FIBRIN GEL STRUCTURE: INFLUENCE OF CALCIUM AND COVALENT CROSS-LINKING ON THE ELASTICITY

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SUMMARY: Calcium ion causes the development of a higher elastic modulus in fibrinogen solutions clotted by thrombin. By also measuring the development of covalent crosslinks introduced by activated Factor XIII, we find that (a) calcium ion causes an overall increase of the modulus, (b) covalent crosslinking of α-chains causes an increase of the modulus, while (c) covalent crosslinking of γ-chains does not.

Introduction: The use of elasticity measurements as a tool in studying the clotting of fibrinogen solutions was initiated by Ferry and coworkers (1,2). The theoretical basis for this work is that the elastic modulus is a measure of 1) the stiffness of the strands making up the network of molecules in the gel and 2) the concentration of branchpoints of the network*. Both quantities are of interest, in that they tell us about the structure of the gel; and, furthermore, the elasticity can be measured readily in gels which are too opaque for the optical methods commonly used to study protein solutions. In order to make the elastic measurement a useful tool in a study of the clotting of fibrinogen, we have used a basically simple, but sensitive Couette elastometer, and have measured the elastic modulus of fibrin gels and of fibrinogen solutions clotting under the action of thrombin. We report here the difference in the elastic modulus when the fibrin molecules are covalently crosslinked, and when they are not. These crosslinks are introduced by an enzyme, activated plasma Factor XIII (3-5), which catalyzes the formation of

*Network branchpoints are commonly called crosslinks by polymer physicists. However, the word crosslinks is used by biochemists, and here by us, to describe the linkage of two protein molecules.

isopeptide bonds between ε-amino groups of lysine and γ-carboxyamide groups of glutamine and requires calcium ion for activity. Our fibrinogen preparation contains both Factor XIII and calcium. Thus, steps must be taken to remove all the free and tightly bound calcium. Then, by adding $CaCl_2$ to the solution, one can control the formation of covalent crosslinks in the clot.

Materials & Methods: Bovine fibrinogen was obtained from Miles laboratory. Most experiments reported here were done with lot 15, which we found to contain approximately three calcium ions per fibrinogen molecule. Earlier experiments were done with lot 14, which is calcium free by our assay. Both preparations were contaminated by Factor XIII as evidenced by the formation of covalent crosslinks upon clotting with purified thrombin (Parke-Davis). The tightly bound calcium was removed by dialysis of the fibrinogen stock solution (approximately 15 mg/ml) with 0.02 M EDTA at pH 8.0 for 10 hours at 4°C. The calcium content was assayed using a Model 306 Perkin-Elmer flame spectrophotometer. The elastic moduli were measured with a Couette elastometer (6) which had been slightly modified to permit measurements of lower moduli. Polyacrylamide gel electrophoresis in phosphate buffer pH 7.0, containing sodium dodecyl sulfate and dithiothreitol was performed according to described procedures (7).

Results & Discussion: It has been reported that calcium increases the rigidity of fibrin clots (1,2,8-10), and therefore effects due specifically to the covalent crosslinks and to differences in calcium concentration must be separated. Dialysis affects the structure of the fibrin clot. We find that the removal of tightly bound calcium ions irreversibly reduces the elasticity, in the presence of added calcium, to about 35% of the original value. The elasticity of the fibrin gel clotted from EDTA-treated fibrinogen (lot 15) is the same as that of a calcium-free fibrinogen preparation from the same source (lot 14). The existence of tightly bound calcium in fibrinogen has been demonstrated by many investigators. Endres and Scheraga (11) indicated that the fibrinogen molecule may possess multiple calcium binding sites, differing

in affinity. The work of these authors, as well as that of Godal (12,13), of Bithell (14) and of Blombäck et al. (15) indicates that the tightly bound calcium ions are essential constituents of fibrinogen and fibrin. Reversible changes occur in the fibrinogen molecule in the presence of EDTA, presumably because of the removal of calcium, which lead to an increase of the clotting time and a change in the sedimentation constant. The elasticity data show that there is an irreversible change, besides the reversible change noted by these authors, which persists when EDTA is removed and calcium is added back. All experiments described below regard the behavior of EDTA-treated fibrinogen, but in the absence of EDTA.

Fig. 1 shows the results of two parallel experiments, in which fibrinogen solutions were clotted in the elastometer in the presence and absence of calcium. At short times, the difference between the two curves increases approximately as the modulus itself. At longer times, the modulus of the

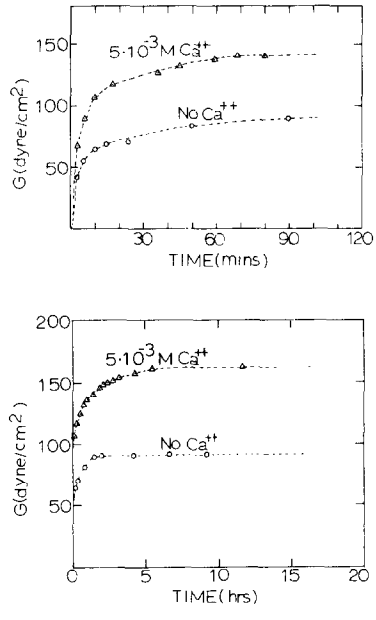

Fig. 1. Elastic modulus of fibrinogen solutions (1.5 mg/ml) clotted by the action of thrombin (1.25 units/ml), in the presence of $CaCl_2$ and without calcium ion, as a function of time (plotted using two different scales). Solvent: 0.1 M NaCl, 0.05 Tris buffer pH 7.4.

gel containing calcium continues to increase slowly when there no longer is any formation of fibrin from fibrinogen, as evidenced by the constancy of the modulus in the absence of calcium. The simplest explanation of these observations is that 1) the strands of fibrin molecules are stiffer when formed in the presence of calcium ion or of active Factor XIII and 2) the strands are further stiffened by chemical crosslinking after the network is complete. One could, for either or both explanations, suppose the presence of a larger concentration of branchpoints in the network for the same concentration of fibrin.

These conclusions are further clarified by polyacrylamide electrophoretic analysis of the covalent crosslinking of fibrin as a function of time, which allows the determination of relative concentrations of α-, β- and γ-chains of which the fibrin molecule is composed. In fibrin not containing added calcium, the concentration of α- and γ-chains varies with time; the γ-band disappears because of the formation of γ-dimer and the α-band disappears because α-polymers are formed. It has been shown that the β-chains of fibrin are not involved in crosslinking (16-18). In Fig. 2 we show photometer tracings of a series of runs as a function of time. Quantitative analysis of these data and another series gave the fraction of α- and γ-chain remaining as a function of time, reported in Fig. 2B and C.

In Fig. 2B and C, we also show the excess modulus due to the presence of calcium and the action of Factor XIII as obtained from the data of Fig. 1, plotted to such a scale as to obtain the best correspondence with analytical data. The excess rigidity observed at short times, which parallels the increase of the rigidity as clotting progresses, is not correlated with the progress of the chemical crosslinking reactions; neither is the disappearance of γ-chains correlated with differences in rigidity. However, the α-polymerization reaction progresses as does the excess rigidity observed at longer times, when the conversion of fibrinogen to fibrin is complete. Thus, we conclude that the modulus of the fibrin network is increased both by calcium

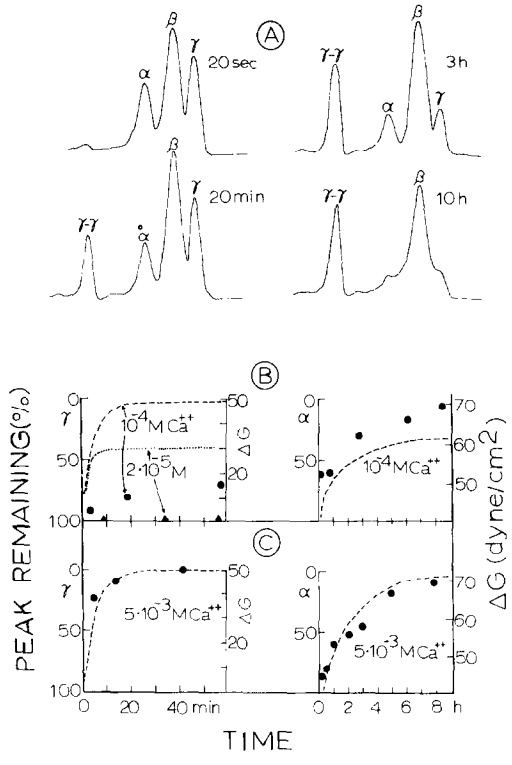

Fig. 2(A). Photometer tracings of 7% polyacrylamide gels containing sodium dodecyl sulfate and dithiothreitol (7) after electrophoresis of fibrinogen clotted with thrombin in the presence of active Factor XIII (10^{-4} M $CaCl_2$). (B). Points indicate fraction of non-crosslinked fibrin α- and γ-chains, versus time, as determined by SDS polyacrylamide gel electrophoresis for solutions containing 10^{-4} M and 2 x 10^{-5} M $CaCl_2$. α- and γ-chain quantities were determined by planimetry of gel scans taken at 570 nm of the Coomassie-blue stained gels; β-chain was used as internal standard. Curves show the difference in elastic modulus from that observed without added $CaCl_2$ (right hand scales). In the presence of 2 x 10^{-5} M $CaCl_2$ very little α-polymer is formed, and the modulus is almost the same at 8 h as at 1 h after clotting. (C). Same as for B, for solutions containing 5 x 10^{-3} M $CaCl_2$.

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ion and by α-α crosslinks, but not by γ-γ crosslinks. In either case, the network stiffening may be the result of an increase in chain stiffness or of an increase in the concentration of network branchpoints. Above we noted an irreversible effect on the elastic modulus due to calcium removal by EDTA. Here we observe a reversible effect on the modulus, in line with the observation of other reversible effects of EDTA noted by several authors (11-15).

The rate of α-polymerization is slow and is not sensitive to calcium

concentration at moderate levels, while the rate of γ-dimerization is much more rapid and is sensitive to calcium concentration. These observations lead us to believe that 1) α-chain crosslinking by activated Factor XIII involves a slow reorganization of the fibrin network as the rate limiting process, either to give sideways thickening of strands or an increase in branchpoints, resulting in the observed network stiffening, but 2) γ-dimerization involves an end-to-end linking of fibrin molecules, which may occur as soon as the fibrin molecules associate (19,20), so that the rate is limited either by the rate of clotting or by the concentration of active enzyme causing the dimerization (Factor XIII-calcium complex), and therefore sensitive to the calcium concentration.

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