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Review of some unusual effects of calcium binding to fibrinogen

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Abstract

Calcium binding curves of human and bovine fibrinogen were obtained by using a calcium sensitive electrode. The two were identical and showed 2 high, 2–3 medium and more than 15 low affinity sites. Differential scanning calorimetry at neutral pH demonstrated the presence of the D and E domains of fibrinogen; however, at pH 3.5 the D-domain was split into two. The presence of the subdomains was demonstrated also by digestion by pepsin at this pH. Combination of digestion of fibrinogen and of its fragments with different enzymes and temperatures identified up to 12 subdomains in the original molecule. Clotting of fibrinogen by thrombin at pH 7.0 was investigated also by differential scanning calorimetry. In the absence of Ca^{2+} clotting elicited a 40% increase in the enthalpy of thermal denaturation of the D domain of fibrinogen, but the position of the peak increased only by 0.4 °C. However, with clotting in the presence of 10^{-3} M calcium the former increased by 70–75% and the latter by 11.0 °C, while these parameters of the E-domain remained unchanged. Changes of bound calcium during clotting were also measured with the calcium sensitive electrode. These had to be corrected, because the drop in free calcium was partly compensated by release of some calcium that was already bound to fibrinogen. Log of the half time of calcium uptake plotted against log thrombin concentration indicated a first order process with respect to thrombin concentration, moreover, the rate determined corresponded to that of the conformation change measured by calorimetry. The calcium uptake was correlated with release of the fibrinopeptides. Release of fibrinopeptide B follows parallel to binding of calcium and that of fibrinopeptide A is about fourfold faster. Polymerization and formation of thick bundles of fibrin is connected with release of fibrinopeptide A. Clotting with Ancrod, an enzyme that releases only fibrinopeptide A, showed only minimal binding of calcium. The polymerization inhibiting tetrapeptide Gly-Pro-Arg-Pro also depressed binding of calcium. These data suggest that a calcium-binding site must be in the proximity of the site of release of fibrinopeptide B and of a polymerization site.
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Keywords: Fibrinogen; Fibrin; Thrombin; Clotting; Calcium binding; Calorimetry

1. Introduction

Numerous papers have been published on the effect of calcium on the resistance of fibrinogen to thermal denaturation, acceleration of the overall clotting reaction, protection from reduction of certain disulfide bonds or from plasmin cleavage at some specific peptide bonds. But, certainly more numerous papers were about physicochemical changes like elasticity, opacity of the fibrin clot brought about by the presence of calcium ions.

John D. Ferry became interested in fibrinogen early in his career while associated with E.J. Cohn's group at Harvard Medical School. Soon his interest was directed toward the role of calcium in affecting the properties of fibrin. His attention remained focused on fibrinogen and especially on the polymerization of fibrin monomers, both in experimental and theoretical aspects, through all his life. Therefore, the following lines on some modest contributions to these subjects are dedicated to the memory of this distinguished scientist.

The papers mentioned above are not relevant to the present discussion and will not be quoted. The discussion will be limited to the results obtained with two methods not generally used: differential scanning calorimetry and cal-

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cium binding determined by the calcium electrode, two methods that permit continuous monitoring of the changes brought about by calcium binding, especially during clotting of fibrinogen by thrombin.

1.1. Binding of calcium to fibrinogen

Most of the material that will be presented subsequently involves one way or another, binding of calcium ions to either fibrinogen or fibrin. Therefore, it is in order to start here with the description of the use of the calcium selective electrode for the binding studies, a method that has not been used so far for this purpose.

Calcium selective electrodes of much improved quality are available commercially. They are used the same way, as are the glass electrodes for hydrogen ion titration. Successive aliquots of standard calcium chloride solution are added to the solution to be titrated and the free calcium concentration of the mixture measured with the calcium electrode. The bound calcium is calculated as the difference between the concentrations of added and the measured free calcium. The only difficulty with this method is the standardization of the electrode. From 0 to −5 log Ca^{2+} concentration the response is linear with a slope close to the theoretical 29.58 mV. From −5 to −6 the slope is less than the theoretical and below −6 decreases rapidly so the electrode is not reliable. A further inconvenience is that the electrode deteriorates with time and the standardization has to be repeated daily. The whole standard curve is lowered unchanged so one or two points are sufficient to establish its position. The main source of instability is usually the reference electrode.

A systematic study of binding of calcium to fibrinogen was first performed by Marguerie et al. [1] in 1977 with equilibrium dialysis and concentration measurements using radioactive calcium. He demonstrated the presence of two classes of binding sites: one of high affinity containing two or three sites and another one of low affinity, not well defined 10–20 sites. Numerous other studies followed that need not be enumerated here.

Titrations of bovine and of human fibrinogen with the calcium electrode are shown on Fig. 1 (E. Mihalyi and M.K. III Horne, in preparation). The amount of calculated bound calcium is plotted against the logarithm of free calcium concentration. At about log free Ca^{2+} of −5 the curve levels off and below this the measurements become uncertain because of the electrodes' deviation from ideal response. The latter is the region where the high affinity sites are expected. Indeed, Dang et al. [2] and Váradi and Scheraga [3] demonstrated that the C terminal portion of the γ-chains, between residues 315 and 329, possesses a sequence homologous to the calcium binding site of calmodulin and other proteins with high affinity for calcium. All these proteins bind calcium with a binding constant (Kd_{Ca}^{2+}) of about 10^{-7} [4]. Therefore, it may be assumed that the two high affinity binding sites of fibrinogen are also titrated in this region, at more than one pK_{Ca}^{2+} unit lower than the value given by Marguerie et al. [1]. The calcium-binding curve levels off and forms a plateau corresponding to two calcium atoms bound per molecule of fibrinogen. This finding is corroborated also by the calcium titration of isolated fragment D that has only a single γ-chain. The curve for this fragment levels off at one calcium atom per molecule exactly in the same region where intact fibrinogen showed two.

At the low affinity portion of the binding curve presumably are the sites provided by the 10–12 sialic acid residues which have a Kd_{Ca}^{2+} of about 10^{-2} [5]. Titration of

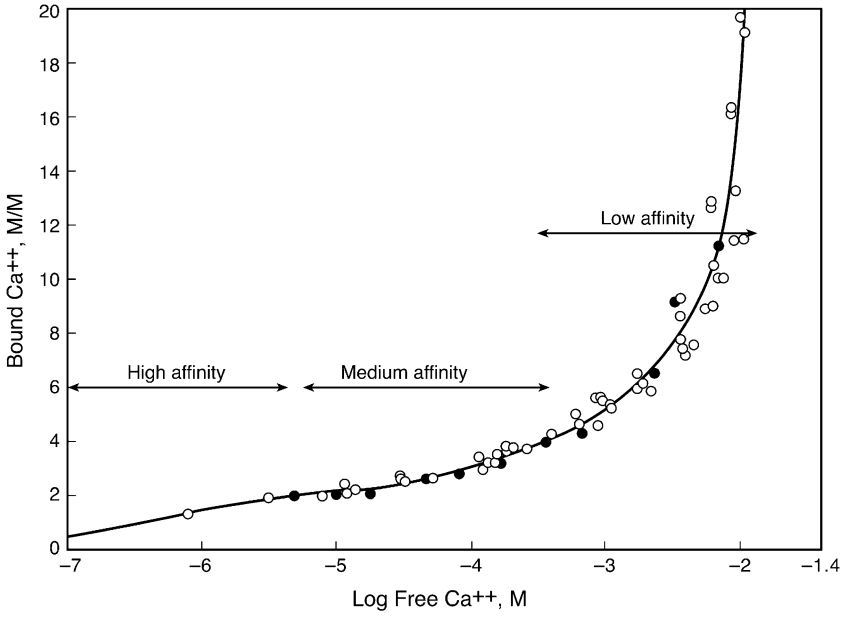

Fig. 1. Binding of Ca^{2+} to fibrinogen. Symbols: ● Human, ○ Bovine fibrinogen.

these is uncertain because it involves a small difference of two large numbers. Between these two extremes are situated two to three sites with an intermediate affinity. The experimental points of both bovine and human fibrinogens are plotted also on this figure showing that these two proteins are identical as far as their calcium binding affinities are concerned.

Calcium binding proteins were discovered in large numbers and the structure of some of them was determined by X-ray crystallography. A masterly review of these developments was presented by McPhalen et al. [6]. Perhaps perusal of these will enable localization of medium affinity calcium binding sites in fibrinogen.

1.2. Differential scanning calorimetry (DSC)

Since this technique is not widely used, perhaps it would not be superfluous to describe in a few words the principle of the instrument and of its use. The calorimeter part is essentially a hot plate that can be heated at a set rate. The sample and solvent are pipetted each in small aluminum pans that can be sealed quickly. These are placed on the plate over sensitive thermocouples and the temperature difference between the two is recorded against the temperature of the plate. The instrument used in the experiments described in the following was Du Pont Model 990 differential scanning calorimeter modified to increase sensitivity and signal to noise ratio [17]. If the sample undergoes an exothermic reaction its temperature increases above that of the solvent and the increase will be proportional to the rate of heat production. The latter increases to a maximum and then returns to the base line. With an endothermic reaction the reverse will be true. The area under the curve will be proportional to the total heat produced. The instrument can be calibrated by the relation of the known heat absorbed by melting of a weighed amount of a crystalline material to the area of the recorded curve.

With the above instrument, the measurements can be performed with very small amounts of material, of the order of a few milligrams and because of this; the time of temperature equilibration will be short. Consequently relatively fast heating rates can be used, up to 10 °C/min. These characteristics make this system ideally suited for kinetic experiments.

Donovan and Mihalyi [7] used the above instrument to record the heat flow during thermal denaturation of fibrinogen. The electron microscopic studies suggested that the fibrinogen molecule was composed of three nodules. Also, proteolytic fragmentation of the molecule produced two kind of large fragments, denoted D and E, and their stoichiometry indicated two D and one E nodule per molecule (for a review see Doolittle [8]). What was not clear, however, was the existence of these nodules within the native molecule. The thermogram shown in Fig. 2, taken from Ref. [7], clearly shows two inverted peaks: one at 60 °C and another close to 100 °C. The peaks point down

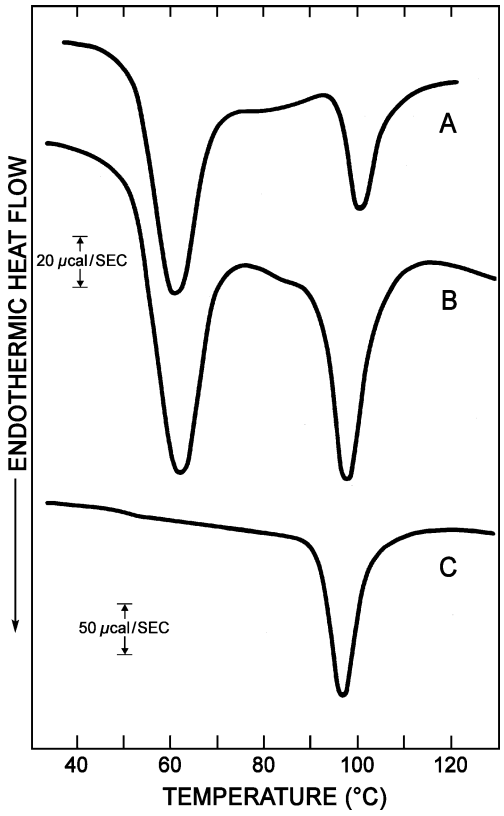

Fig. 2. Differential scanning calorimetry. (A) Native fibrinogen with the two cooperative subunits; (B) mixture of the isolated fragments D and E; (C) isolated fragment E.

because the reaction is endothermic. Runs with a mixture of isolated D and E fragments reproduced exactly the thermogram of the denaturation of the original molecule. Also, isolated fragments appeared in the same position as in the mixture, or in the native molecule. Therefore, it appears that these nodules are preexistent in the native molecule and form cooperative unfolding units.

Privalov and Medved' [9] continued these studies using a differential scanning microcalorimeter of different design, Model DASM-1M [10]. The calorimeter was used at a slower scanning rate of 1 °C/min. The thermogram obtained with fibrinogen at pH 8.5 was identical with that of Donovan and Mihalyi obtained at pH 7 and at a scanning rate of 10 °C/min, showing that the higher rate is still acceptable. The thermograms were analyzed by Privalov and Medved' with the use of theoretical thermodynamic relationships. The enthalpy of a single cooperative transition can be calculated with the van't Hoff equation (ΔH_{vh}) from the sharpness of the transition, i.e. the ratio of peak height to its width. If the experimentally determined enthalpy (ΔH_{cal}) is larger than the calculated one, this indicates more than one transition and the number of these is given by the ratio of the two enthalpies: $\Delta H_{cal}/\Delta H_{vh}$. This ratio, with the entire molecule of fibrinogen, for the D nodule was 2.4 indicating two cooperative transitions within the envelope of the D nodule. When the heat denaturation of isolated fragment D

was followed at pH 3.5 two peaks were observed indicating that at this pH fragment D was cleaved into two components [9]. It should be mentioned here that optical rotation studies of Mihalyi [11] indicated that the stability of fibrinogen below pH 4 is decreasing sharply, thus the separation of the subnodules of fragment D may be increased at the low pH, facilitating their separate unfolding. The two components were demonstrated by digestion with pepsin at pH 2.8 [9]. Thermogram of native fibrinogen at pH 3.5 showed also the splitting of the D nodule. Two components appeared: the one unfolding in the low temperature range was digested entirely by prolonging the pepsin digestion, the other one, resistant to digestion, designated TSD, was unfolded reversibly at higher temperatures. The latter had a molecular weight of 28,000 and upon reduction yielded three fragments of equal weight; therefore, it should be the disulfide knot, binding the N-terminal portions of two D nodules. The existence of the subnodules was demonstrated also by cleavage of the isolated D nodule by pepsin at pH 2.5 and 25 °C [12].

Marguerie and Ardaillou [13] reported the loss of one high affinity Ca^{2+} binding site with fibrinogen in which the Aα-chain was partially degraded. This may be the third high affinity site, which appears repeatedly in the literature. Given the symmetrical structure of the molecule, Ca^{2+} may be a bridge between the two Aα-chains, but its presence certainly should be supported by more investigations. Medved' et al. [14] combining further degradation of fragment X with differential calorimetric data concluded that each of the C-terminal parts of the two Aα-chains form domains that strongly interact with each other.

Further investigations [15] of the digestion of the 95,000 MW D fragment, using various enzymes under different conditions, showed that a series of products of diminishing molecular weights can be obtained, corresponding to the successive removal of the labile domains. The end result was the already mentioned thermostable TSD component. The C-terminal parts of both β-chains and γ-chains also appeared to form two thermolabile calorimetric domains, which can be separated by digestion with elastase.

Taking into account all the unfolding data of native fibrinogen and of its proteolytic fragments Privalov and Medved' [9] identifies 12 cooperative regions in the native fibrinogen molecule and their spatial relationships. The arguments leading to this picture are too lengthy to summarize here and the reader is referred to the original publication. It is noteworthy that Weisel et al. [16], examining electron microscopic pictures of fibrinogen and fibrin found a striated pattern suggesting at least seven nodular structures within the molecule.

Binding of calcium has very little effect on the thermogram of either human or bovine fibrinogen between log free Ca^{2+} concentrations of −7 to −4. At higher concentrations ΔT_d, the shift of the peak temperature, approaches asymptotically 11.5 °C for both human and bovine fibrinogen. The increase follows binding of calcium to the low affinity sites. The enthalpy of denaturation remains constant through the whole range of pCa^{2+} of 7–1 [17].

In the following, studies of the action of thrombin on fibrinogen and the effect of calcium ions upon this will be described [17,19].

Typically, to 0.2–0.3 ml of 6–10% fibrinogen solution 2–3 units of thrombin were added that resulted in a clotting time of approximately 200 s. After thorough mixing approximately 20 μl samples were pipetted with a glass capillary pipette into a series of preweighed pans. Usually up to eight pans could be filled before clotting set on. The pans were sealed in a press, reweighed, and then let to stand at room temperature and successively run in the calorimeter at times appropriate for the reaction observed.

1.2.1. Differential scanning calorimetric thermograms of bovine fibrinogen clotted by thrombin in the absence of calcium ion

An experiment with 0.3 M NaCl and 0.025 M phosphate, pH 7.0 as solvent, with 1.6×10^{-3} M EDTA to chelate any Ca^{2+} present, is depicted on the A panel of Fig. 3. Successive recordings are plotted on the same graph, with the time after thrombin addition written above each graph. The reaction is endothermic and causes the recordings of the peaks to point down [7]. The thermograms show the familiar denaturation endotherms of the D nodules at approximately 60 °C and of the E nodules at 100 °C identified in a previous study [7]. Another more diffuse peak appears on Fig. 3 at 125 °C whose provenance was not yet definitively established. The characteristics defining these endotherms are the position of the peak on the temperature scale and the area under the peak. The later can be converted into ΔH using the conversion factor determined with the calibration of the instrument.

The position and area of the E nodule and of the 125 °C endotherms remain unchanged in all the runs depicted on the A panel of Fig. 3. However, the area and the position of the peak of the D nodule both show a small change upon clotting with thrombin. Initially, the peak temperature (T_d) decreases about 1°, then rises slowly to a fraction of a degree above the starting position and remains stable there for the rest of the experiment. The area follows a similar course and levels off at approximately 40% above the original enthalpy of unfolding (ΔH_d) of the D nodule. While clotting of the fibrinogen occurred in this experiment at about 200 s after addition of thrombin to approximately 2 units/ml, the changes noted above were much slower, taking 200–250 min.

The results were identical with fibrinogen to which 1.6×10^{-3} M EDTA was added, or with fibrinogen that was treated with EDTA and then dialyzed exhaustively against 0.3 M NaCl prepared with specially purified NaCl, "Aristar" grade, free of Ca^{2+} from BDH Chemicals, Poole England.

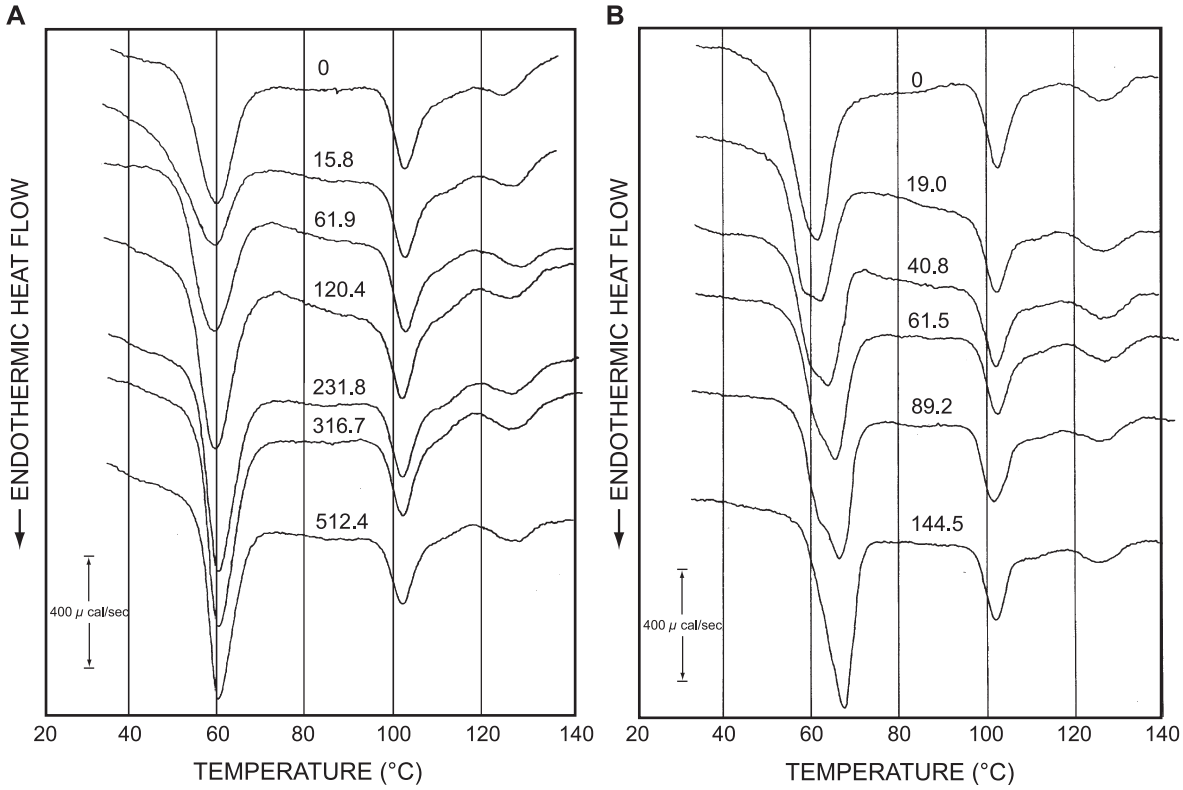

Fig. 3. Differential scanning calorimetric thermograms of fibrinogen after addition of thrombin. Numbers above each curve refer to the time elapsed after thrombin addition. Panel A, solvent was 0.3 M NaCl, 0.025 M phosphate, pH 7.0. Panel B, same solvent with 2×10^{-4} M $CaCl_2$.

1.2.2. Changes in the thermograms of bovine fibrinogen clotted by thrombin in the presence of increasing amounts of calcium ions

Panel B of Fig. 3 shows a series of thermograms under identical conditions to those on Panel A, but with Ca^{2+} added to 2.0×10^{-4} M concentration [17]. The D endotherm in the successive tracings appears broader then split as if were composed of two peaks and the area of the lower temperature peak is progressively shifted into that of the higher temperature one. Finally, only the much sharper higher temperature peak persists. The temperature of the peak shifts 7.4 °C from the initial position and its area is increased by 26% in this experiment. There is no change in either of these for the endotherm of the E nodule or of the one at 125 °C.

The results of a series of experiments performed with increasing Ca^{2+} concentrations with both bovine and human fibrins were reported in Ref. [17]. The asymptotic values of the shift of the peak temperature of the D endotherm, reached at high Ca^{2+}, was 11.0 °C for the bovine and slightly higher for the human material. These data were related to the calcium binding curves reported in Fig. 1. It appears that the temperature shift is caused by binding to sites with an intermediate affinity between the high and low ones.

Similar considerations suggest that the much smaller shift of T_d in bovine or human fibrinogen be also caused by calcium binding to intermediate sites.

Experiments with clotting of bovine fibrinogen with varying thrombin concentrations showed that the increase of the peak temperature of the thermal unfolding of the D nodule follows a first order course. The rate constant of this process plotted against the thrombin concentration was strictly linear.

The fibrinogen preparations, either bovine or human, contained as an impurity inactive factor XIII, which could be activated in the presence of Ca^{2+} and the introduced cross-links of the chains could affect the denaturation of the molecule. This possibility was eliminated by introducing a large amount of activated factor XIII in the reaction mixture, which would have accentuated the effect, or iodoacetamide that would have inhibited it completely. Neither had affected the changes associated with the thermal unfolding of the molecule.

1.2.3. Changes in the thermograms of fibrinogen associated with clotting with Ancrod

Ancrod is a clotting enzyme isolated from the venom of the Malayan pit viper *Agkistrodon rhodostoma*, which cleaves off only fibrinopeptide A from fibrinogen [18]. With this enzyme the endotherm of the D nodule widens to its final value as fast as all the molecules loose fibrinopeptide A and remains constant after this [19]. In contrast, with thrombin an initial widening is followed by a long period when the width decreases slowly. This is illustrated in Fig. 4,

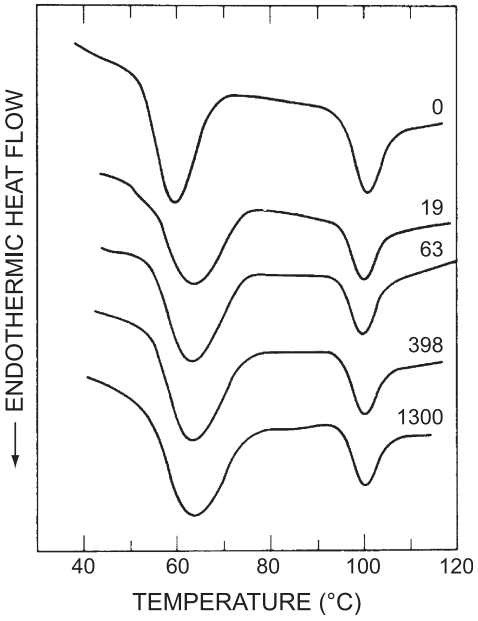

Fig. 4. Clotting of fibrinogen with Ancrod in the presence of 5×10^{-3} M calcium. Numbers above each curve refer to the time (min) elapsed after Ancrod addition.

taken from Ref. [19]. In the same way the peak temperature and the area (ΔH) rises moderately and very early with Ancrod, then remains constant, while with thrombin these changes are larger and continue for a considerable time. All the experiments described above were run at a relatively high Ca^{2+} concentration from 5×10^{-3} to 10^{-2} M. As it will be apparent later in the text, with Ancrod not only fibrinopeptide B is not released but also with this, Ca^{2+} binding by the fibrinogen molecule is blocked.

1.3. Binding of calcium, release of fibrinopeptides and opacity increase during clotting of fibrinogen by thrombin

1.3.1. Recording of the binding of calcium

In the studies on calcium binding with individual samples, described in the preceding sections this was calculated from the drop in free calcium derived from the decrease of the electromotive force of a calcium electrode. In the following section the interest was in the kinetics of the processes and the binding of calcium was continuously recorded.

Free calcium in a series of fibrinogen samples was adjusted to various levels by adding 0.1 M $CaCl_2$. After measuring the electromotive potential of a calcium electrode, thrombin was added to each sample and then the change of the potential recorded until a stable value was reached. The first reading corresponded to the free calcium level with fibrinogen, the second one with fibrin [20]. With these two values, knowing the amount of fibrinogen present and that of calcium added to each sample, the amount of calcium bound to fibrinogen or fibrin can be calculated. The results of these calculations are plotted on Fig. 5 against log of the free calcium concentration. The vertical distance between the two curves, shown on the lower panel of this figure, corresponds to the number of calcium ions bound by the clotting at the respective free calcium concentration. It appears that binding is restricted to the 10^{-5} to 10^{-3} M zone and eventually the two curves rejoin each other at higher concentrations of free calcium. This means that what happens is a shift of some sites from lower to higher affinity. The difference curve, shown on the lower panel of this figure, has no plateau because for this the shift should be of the order of 4 pK units and is evident that actually it is less than one unit. The dashed curve in the figure is an ideal titration curve with a pK of 4.3, with its height adjusted to coincide with the initial segment of the difference curve. This curve suggests that two sites are taking part in this reaction. It may be recalled at this point that Donovan and Mihalyi [17] estimated that the pK of the Ca^{2+} binding groups that govern the conformational change on clotting of fibrinogen was also 4.3.

The calcium shifts actually measured by the calcium electrode cannot be used directly to follow kinetics, because the situation is complicated by the fact that fibrinogen, or fibrin, act as a calcium buffer: the decrease of free calcium is countered by release of some of the calcium bound to the protein. The procedure for calculating the corrected free Ca^{2+} level is described in the original publication. The kinetics of the process is also complicated by the presence of a lag period. Therefore, instead of calculating rate constants the reciprocal of half-reaction times were used.

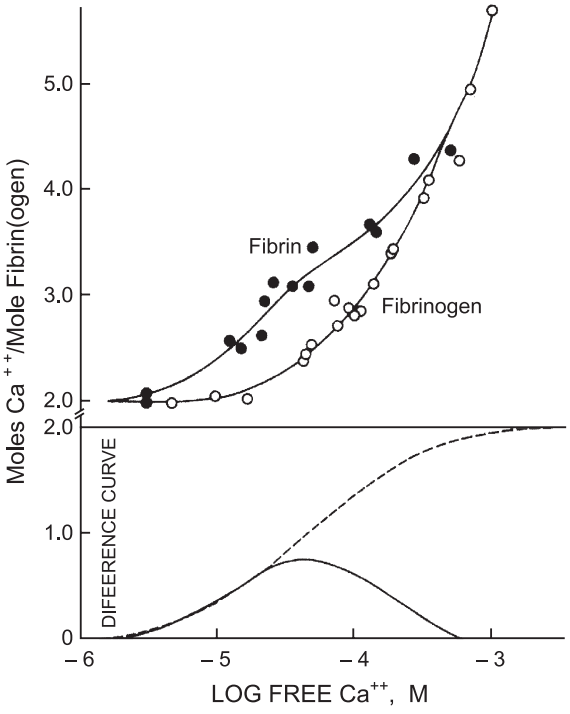

Fig. 5. Upper panel: Calcium bound to fibrinogen or fibrin, plotted against log free Ca^{2+} concentration. Symbols: ○ fibrinogen, ● fibrin. Lower panel: Full line: Difference of the fibrinogen and fibrin curves plotted against logarithm of free Ca^{2+}. Broken line: Theoretical titration curve with pK of 4.3.

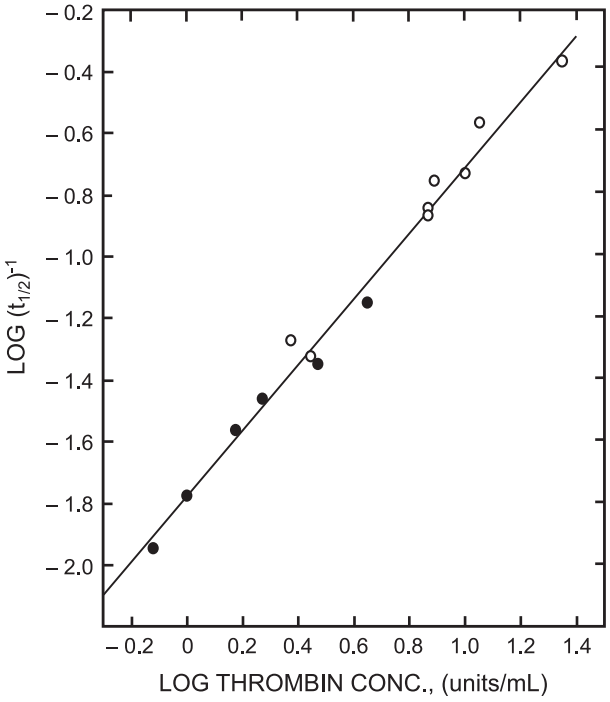

Fig. 6. Logarithm of the reciprocal half time plotted against logarithm of thrombin concentration. Symbols: ○ calcium uptake, ● conformational change.

Experiments with varying thrombin concentrations showed that log half-time plotted against log thrombin concentration gave a straight line, demonstrating that the rate limiting process is of the first order with respect to thrombin. Moreover, the rate constants of the conformational change determined in our previous publication [17], plotted the same way, fell on the same straight line (Fig. 6). This further demonstrated that the conformational change and binding of calcium are two sides of the same process.

The effect of fibrinogen concentration on the rate of calcium binding was also investigated. As expected, the drop in free calcium concentration increased with increase of the former. However, the increase was not linear, but progressively declined and the asymptotes seemed to reach a limiting value at high fibrinogen concentrations. This is expected knowing the calcium buffering capacity of this protein. At the leveling point loss of free calcium accompanying the specific binding is compensated exactly by release from other sites. On the other hand, the half-reaction times read on the corrected curves increased linearly with the fibrinogen concentration.

Both of the above two studies were performed at log free Ca^{2+} concentration of −4.00, 0.3 ionic strength, pH 7.26 and 25 °C.

1.3.2. Release of fibrinopeptides and opacity increase

In the experiments performed to establish the relationships between calcium binding and release of fibrinopeptides [20] again individual samples had to be prepared. To fibrinogen solution of 8.11%, in the solvent given in the preceding section, thrombin was added to 1.36 NIH units/ml concentration. After increasing reaction times the free Ca^{2+} concentration and opacity of the sample were determined, the latter by measuring the optical density at 450 nm, and immediately after this the tube was transferred in boiling water bath. The sample was centrifuged and the supernatant, free of proteins, was analyzed by HPLC. Polymerization of the fibrinogen molecules, activated by removal of the fibrinopeptides, follows in two steps: first they are associated end-to-end forming thin protofibrils and then these are bound laterally into thick fibers. The opacity measurements are not sensitive enough and pick up only the formation of fibers, but at the high fibrinogen concentration used fiber formation is the dominant step of the clotting. In Fig. 7, taken from Ref. [20], the normalized values of opacity, release of fibrinopep-

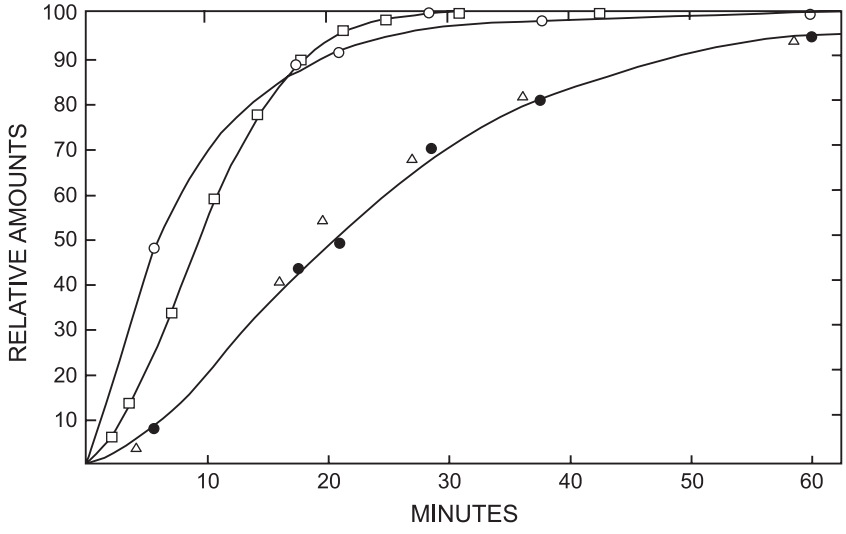

Fig. 7. Turbidity increase, calcium uptake and release of fibrinopeptide A and B, plotted against reaction time. Symbols: □ turbidity, Δ calcium uptake, ○ fibrinopeptide A, ● fibrinopeptide B.

tide A, fibrinopeptide B and uptake of calcium are plotted against reaction time. It is apparent that polymerization follows closely removal of fibrinopeptide A. The lag period corresponds to the time required for formation of enough molecules of fibrinogen activated by removal of this peptide to form the thick fibers. Removal of fibrinopeptide B is much slower and follows parallel to binding of calcium. The resolution of this experiment is not sufficient to decide which one is first between the last two. A similar experiment was performed at lower fibrinogen concentration of 1.24%. Opacity measurements were omitted, but calcium uptake again ran a parallel course with release of fibrinopeptide B. Judging from the half-reaction times, release of fibrinopeptide A was 4 times faster than that of fibrinopeptide B.

The most remarkable fact in these experiments was the parallelism of calcium binding and release of fibrinopeptide B. This suggested for further investigations the use of Ancrod, which cleaves off only fibrinopeptide A [20]. Fig. 8, also taken from Ref. [20], shows on the one hand recordings of the calcium uptake during clotting with either thrombin or Ancrod, on the other, the release of the fibrinopeptides with either enzyme are illustrated by the HPLC chromatograms at the end-stage of the reaction, shown on panels A and B. With thrombin the calcium uptake is at the expected level, with Acrod is minimal and corresponds to the reduced release of fibrinopeptide B shown on the chromatogram. Apparently, the specificity of Ancrod is not absolute, or there might be an enzyme impurity responsible for the B peptide released.

1.3.3. Effect of the polymerization inhibitor tetrapeptide Gly-Pro-Arg-Pro on calcium uptake during clotting

Laudano and Doolittle [21], assuming that removal of the fibrinopeptides by thrombin uncovers binding sites for the polymerization of fibrin monomers, synthesized a series of peptides corresponding to the N-terminal sequences of the stumps of the two chains involved. Investigation of the binding of these to fibrinogen revealed that the terminal dipeptides were ineffective, but the tripeptide corresponding to the terminus of the α-chain, Gly-Pro-Arg, inhibited polymerization and this effect was greatly enhanced when proline, as a fourth residue, was attached to it. The tetrapeptide Gly-Pro-Arg-Pro bound to two sites per mole of fibrinogen and one per mole of fragment D, with an association constant of 4×10^4. The association constant of the analogous tetrapeptide derived from the residues of the N-terminus of the β-chain, Gly-His-Arg-Pro, was less than one fourth

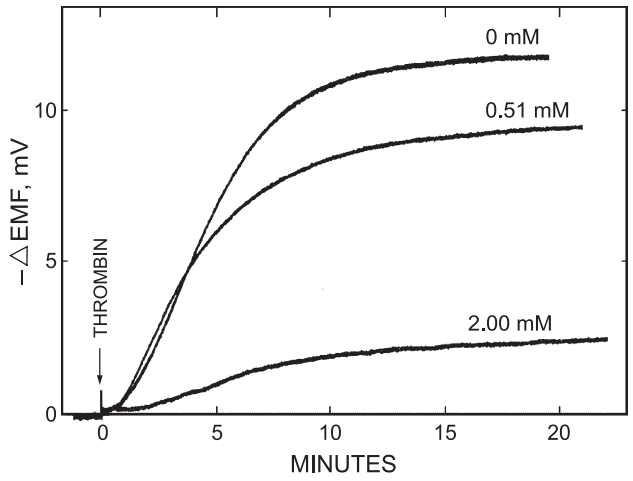

Fig. 9. Effect of the polymerization inhibitor Gly-Pro-Arg-Pro on calcium uptake during clotting of fibrinogen by thrombin. Numbers above each curve give the final concentration of the tetrapeptide.

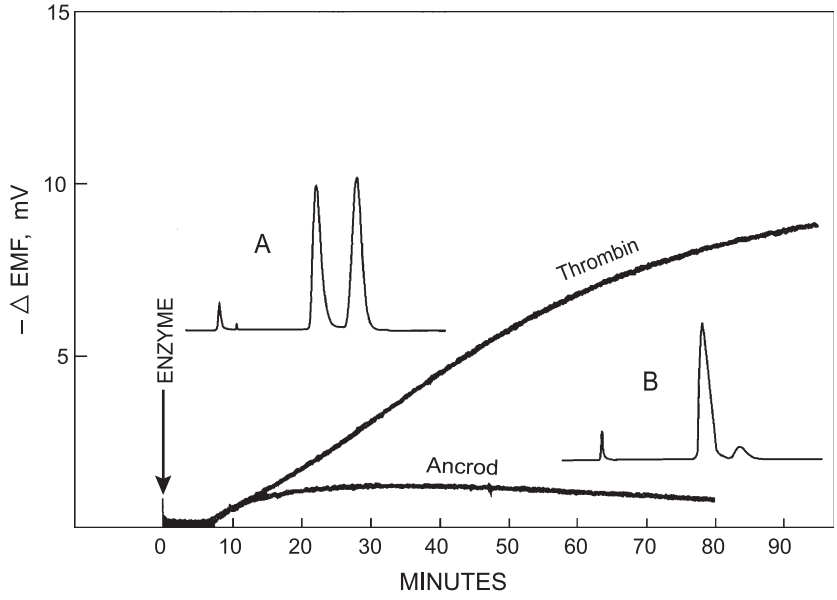

Fig. 8. Recordings of calcium uptake with clotting of fibrinogen with either thrombin or Ancrod. Panel A: HPLC pattern of the peptides liberated by thrombin, Panel B: the same with Ancrod.

of that of Gly-Pro-Arg-Pro. Further studies [22] demonstrated that the presence of 2 mM calcium ions increased ten fold the association constant of Gly-His-Arg-Pro and left practically unchanged that of Gly-Pro-Arg-Pro.

The binding of Gly-Pro-Arg-Pro interfered also with binding of Ca^{2+} to fibrinogen [20]. Fig. 9 shows this effect. At 2 mM concentration, a 20-fold excess over Ca^{2+}, binding of the latter was nearly abolished.

2. Conclusions

Binding of calcium to two of the intermediate affinity sites of fibrinogen has an effect on a number of properties of the molecule. These become more accentuated during the clotting elicited by thrombin. The apparent heat of unfolding of the D nodule of the fibrinogen molecule is approximately 350 kcal/mol and this is increased by clotting in the absence of Ca^{2+} by 40%. At high calcium concentrations the increase reaches asymptotically 90–95%. The peak temperature of the D domain increases on clotting by 2.2 °C without and 11.5 °C with calcium present [17,19]. This is the result of a conformation change of the molecule and of secondary bonds produced by their association in the fibrin network.

When Ca^{2+} binding at 10^{-4} M was followed during clotting, this showed two sites involved [20]. The localization of these was not possible from these experiments; however, this was apparent when the relationship of release of fibrinopeptides during clotting with thrombin and binding of calcium was studied. It appeared that binding of calcium and release of fibrinopeptide B are strongly connected. Calcium binding was reduced, if not abolished, when the peptide remained attached to its original position. When released, a parallel calcium binding followed. This suggested that the calcium-binding site and the peptide bond cleaved by thrombin to release fibrinopeptide B must be close neighbors. Fibrinopeptide A and its release did not appear to be involved with calcium binding, however, a more complex kinetic analysis of data obtained at high fibrinogen concentrations [23] indicated that some involvement of this in calcium binding is possible, but the data are not precise enough to prove it. Therefore, the original claim that release of fibrinopeptide B and calcium uptake follow identical course appears to be valid.

Finally, reduction of calcium binding by the polymerization inhibitor tetrapeptide suggested that the sites taking part in either of these must be also in proximity [20].

All the above facts suggest that the tight structure of the N-termini of the fibrinogen molecule contain the two crucial Ca^{2+} binding sites, the polymerization site and possibly the sites governing the conformational change of the fibrinogen molecule after thrombin action in the presence of calcium.

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