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Elasticity of Fibrin and Protofibrin Gels Is Differentially Modulated by Calcium and Zinc

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Key words

Elasticity – Fibrin – Protofibrin – Calcium – Zinc – Fibre thickness

Summary

The mechanical properties of fibrin and protofibrin gels in the presence of physiologic levels of Ca(II) and Zn(II) are described. As monitored with a thrombelastograph, Ca(II) (0.5–2 mM) increases the rate of development and the maximum level of gel elastic modulus (G) of fibrin and protofibrin gels. Zn(II) (10–50 μM) decreases the elastic modulus of those gels, even in the presence of a large excess of Ca(II). This contrasts with the ability of both divalent cations to increase fibrin and protofibrin gel turbidity. Unlike the turbidity or fibre thickness of fibrin and protofibrin gels, both of which are increased by these cations, gel elasticity is increased by Ca(II) but decreased by Zn(II). It is demonstrated that Ca(II) and Zn(II) modulate fibrin and protofibrin gels independently of one another, and that they have opposing effects on the mechanical properties of the gels. The disparity between the visual (turbidity, TEM) and the mechanical (elasticity) properties of (proto)fibrin gels indicates the need for new conceptual and analytic paradigms.

Introduction

The earliest phase of fibrin polymerization is the linear assembly of fibrin oligomers and linear polymers. Subsequently, the polymer chains interact laterally, and thereby form a branched gel structure. The critical feature of coagulation is the formation of a 3-dimensional gel network. This step is particularly sensitive to divalent cations.

Calcium and zinc independently exert great effect on the rate of formation of fibrin and on its ultrastructure (1–4). Both cations accelerate gelation and increase fibre thickness. A consistent feature of the sensitivity of fibrin gel to the divalent cations is that Zn(II) is much more effective than Ca(II) in modulating gel parameters.

Zn(II) does not displace ^{45}Ca(II) during the formation of fibrin (5), and is effective even in the presence of a large excess of Ca(II). This and other evidence (6, 7) indicates that the two divalent cations have independent modes of binding to fibrinogen and of regulating fibrin structure.

Recent investigations show that soluble protofibrils can be induced to undergo a change of state (gelled) by 0.5–2 mM Ca(II) and 10–50 μM Zn(II) without the intervention of active thrombin. Scanning electron microscopy (SEM) of protofibrin gels instigated by divalent cations, were indistinguishable from fibrin, both exhibiting branching, merging, supercoiling and thickening of individual fibres (6). Moreover, the striations observed in transmission electron microscopy (TEM) of negatively stained fibrin and protofibrin gels were identical (~230 Å) and not altered by either Ca(II) or Zn(II) (7). In all, the evidence assembled to date indicates that the underlying mechanism of gelation relates to the cation-sensitive augmentation of the lateral association of relatively short fibrin oligomers (protofibrils).

One might expect other properties of fibrin and protofibrin gels to be affected by Ca(II) and Zn(II). For example, the mechanical properties of fibrin gels are essential for their biologic functions. This report compares the ability of the divalent cations to modulate the elastic modulus (G) and the fibre cross-sections of fibrin and protofibrin gels.

Materials and Methods

Purified human fibrinogen was from Immuno AG (Vienna) and Kabi AG (Stockholm, Sweden); bovine thrombin was Topstasin (Hoffman-La Roche, Basel, Switzerland); hirudin, Tris, and analytic grade reagents were from Sigma (St. Louis, MO). Unless otherwise indicated, reagents and salts were diluted with 0.015 M Tris, 0.15 M NaCl, pH 7.4 buffer. Fibrinogen was dialyzed in Tris buffer under nitrogen at ambient temperature. In order to minimize the effect of factor XIII-induced cross-linking, for some experiments, fibrinogen was first incubated with 3.3 N urea, 4 h, 25° C (8), and extensively dialyzed under nitrogen. Atomic absorption analysis indicated that only traces of Ca(II) (less than 10 μM) remained in the dialyzed fibrinogen solutions. Fibrinogen concentrations were determined by measuring the Abs_{280} (Zeiss PNQ 2 spectrophotometer) using a conversion factor of $E^{1\%}_{280} = 15$.

For the purposes of nomenclature, *protofibrin* is generated by adding cations to soluble *protofibrils*. The latter are formed by activating fibrinogen with thrombin, and inhibiting thrombin by adding hirudin prior to gelation. In a typical protocol, 3 mg/ml fibrinogen is incubated at 25° C with 0.1 U/ml thrombin. At some point prior to gelation (i.e. clotting time [CT] = 140 sec), 0.2 U/ml hirudin was added (i.e. activation time [AT] = 15–110 sec).

The relative turbidity (695 nm, 37° C) of the protofibril solution was monitored prior to, and after, addition of Ca(II) and/or Zn(II) in a 4-channel P-4 aggregometer (Biodata, Hatboro, PA) as previously described (6, 7). The 100% turbidity value was set using 2 mg/ml fibrin with 2 mM Ca and calculating: %TURBID = (100% – %AGGREG).

Elasticity (G) was measured in a 3-channel Helliger thrombelastograph (Freiburg im Breisgau, W. Germany). 9 sec/oscillation, 37° C, with measurements recorded manually at various time points. Generally, coagulating mixtures were prepared in small conical tubes, immediately pipetted into the oscillating well, and the plunger was lowered into the mixture. In a typical procedure used to follow the development of elasticity of cation-induced protofibrin gels, 20 μl of stock solutions of Ca(II) or Zn(II) was placed in the thrombelastograph well, and 340 μl protofibril was added, and the plunger engaged. Final concentration ranges of Ca(II) and Zn(II) were 0–2 mM and 0–50 μM respectively. Elasticity (G; dyne/cm^2) was calculated from the deflection amplitude, using a conversion table supplied by the manufacturer.

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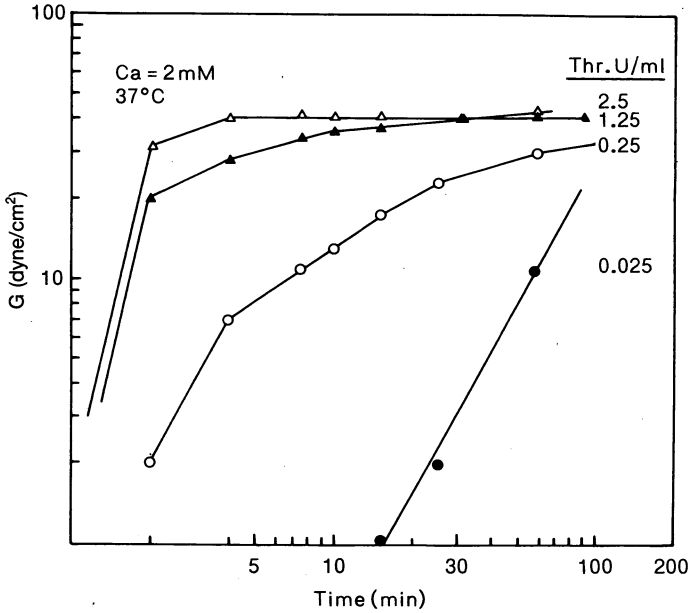

Fig. 1 Elasticity (G, dyne/cm^2) of urea-treated fibrinogen (2 mg/ml), 2 mM Ca(II) versus time, at different levels of thrombin (0.025–2.5 U/ml)

Results

Elasticity of Fibrin Gels

The rate of development of fibrin elasticity (G) is dependent on the concentration of thrombin (Fig. 1). At low levels (less than 0.2 U/ml) of thrombin, the initial rate of G development is linear with time. In the example shown here, gels obtained with more than 0.25 U/ml thrombin achieved G_{max} (~45 dyne/cm^2) within 60 min. Possibly, the lowest curve (with 0.025 U/ml thrombin) would also approach the same G_{max} asymptotically, though a greater time frame would be required. One should note that initial gelation (CT) and the development of maximum clot turbidity are both much more rapid than the evolution of measurable elasticity. For example, with 0.25 U/ml thrombin, CT is on the order of 90 sec, and turbidity plateaues after ~10 min, whereas measurable G requiring at least 2 min to observe and G_{max} requires more than 100 min. This pattern is typical of different stocks and concentrations of fibrinogen.

In agreement with previous reports (9–12), Ca(II) increases the rate of development of G and the G_{max} (Fig. 2A). By contrast, Zn(II) decreases the initial development of fibrin gel elasticity and its maximal value (G_{max}) in a concentration dependent manner (Fig. 2B). A summary of the dependence of fibrin elasticity on the concentration of Ca(II) and Zn(II) is presented in Fig. 3. Here, we observe the individual and combined effects of each divalent cation. Below 0.1 mM, Ca(II) has little effect, whereas above 0.25 mM, it significantly increases G_{max}. By contrast, less than 50 μM Zn(II) decreases the elasticity of fibrin. Even with 2 mM Ca(II), less than 50 μM Zn(II) decreases the elasticity of fibrin in a concentration-dependent manner. Similar results were also observed with fibrinogen which had been treated to remove possible cross-linking by contaminating factor XIII (not shown). In all instances, Ca(II) and Zn(II) have opposing and independent influence on fibrin elasticity.

Elasticity of Protofibrin Gels

The development of Ca(II)-induced protofibrin elasticity is relatively slower than that of fibrin formed with equivalent levels of Ca(II). In the example shown here, 3 mg/ml protofibril (AT 110 sec; CT 140 sec) was gelled by the addition of 2 mM Ca(II) without or with 20 μM Zn(II) (Fig. 4, lower two curves). Measurable G values for the protofibrin gel developed only after 20 min, and eventually plateaued after 200 min. By contrast, the turbidity of the cation-induced protofibrins plateaued by ~4 min (not shown). For the purposes of comparison, the development of 3 mg/ml fibrin initial elasticity at low level of thrombin (0.25 U/ml), ±2 mM Ca(II) and ±20 μM Zn(II), are shown in the upper curves. The ability of Zn(II) to decrease fibrin and protofibrin elasticity is quite clear. It is also apparent that for equivalent protein levels, fibrin gels exhibit greater elasticity than protofibrin gels. Qualitatively similar results were obtained with fibrinogen which had been rendered free of XIII. In all experiments, it was observed that Ca(II) increases, and Zn(II) decreases the elasticity of fibrin and protofibrin gels.

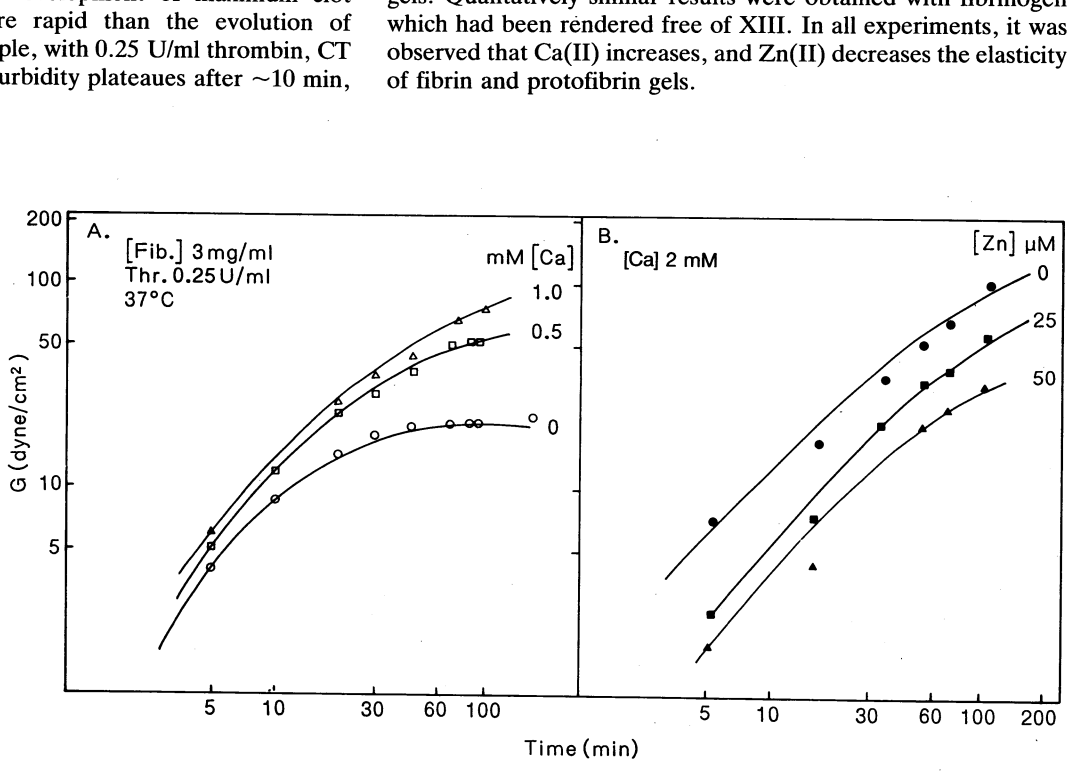

Fig. 2 Elasticity (G, dyne/cm^2) of fibrinogen (3 mg/ml) and thrombin (0.25 U/ml) versus time: (A) With 0, 0.5 and 1 mM Ca(II). (B) With 2 mM Ca(II) and 0, 25 and 50 μM Zn(II). It is apparent that Ca(II) increases, and Zn(II) decreases, the rate of development of G

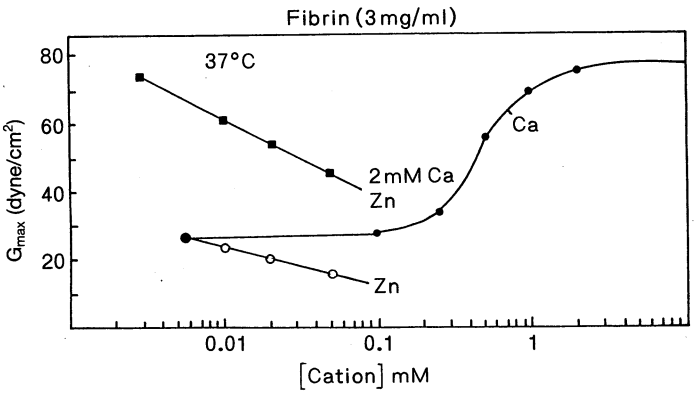

Fig. 3 Maximal elasticity of fibrin (as in Fig. 2) at different levels of Ca(II) (●—●), Zn(II) in the absence (○—○) or presence of 2 mM Ca(II) (■—■). Note that Ca(II) significantly increases G_{max} above 0.25 mM Ca(II), whereas Zn(II) decreases G_{max} without or with a large excess of Ca(II)

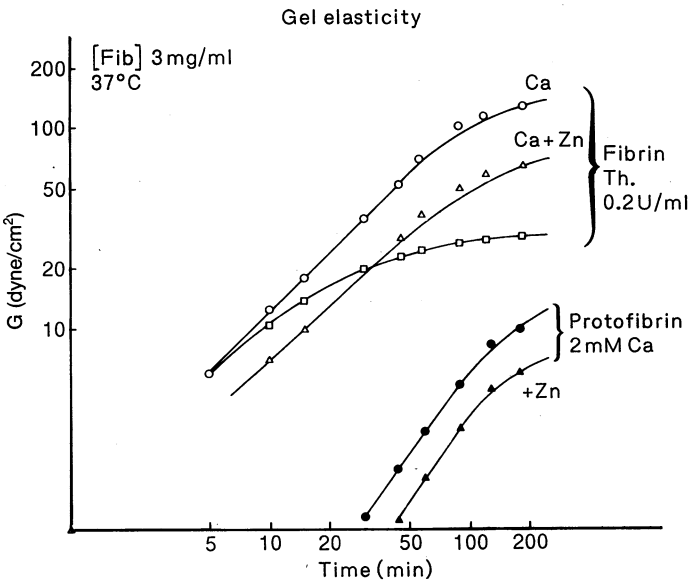

Fig. 4 Elasticity (G, dyne/cm^2) of fibrin (as in Fig. 2), and of protofibrin (3 mg/ml; AT 100 sec; CT 140 sec), 2 mM Ca(II) ± 20 μM Zn(II). Note that G development for protofibrin is much slower and weaker than for fibrin at equivalent levels of divalent cations

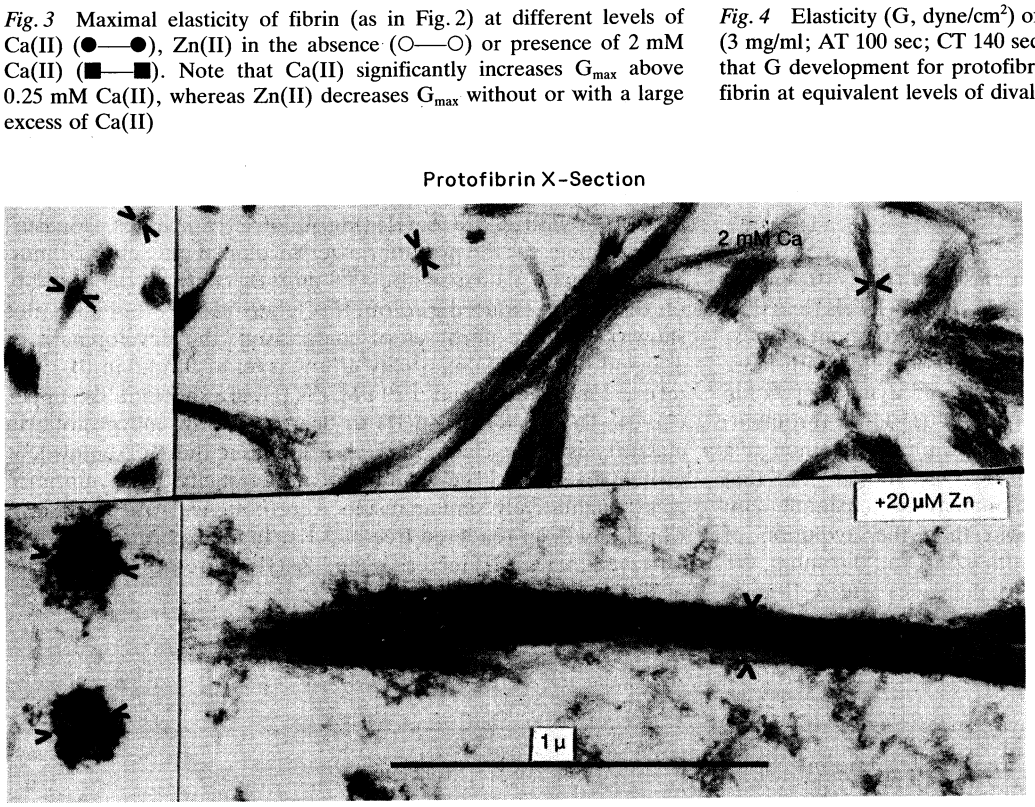

Fig. 5 TEM of protofibrin cross-sections formed with 2 mM Ca(II) with 0 and 20 μM Zn(II). It is apparent that 20 μM Zn(II) increases fibre cross-section from 240–600 Å to 1500–2400 Å, equivalent to the lateral packing of 4 to 10 and 25 to 40 fibrin monomers respectively

TEM of Cross-Sections of Protofibrin Formed with Ca(II) and Zn(II)

Fibre cross section of protofibrin gels formed with 2 mM Ca(II) range between 240–600 Å (Fig. 5 A). With the addition of Ca(II) and 20 μM Zn(II), fibre cross-sections increase to 1500–2400 Å (Fig. 5 B). Thus, the protofibrin fibres are composed of between 4 to 10 packed monomers, and the fibres formed with Zn(II) contain 25 to 40 laterally packed monomers (60 Å monomeric cross-section). These changes are in agreement with the turbidity of the protofibrin gels, which increase with Zn(II) levels. As with fibrin, both turbidity and TEM indicate that Zn(II) increases protofibrin fibre diameter.

Discussion

"Physicality" is the essential property of fibrin which dictates its importance in coagulation. While visual studies are often more convenient for determining initial gelation times, direct viscoelastic measurements are required in order to understand the relation between the molecular organization of fibrin gel and its mechanical properties. In agreement with other studies (10, 11), we note that the rate of development of elasticity (G) is proportional to thrombin levels (Fig. 1) and that Ca(II) increases the elasticity of fibrin (Fig. 2). These experiments serve to orient us with the work of other laboratories (9–12). Within this

framework, it is apparent that Zn(II) decreases the elasticity of fibrin gels (Figs. 2, 3).

Protofibrin gels induced by Ca(II) also develop elasticity (Fig. 4), albeit at a slower rate and with generally lower absolute values, than those directly formed as with fibrin. Here too, Zn(II) significantly decreases protofibrin elasticity (Figs. 2–4) even with much larger levels of Ca(II). This is contrary to the intuition gained from visual inspection of fibrin and protofibrin gel cross-sections. Here, both Ca(II) and Zn(II) increase gel turbidity, which indirectly reflects fibre thickness (1, 14). This is also verified by the TEM of cross-sections of protofibrin fibres (Fig. 5). Relatively small levels of Zn(II) (i.e. 20 μM) result in greatly increased fibre cross section, from 4 to 8 monomeric units to over 25 (using 60 Å as the monomeric dimension). It is apparent that fibre diameter increases in proportion to Zn(II) levels. Notwithstanding, the elasticity of the larger fibres formed with Zn(II) decreases.

Such opposing trends with fibrin gels have been reported before with dextran (13, 14), and with a case of dysfibrinogenemia (15). In those reports, fibrin turbidity also increased with a concomitant decrease of mechanical strength. No rationale for those paradoxical findings were suggested. Here too, the observation presented for consideration are similar in that Zn(II) increases fibrin and protofibrin fibre diameter, but decreases gel elasticity.

This may relate to physiologic function. The release of calcium from activated platelets may accelerate the formation of protofibrin clots near their surface. Pössibly because activated platelets also release Zn(II) (16), the earliest formed protofibrin gels would be thick and coarse, but relatively weak. Such gels would be porous, and allow easy flow-through of fluids. Subsequently, more protofibril units would augment the nucleating gel and reinforce its original structure.

In any case, the above experiments illustrate that protofibrin clots are substantial enough to exhibit viscoelastic properties. It is apparent that Ca(II) and Zn(II) modulate fibrin and protofibrin independently, and that they have opposing effects on the mechanical properties of the gels. No obvious rationale for this is apparent. Possibly, the lateral packing density of (proto)fibrin strands is increased or decreased by Ca(II) and Zn(II) respectively. X-ray diffraction might be helpful for evaluating this though previous X-ray studies have shown the difficulties and limitations of this technique when applied to fibrin gels (17, 18). In all, it seems that under physiologic concentrations of calcium and zinc, initial gelation of thrombin-activated fibrinogen may actually be due to the cation-induced lateral association of protofibrils. The aim of future efforts should be to formulate a fibrin model which incorporates the effects of various cations and metabolites on the kinetics of formation, on the ultrastructure and on the mechanical properties of fibrinogen-derived gels.

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