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Influence of Ca^{2+} on the structure of reptilase-derived and thrombin-derived fibrin gels

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The effects of Ca^{2+} ion on the structure of thrombin-derived and reptilase-derived fibrin gels formed at various ionic strengths were studied turbidimetrically. For both enzymes clotting times were shorter, final gel turbidities were higher and fibre mass/length ratios were increased as the ionic strength was lowered. The addition of 5 mM-Ca^{2+} augmented each of these effects for any given ionic strength. In the thrombin system, Ca^{2+} increased the final gel turbidity from 0.04 to 0.26 $A_{632.8}$ at ionic strength 0.15. Under identical conditions in the reptilase system, the final gel turbidity increased from 0.03 $A_{632.8}$ in the absence of Ca^{2+} to 0.345 $A_{632.8}$ in the presence of 5 mM-Ca^{2+}. In the thrombin system, fibre mass/length ratios increased from 0.4×10^{12} to 6.9×10^{12} Da/cm in the absence of Ca^{2+}, and from 4.4×10^{12} to 7.9×10^{12} Da/cm in the presence of Ca^{2+}, as the ionic strengths were decreased from 0.15 to 0.08 and to 0.11 respectively. In the reptilase system, the mass/length ratios increased from 0.9×10^{12} to 5.8×10^{12} Da/cm in the absence of Ca^{2+}, and from 4.8×10^{12} to 8.7×10^{12} Da/cm in the presence of Ca^{2+}, as the ionic strengths were decreased from 0.15 to 0.08 and to 0.10 respectively. At ionic strengths below 0.10, the presence of 5 mM-Ca^{2+} caused precipitation and macroscopic aggregation of fibrinogen upon the addition of either enzyme. In the presence of 5 mM-Ca^{2+}, the fibres composing thrombin-induced and reptilase-induced gels were virtually identical.

INTRODUCTION

The final stages of blood coagulation involve the thrombin-mediated cleavage of two pairs of fibrinopeptidase, A and B, from the α- and β-chains of the fibrinogen molecule (Bettelheim & Bailey, 1952; Lorand, 1952). Since fibrinopeptide A is removed at a higher rate (Bettelheim, 1956; Blombäck & Vestermark, 1958), it was initially postulated that fibrinopeptide A removal was responsible for fibre lengthening, whereas fibrinopeptide B removal resulted in lateral association and fibre thickening (Laurent & Blombäck, 1958; Laki & Gladner, 1964). The discovery and application of snake-venom enzymes such as reptilase, which only removes fibrinopeptide A from fibrinogen, demonstrated that fibrinopeptide B removal was not a prerequisite to gel formation (Blombäck *et al.*, 1957; Laurent & Blombäck, 1958; Bilezikian *et al.*, 1975). Although under similar conditions reptilase-induced gels are less turbid than thrombin-induced gels (Laurent & Blombäck, 1958), gels composed of thick fibres are possible with reptilase under conditions of low ionic strength or high Ca^{2+} concentraton (Shen *et al.*, 1977). Thus, as with gelation, fibrinopeptide B removal, though favouring it, is not a prerequisite for the formation of thick fibres.

The role of Ca^{2+}, a known enhancer of clotting, in thrombin-induced fibrin polymerization appears to be multifaceted. Early observations of enhanced monomer aggregation in the presence of Ca^{2+} (Lorand & Konishi, 1964; Boyer *et al.*, 1972; Endres & Scheraga, 1972; Brass *et al.*, 1978) have been followed by the demonstration of specific Ca^{2+}-binding sites on the fibrinogen molecule (Marguerie *et al.*, 1977; Purves *et al.*, 1978; Van Ruijven-Vermeer *et al.*, 1978; Nieuwenhuizen & Haverkate, 1983). The presence of Ca^{2+} on these binding sites imparts new properties to the molecule, increasing its heat-stability (Ly & Godal, 1973; Marguerie, 1977), altering its degradation by plasmin (Haverkate & Timan, 1977; Lawrie & Kemp, 1978; Purves *et al.*, 1978; Nieuwenhuizen & Haverkate, 1983) and changing its electron-microscopic appearance (Williams, 1981). Ca^{2+} at higher concentrations shortens clotting times and enhances fibre size (Okada & Blombäck, 1983*a*).

The present paper defines the influence of fibrinopeptide B release, in the presence and in the absence of Ca^{2+}, on the size of fibrin fibres formed at various ionic strengths. The results are interpreted in the light of current kinetic theory of fibrin polymerization (Hantgan & Hermans, 1979; Hantgan *et al.*, 1980, 1983) and of thermodynamic data on the impact of Ca^{2+} binding on fibrinogen stability (Donovan & Mihalyi, 1985; Mihalyi & Donovan, 1985).

EXPERIMENTAL

Human fibrinogen (grade L; A.B. Kabi, Stockholm, Sweden) was dissolved in 0.3 M-NaCl at 25 °C. The solution was clarified by centrifugation at 37000 *g* for 20 min, dialysed against 0.3 M-NaCl for 24 h to remove free Ca^{2+}, and frozen at −70 °C. Clottability was 97% by the method of Laki (1951). Fibrinogen concentration was determined from absorbance at 280 nm, with an absorption coefficient of 1.6 $ml \cdot mg^{-1} \cdot cm^{-1}$. This fibrinogen preparation contained trace amounts of Factor XIII. This contamination was considered to be acceptable

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given the minimal impact of Factor XIII on fibrin fibre size (Carr *et al.*, 1985).

Reptilase was purchased as Atroxin (*Bothrops atrox* venom protein) from Sigma Chemical Co., dissolved in deionized water and used without further purification. It was maintained in an ice/water bath and used within 2 h of being dissolved.

Purified human thrombin (2000 NIH units/ml) was kindly provided by Dr. John Fenton, New York State Department of Health (Fenton *et al.*, 1977). It was diluted with 0.3 M-NaCl to a concentration of 20 NIH units/ml and frozen at −90 °C until use. Upon thawing, it was maintained in an ice/water bath and used within 2 h. Gels formed by the combination of purified fibrinogen and thrombin did not dissolve when left at room temperature for 7 days, illustrating the virtual absence of plasmin and plasminogen from the system.

Turbidity measurements were made at 22 °C with a Cary 118C spectrophotometer. Gels were formed at pH 7.4 in 0.05 M-Tris/HCl buffer. The ionic strength of the clotting solution was adjusted by varying the amount of NaCl. The effects of Ca^{2+} were investigated by forming gels of various ionic strengths in the presence or in the absence of 5 mM-$CaCl_2$ and by varying the Ca^{2+} concentration at a constant ionic strength (0.15). Gels were formed directly in the cuvette by the addition of the appropriate enzyme at time zero. Thrombin was added to a final concentration of 1.0 NIH unit/ml, and reptilase was added to a final concentration of 0.25 μg/ml. The kinetics of early turbidity increase during clotting were measured at the HeNe laser line (632.8 nm). Turbidity was monitored for 10 min, after which time gelation was allowed to go to completion unobserved. After 24 h the gels were scanned from 400 to 800 nm and the mass/length ratios of the fibrin fibres were determined according to the following equation (Carr & Gabriel, 1980*a*,*b*):

$$\tau = [(88/15)\pi^3 n(dn/dc^2 C\mu]/N\lambda^3$$

where τ is the turbidity, n the solution refractive index, dn/dc the refractive-index increment, λ the wavelength, C the concentration of fibrinogen in g/ml, N Avogadro's number and μ the mass/length ratio. For clear gels μ was determined from the slope of a plot of τ versus $1/\lambda^3$. For more turbid gels, where the radius of the fibres is no longer small relative to the incident wavelength, μ was obtained from the inverse of the intercept of a plot of $C/\tau\lambda^3$ versus $1/\lambda^2$ (Carr & Hermans, 1978).

RESULTS

The influence of ionic strength and Ca^{2+} on the kinetics of fibrin gels formed by the action of thrombin is illustrated in Fig. 1. Fig. 1(*a*) is for gels formed in the presence of 5 mM-$CaCl_2$, and Fig. 1(*b*) presents data from gels formed in the absence of added Ca^{2+}. In both instances the initial rate and the final turbidity increase with decreasing ionic strength. In this system turbidity continued to increase for several hours. The presence of Ca^{2+} augments the effect of ionic strength on initial rate of turbidity increase and enhances the final gel turbidity. The Ca^{2+}-induced enhancement of gel turbidity is greatest at higher ionic strength, 0.15, as illustrated by an increase in final turbidity from 0.04 $A_{632.8}$ in the absence of Ca^{2+} to 0.26 $A_{632.8}$ in the presence of Ca^{2+}. This represents a 6.5-fold increase.

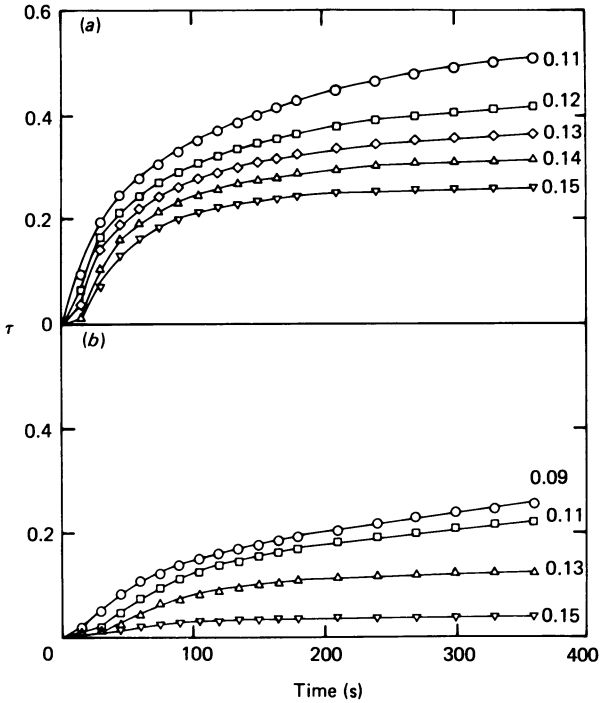

Fig. 1. Influence of Ca^{2+} concentration and ionic strength on the kinetics of turbidity increase during thrombin-mediated fibrin assembly

Turbidity was measured at 632.8 nm with time zero being the moment of thrombin (1 unit/ml) addition. Gels in (*a*) were formed in the presence of 5 mM-$CaCl_2$, whereas those in (*b*) were formed in the absence of added Ca^{2+}. The ionic strength is indicated at the far right in each panel. Other clotting conditions include: pH 7.4, 0.05 M-Tris/HCl and 1 mg of fibrinogen/ml.

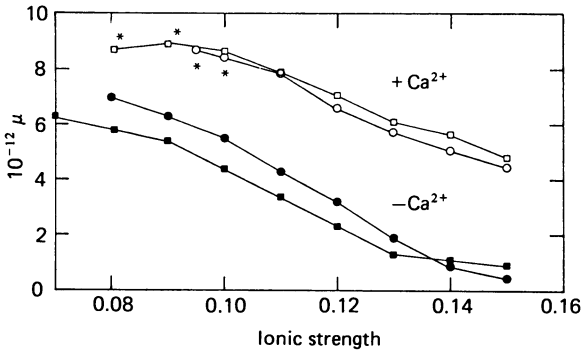

Fig. 2. Effects of ionic strength and Ca^{2+} on thrombin-derived and reptilase-derived fibrin fibre mass/length ratio, μ

Data for gels induced by thrombin are plotted as circles (○ and ●) and those mediated by reptilase are depicted as squares (□ and ■). The ○ and □ symbols are for gels formed in the presence of 5 mM-$CaCl_2$, and the ● and ■ symbols are for gels formed in the absence of added Ca^{2+}. Precipitation was noted in the asterisked data points. Other clotting conditions are the same as for Fig. 1.

The effect of ionic strength and Ca^{2+} on thrombin-derived fibrin fibre mass/length ratio, μ, is illustrated in Fig. 2. In the absence of Ca^{2+} (●) μ increases from 0.4×10^{12} to 6.9×10^{12} Da/cm as the ionic strength is decreased from 0.15 to 0.08. In the presence of Ca^{2+} (○)

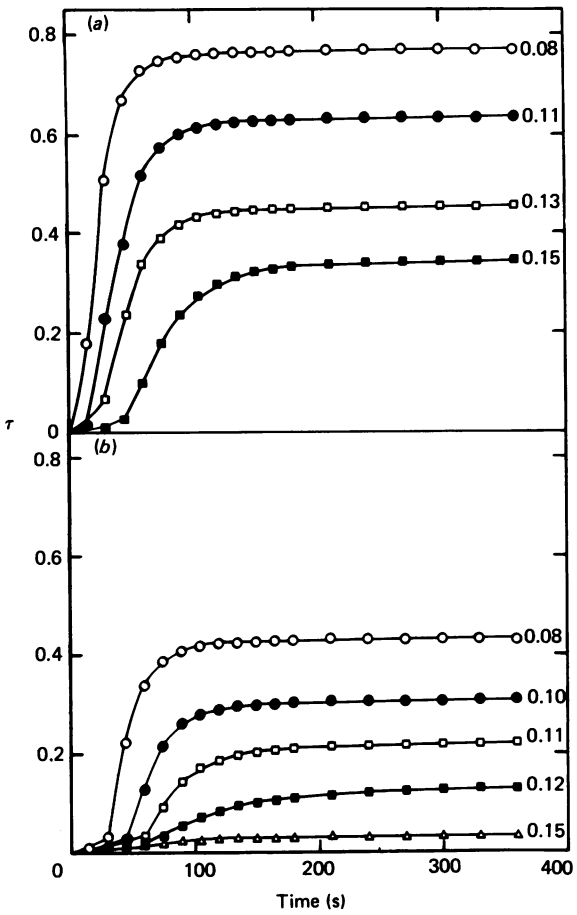

Fig. 3. Influence of ionic strength and Ca^{2+} on the kinetics of reptilase-induced turbidity increase during fibrin gel assembly

Turbidity was measured at 632.8 nm with time zero being the moment of reptilase (0.25 g/ml) addition. Gels in (*a*) were formed in the presence of 5 mM-$CaCl_2$, and those in (*b*) were formed in the absence of added Ca^{2+}. The ionic strength is indicated at the far right in each panel. Other clotting conditions include: pH 7.4, 0.05 M-Tris/HCl and 1 mg of fibrinogen/ml.

the fibres are much larger for any given ionic strength. For Ca^{2+}-containing gels μ increases from 4.4×10^{12} to 7.9×10^{12} Da/cm as the ionic strength is decreased from 0.15 to 0.11. Below an ionic strength of 0.11, precipitation and macroscopic aggregate formation were noted during clotting. This process produced inhomogeneous gels, preventing the measurement of mass/length ratios by turbidity.

The influence of ionic strength and Ca^{2+} on the early kinetics of fibrin gels formed by the action of reptilase is illustrated in Fig. 3. Fig. 3(*a*) is for gels formed in the presence of 5 mM-$CaCl_2$, and Fig. 3(*b*) shows data from gels formed in the absence of added Ca^{2+}. As was the case with thrombin, in both instances the initial rate of rise and the final turbidity increase with decreasing ionic strength. Although the initial rise in turbidity after addition of reptilase occurs later than with thrombin, the turbidity plateaus sooner. At 300 s the turbidity of reptilase-induced gels was frequently higher than that of thrombin-induced gels. However, there was minimal increase in gel turbidity after this period. Thus, for similar conditions, the final turbidity of gels formed with the use of thrombin was higher than that of gels formed with the use of reptilase. As in the thrombin system, the presence of Ca^{2+} augments the effect of ionic strength on the initial rate of turbidity increase and enhances the final gel turbidity. When 5 mM-Ca^{2+} was added, the final turbidity increased from 0.03 to 0.345 $A_{632.8}$ at an ionic strength of 0.15 and from 0.43 to 0.77 $A_{632.8}$ at an ionic strength of 0.08.

The effects of ionic strength and Ca^{2+} on the mass/length ratio of reptilase-induced fibrin are illustrated (□) in Fig. 2. In the absence of Ca^{2+} (■) the mass/length ratio increases from 0.9×10^{12} to 5.8×10^{12} Da/cm as the ionic strength is decreased from 0.15 to 0.08. In the presence of Ca^{2+} (□) the fibres are much larger for any given ionic strength. For Ca^{2+}-containing gels μ increases from 4.8×10^{12} to 8.7×10^{12} Da/cm as the ionic strength is decreased from 0.15 to 0.10. Below an ionic strength of 0.10, as seen with thrombin, precipitation and macroscopic aggregate formation were noted during clotting. This prevented the measurement of μ below this point. Comparison of μ from thrombin-induced and reptilase-induced gels reveals that, in the absence of Ca^{2+}, for any given ionic strength fibres are larger in the thrombin system. In the presence of Ca^{2+} the fibres are virtually identical in both systems.

DISCUSSION

The finding that reptilase-mediated fibrin gels are very similar to thrombin gels is in good agreement with the report by Shen *et al.* (1977). The structures of both types of gels depend on ionic strength and Ca^{2+}. In the presence of millimolar concentrations of Ca^{2+} the gross fibre size is virtually the same for both types of gels. This is in agreement with the report by Shainoff & Dardik (1983) of minimal contribution of b–b coupling to monomer aggregation.

The apparent minimal role of fibrinopeptide B in fibrin assembly has prompted speculation as to its physiological purpose. Several authors have postulated that slow fibrinopeptide B release allows removal of partially cleaved fibrinogen from the circulation and thus serves to prevent intravascular coagulation (Muller-Berghaus *et al.*, 1976; Shen *et al.*, 1977; Dardik & Shainoff, 1980; Gonda & Shainoff, 1982). Additionally, fibrinopeptide B release may cause a subtle intrafibre molecular rearrangement serving to strengthen molecular contacts between the constituent polymer elements while enhancing contact site orientation for enzymes such as Factor XIII and plasmin. Studies consistent with this hypothesis have already been reported (Furlan *et al.*, 1976; Okada & Blombäck, 1983*b*).

Enhancement by Ca^{2+} of fibrin polymerizaton has been studied from several approaches. Except for one report of Ca^{2+}-dependent proton release during peptide cleavage (Marguerie *et al.*, 1979), all studies have failed to demonstrate an effect of Ca^{2+} on thrombin-induced peptide release (Elias & Iyer, 1967; Boyer *et al.*, 1972; Blombäck *et al.*, 1978; Hardy *et al.*, 1983). Fibrin–fibrinogen dimer formation (Marguerie *et al.*, 1979), the ultimate size of fibrin fibres induced by thrombin and reptilase (Okada & Blombäck, 1983*a*) and Factor XIII induced cross-linking are all Ca^{2+}-dependent events (Folk & Finlayson, 1977; Credo *et al.*, 1978). The final result of these Ca^{2+}-mediated processes is a fibrin gel with increased elastic modulus and strength (Shen *et al.*, 1974).

It has been shown that the enthalpy of thermal denaturation and the denaturation temperature of the D-domain increase, in the presence of Ca^{2+}, during clotting with thrombin (Donovan & Mihalyi, 1985). Clotting with reptilase, which does not remove fibrinopeptide B, failed to lead to increased enthalpy (Mihalyi & Donovan, 1985). The altered thermodynamic stability was not due to Factor XIII-induced cross-linking. The authors concluded the changes in enthalpy were the result of intermolecular interactions of the D nodules in the clot, which are hindered by the presence of fibrinopeptide B and strengthened by Ca^{2+} bound to the high-affinity binding sites of fibrin.

If, as proposed by Hantgan and co-workers (Hantgan & Hermans, 1979; Hantgan *et al.*, 1980, 1983), the length of the photofibril is the key to lateral association, the impact of fibrinopeptide B release and Ca^{2+} concentration on fibrin assembly can be put in perspective. When fibrinopeptide A is removed, monomer–monomer and monomer–fibrinogen interactions are favoured and protofibril formation begins. Although not a prerequisite for monomer–monomer interaction, the additional removal of fibrinopeptide B further enhances and stabilizes these interactions, leading to more rapidly produced and longer protofibrils. The addition of Ca^{2+} enhances the binding site for the newly exposed *N*-terminal portion of the β-chain (Laudano & Doolitte, 1981). This further stabilizes the protofibril and augments its growth. At higher Ca^{2+} concentrations the relatively weak electrostatic forces involved at the D–D lateral contact sites are enhanced and lateral growth is further augmented. Thus the eventual size of a fibre is dependent on the interplay of forces favouring the production of long protofibrils and forces strengthening the D–D lateral contact site. Ca^{2+} is involved at all stages of the process promoting monomer association, enhancing β-epitope binding, and augmenting D–D-lateral-contact-site binding. Combined with its cofactor role for Factor XIII activity, this constellation of activities makes Ca^{2+} a prime determinant of clot strength as demonstrated by enhanced elastic modulus.

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