 60°C) utilizing as eluent a sodium citrate buffer gradient from 0.2M (pH 2.9) to 0.8M (pH 5.0) in a nine chamber varigrad. 10% of the column effluent was monitored for ninhydrin positive material in the automated analyzer, the remaining 90% being collected in 2 ml fractions. Radioactivity of the fractions was measured by counting 50 aliquots in dioxane scintillation fluid. Some 60 - 90% of the radioactivity could be recovered by this procedure. In a typical experiment 60% of the radioactivity recovered was found in a peak appearing just before aspartic acid, 85 ml after elution began. See Fig. 1 for ninhydrin values (continuous lines) and radioactivity

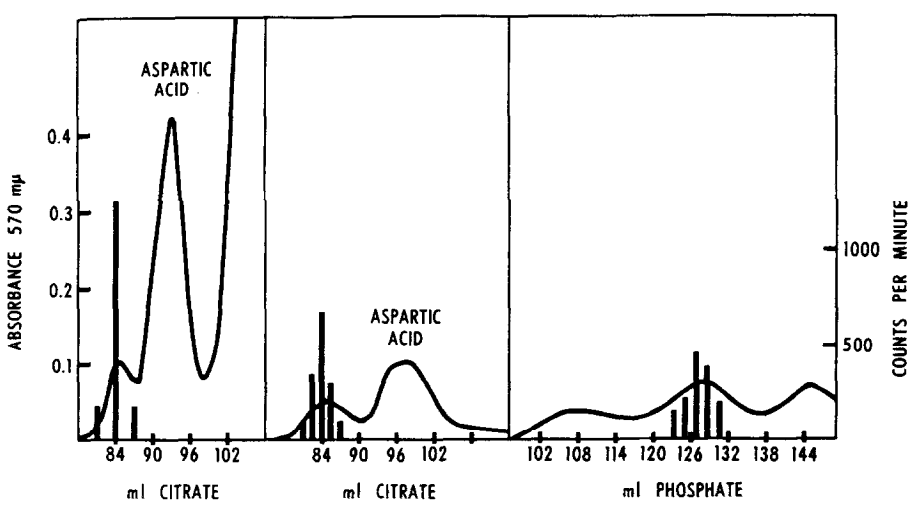


FIGURE 1 FIGURE 2 FIGURE 3

of collected fractions (histograms). Normally all of the remaining radioactivity was found in the glycine peak. At times small amounts of radioactivity appeared in other regions of the chromatogram probably due to incomplete hydrolysis of the γ-glutamyl peptides. In no instance were mobilities of these small amounts of radioactive fractions identical with the mobility of β-aspartyl glycine.

(2) The entire radioactive fraction appearing before the aspartic acid peak was rechromatographed by the same procedure in order to remove some of the aspartic acid contaminant (Fig. 2).

(3) A third chromatographic passage utilized as eluent 0.5M Na_2HPO_4 adjusted to pH 1.82 with phosphoric acid, after the column had been equilibrated with the same eluent (Dorer et al. in press). Three peaks usually appeared, the second peak (125 ml after the beginning of elution) containing all the radioactivity (Fig. 3). The radioactive material was desalted on a Dowex 50 X-8 column (Haley in press), eluted with 1M NH_4OH and freeze dried. Upon hydrolysis, this material always contained glycine and glutamic acid in roughly equal proportions, and at times varying smaller amounts of aspartic acid, alanine and valine (Table 1). These latter contaminants, we concluded, are derived from small amounts of unhydrolyzed peptides composed of aspartic acid and amino acids with non polar side chains which have mobilities very

TABLE I

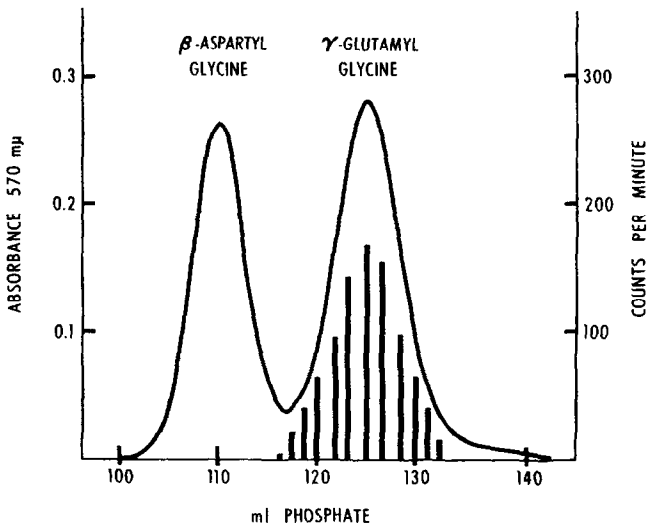
	μ moles				
Experiment	Glutamic acid	Glycine	Aspartic acid	Alanine	Valine
1	0.039	0.041	0	0	0
2	0.031	0.038	0.013	0	0
3	0.030	0.035	0.003	0.003	0.001
4	0.0095	0.012	0	0	0

close to that of γ-glutamyl glycine.

The identity of the radioactive material purified by the three successive column chromatographic passages was established in the following way. A small amount (usually less than 0.02 μ moles) of radioactive material was diluted with 1.0 μ mole of cold synthetic γ-glutamyl glycine (Cyclo Chem. Corp.) and β-aspartyl glycine* previously tested chromatographically for purity. This mixture was then chromatographed according to procedure 3 (i.e. phosphate elution). 10% of the eluent was monitored for ninhydrin positive material, the remaining 90% being collected in 0.5 ml fractions. 200 λ were used for the determination of radioactivity and 200 λ were used for the direct determination of ninhydrin value. This latter device permitted the exact superimposition of the radioactivity over the trace of the monitored ninhydrin value. Figure 4 shows the results of one of several such experiments. It demonstrates the perfect correspondence between the mobility of the synthetic γ-glutamyl glycine and the radioactive material purified from the ^{14}C-GEE fibrin derivative. In all hydrolysates studied by this procedure the fraction containing the highest radioactivity also contained the highest ninhydrin value. In no case did we find radioactivity in the β-aspartyl glycine peak which was identical in mobility with the β-aspartyl glycine marker.

Recently Tyler and Laki (in press) have purified guinea pig liver transglutaminase on DEAE - cellulose and have obtained two peaks, each capable of converting soluble fibrin into insoluble

* We wish to thank Dr. D. L. Buchanan of the Veterans Administration Hospital, West Haven, Conn., for the gift of β-aspartyl glycine and particularly for the valuable advice he offered during this investigation.



fibrin but only one of them capable of acting on CBZ-L-glutaminyl glycine. We have found that the plasma transglutaminase is not capable of using CBZ-L-glutaminyl glycine ethyl ester as a substrate and it is therefore possible that it is identical with or at least similar to one of the two enzymes found in liver by Tyler and Laki.

Studies at present underway carried out in collaboration with Dr. A. F. H. Britten on the plasma and platelets of a patient with a congenital inability to convert soluble fibrin into insoluble fibrin demonstrate that both the plasma and platelets are incapable of incorporating ^{14}C-GEE into fibrin or casein (Britten et al. unpublished).

These recent findings confirm the transamidase theory of insoluble fibrin formation and show that the plasma and the platelet transglutaminase enzymes are coded at least in part by the same genetic locus.

The results we have discussed help to establish the transglutaminase mechanism of insoluble fibrin formation. The identity

of the amino acid residues involved as amine donors in the cross-linking reaction will as yet have to be determined by direct measurement.

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