A TRANSAMIDASE MECHANISM FOR INSOLUBLE FIBRIN FORMATION

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The plasma of blood contains a factor which in the presence of calcium brings about the formation of an insoluble fibrin clot (Barkan and Gaspar, 1923; Robbins, 1944; Laki and Lorand, 1948). It has been shown that this factor is an enzyme which acts on its substrate, soluble fibrin, and converts it into insoluble fibrin (Loewy et al, 1961). Recent work (Buluk, 1961; Lorand and Konishi, 1962) suggests that the enzyme is found both in platelets and in plasma in an inactive form presumably being activated by thrombin during the clotting reaction. Having on previous occasions referred to the enzyme as "fibrinase" (it is also called the Laki-Lorand factor and the fibrin stabilizing factor) we shall for the moment continue to use this term to denote the active form of the enzyme. When the mechanism of its action will have been unambiguously elucidated it may be desirable to seek general agreement on a more descriptive name.

A number of theories have been proposed to account for the transformation of fibrin soluble in a variety of mild solvents, into fibrin insoluble in all reagents which do not split disulfide or peptide bonds. These theories include: disulfide exchange (Loewy and Edsall, 1954), specific partial proteolysis followed by disulfide exchange (Loewy, Gallant and Dunathan, 1961), transpeptidation or peptide bond synthesis with a brief reference to the possibility of transamidation (Lorand, Konishi and Jacobsen, 1962), and an effect on the carbohydrate moiety of soluble fibrin resulting possibly in Schiff-base like bonds (Laki and Chandrasekhar,1963). We shall present evidence which suggests that fibrinase catalyzes a transamidation reaction between the N-terminal

glycine amino groups of one fibrin monomer and the carboxylamide of asparagine or glutamine of the adjacent fibrin monomer.

We were led to this conclusion at first because our search for a proteolytic mechanism of fibrinase action gave a series of negative results (Loewy, 1964). Thus:

(1) Studies in the pHstat of 12 different peptides or peptide derivatives known to act as substrates for a number of proteolytic enzymes, yielded no evidence for any proteolytic activity of fibrinase.

(2) Measurements in the pHstat of the action of fibrinase on a suspension of finely divided soluble fibrin revealed that at pH 8-8.5, instead of the release of protons expected in a proteolytic reaction there was in fact an absorption of protons, some 3-5 protons being absorbed per fibrin monomer.

(3) Measurements of volume changes during the formation of insoluble fibrin, using dilatometers kindly made available by Professor Walter Kauzmann, revealed a volume increase rather than the volume decrease generally observed for the hydrolysis of the peptide bond.

(4) A chromatographic study of the extracts obtained from insoluble fibrin with a number of solvents including trichloroacetic acid, revealed that no new peptides appear after the action of fibrinase on soluble fibrin. In this we have confirmed the work of Laki and Chandrasekhar (1963).

Our search for a new peptide nevertheless did reveal one important difference between the chromatograms of the soluble and insoluble clot liquors. These chromatograms were obtained from clots extracted with 0.2 M sodium formate at pH 7.0. The extract was then acidified to pH 3.0 and placed on a column of Aminex (AG 50W-X2 200-325 mesh). Elution was carried out by raising ionic strength and pH from 0.2 M sodium formate, pH 3.0 to 2.0 M sodium acetate, pH 5.0, a "Technicon" automatic analyzer being employed for the ninhydrin reaction. Five peaks, three large ones and two small ones were resolved under these conditions. The only significant and reproducible difference between the chromatograms of soluble and insoluble fibrin clotted in the presence of EDTA and calcium respectively was in the 4th peak which was consistently

2-3 times larger in the insoluble fibrin extract. This peak proved to be due to ammonia. We estimate that two molecules of ammonia are released per molecule of fibrin monomer. We are at present refining our methods in order to obtain a more reliable value for the stoichiometry of the ammonia release.

The release of ammonia which we have observed is characteristic of a crosslinking reaction catalyzed by certain transamidases (Schweet and Borsook, 1953; Neidle, Mycek, Clarke and Waelsch, 1958). These enzymes have been shown to catalyze a reaction in which the γ-carboxyl amide group of glutamine acts as an acceptor to the ϵ-amino group of lysine with the formation of an interchain peptide bond and the release of ammonia. In addition to the cross-linking action of fibrinase and the release of ammonia, there are a number of other startling similarities between fibrinase and these transamidase enzymes. Thus for both: calcium is a necessary cofactor, sulphydryl compounds are activators, iodoacetate and heavy metals are poisons, diisopropyl fluorophosphate has no inhibitory effect and there is no proteolytic activity on known peptides.

It has been observed by Middlebrook (1955) and confirmed by Lorand, Konishi and Jacobsen (1962) that there is a loss of glycine N-terminal groups when soluble fibrin is converted to insoluble fibrin. The interesting observation by the latter authors that glycyl-glycine and some of its derivatives act as fibrinase inhibitors also suggests that the N-terminal glycine groups might be involved in the cross-linking reaction. Furthermore since the N-terminal glycine amino group has a pK of 7.7-8.2 and ammonia a pK of 9.2, one would expect a transamidation involving the glycyl amino group to be accompanied by an absorption of protons as indeed we have observed. The magnitude of the volume increase during insoluble fibrin formation which we have observed (Loewy, 1964) could be accounted for by the release of ammonia and some carbohydrate molecules (Laki and Chandrasekhar, 1963).

A transamidase theory involving N-terminal glycine amino groups would predict that the inhibitory role of glycyl-glycine is brought about by the incorporation of the dipeptide into the fibrin monomer thus preventing the

cross-linking reaction between monomers. We have been able to show that such an incorporation indeed takes place. We used 19 mg. of fraction 3 (Loewy, Dunathan, Kriel and Wolfinger, 1961) a fibrinase-rich fibrinogen, 39 mg. glycyl-glycine and 0.5 mg. of C^{14} glycyl-glycine (specific activity 0.87 millicuries/millimole) and clotted in the presence of $5x10^{-2}$ M cysteine and $5x10^{-2}$ M calcium. The final volume of the clot was 2 ml of 0.15 M KCl at pH 7.5 The procedure employed was to incubate the clots for 4 hours and then to extract and wash repeatedly with 5% TCA, then to digest the precipitate with 2 ml of 6 N HCl at 100° C for 2 hours and evaporate to dryness in the counting planchets. With such a system we have obtained the incorporation of some 200 cpm (counting efficiency 9.7%) while the controls involving the same components but with $5x10^{-3}$ M EDTA substituted for the calcium gave counts at background levels.

These preliminary results indicate that some 4-5 molecules of glycyl-glycine are incorporated per fibrin monomer (molecular weight $3.2x10^{5}$).

While the transamidases studied by Neidle, Mycek, Clarke and Waelsch (1958) catalyse a reaction involving glutamine, it may well be as is suggested by the work of Chandrasekhar and Laki (this issue) that asparagine is involved in the fibrinase catalyzed reaction. Work is in progress to test this possibility and to obtain further evidence for other aspects of the transamidase theory.

The work of Blombäck and Yamashina (1958), Gladner, Folk, Laki and Carroll (1959), Henschen (1962), Clegg and Bailey (1962) and Haschemeyer and Nadeau (1963) provides us with the following picture of the fibrin monomer: it is composed of two symmetrical halves each consisting of three different peptide chains, a T-chain with an N-terminal tyrosyl group and an A-chain and B-chain each with an N-terminal glycyl group. Both these N-terminal glycyl groups are formed during soluble fibrin formation by the thrombin catalyzed cleavage of peptides A and B from the A and B chains. This picture provides us with an explanation for the specificity of the fibrinase for its soluble fibrin substrate, since only when the action of thrombin has revealed the

4 glycine N-terminal groups is it possible for the transamidation reaction to occur, thus producing the physiologically functional insoluble fibrin network.

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