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Clotting of Fibrinogen. 1. Scanning Calorimetric Study of the Effect of Calcium

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ABSTRACT: The denaturation temperature T_d and the enthalpy of thermal denaturation ΔH_d of the D nodules of fibrinogen increase 12-13 °C and 40%, respectively, when fibrinogen is clotted by thrombin in the presence of 10^{-3} M calcium ion. The rate of change of T_d and ΔH_d is first order in thrombin concentration. In the absence of calcium, little change in T_d is observed, but the increase in ΔH_d still occurs. The shift in T_d as a function of logarithm of calcium concentration is sigmoid, with a half-point at 2.5×10^{-5} M calcium for human and 6.0×10^{-5} M calcium for bovine fibrinogens, suggesting that the shift is due to binding of calcium at the high-affinity binding sites of fibrin. The T_d of the D nodule of native fibrinogen also increases, but not as much, on addition of calcium. This increase in T_d is also sigmoid with log calcium, with a half-point of 1.6×10^{-3} M calcium for human and 3.2×10^{-3} M calcium for bovine fibrinogens, and appears to be due to binding of calcium to the low-affinity binding sites of fibrinogen. At calcium concentrations greater than 10^{-4} M, traces of factor XIII in the bovine fibrinogen preparation become activated and cause cross-linking of the fibrin gel. But the changes in T_d and ΔH_d still occur when factor XIIIa is inactivated by iodoacetamide, and the rate of the changes is not altered by addition of large amounts of factor XIIIa. Thus, the changes in T_d and ΔH_d are not due to cross-linking but result from intermolecular interactions of the D nodules in the fibrin clot, which are strengthened by binding of calcium at the high-affinity binding sites of fibrin.

Fibrinogen is an approximately linear array of three globular regions, or nodules, separated by two triple-stranded, coiled-coil connectors. The larger terminal nodules have been designated D nodules, while the smaller central one is called the E nodule, after the globular fragments obtained by proteolysis. Conversion of fibrinogen into fibrin is a multistep reaction. The first step is the enzymatic cleavage of the fibrinopeptides by thrombin from the N-termini of the Aα and Bβ chains of fibrinogen. This is followed by purely physicochemical steps of polymerization. The gel formed may be cross-linked eventually by another enzymatic step performed by factor XIIIa. The molecular structure of fibrinogen remains essentially intact on conversion to fibrin. For reviews, see Doolittle (1973, 1984) and Hermans & McDonagh (1982).

In our previous study using differential scanning calorimetry (DSC), we showed that the D and E nodules of fibrinogen were heat denatured at different temperatures. During formation of the fibrin gel, after addition of thrombin, the thermal stability of the D nodule increased. The rates of increase of the enthalpy of the transition and the increase in its characteristic temperature appeared to be a function of thrombin concentration. The thermal stability of the E nodule was not affected significantly by clotting (Donovan & Mihalyi, 1974). In a continuation of these studies, we observed that the shift of the denaturation temperature of the D nodule that accompanies clotting is markedly affected by calcium ions (Mihalyi & Donovan, 1978). In the present paper we correlate the changes in the thermal stability of the D nodule with the concentration of free calcium ion and the calcium binding studies of Marguerie et al. (1977) and Nieuwenhuizen et al. (1979, 1981a) and our own. Since our preparations were not free of factor XIII, above a certain free calcium concentration factor XIII became activated and caused cross-linking of the gel. Several different approaches were taken to show that the changes in thermal stability were not caused by the cross-linking. Finally, the order of the reaction with respect to thrombin was established.

The denaturation of the D and E nodules of fibrinogen has been investigated at pH 8.5 and 3.5 by Privalov & Medved' (1982) and by Medved' et al. (1983). Their results, which confirm our previous work, also indicate that substructures, or domains, exist in both the D and E nodules.

EXPERIMENTAL PROCEDURES[1]

Materials. Bovine fibrinogen was purified by the method of Laki (1951) from fraction I from bovine plasma, lot G 10505 obtained from Reheis Chemical Co., a division of Armour Pharmaceutical Co. It was dialyzed exhaustively against 0.3 M NaCl, yielding preparations that were 94–95% clottable by Laki's method. Approximately 1 mol of calcium was present per mole of fibrinogen; this calcium could not be removed by prolonging the dialysis. This fibrinogen will be referred to as "normal" fibrinogen. The analytical reagent-grade NaCl used in these studies, various batches from different manufacturers, contained 6–14 ppm of calcium. This corresponds to $(2.75–6.25) \times 10^{-6}$ M calcium in the 0.3 M NaCl solutions. According to the calcium binding curves that will be presented later, this concentration of calcium should correspond to approximately 0.3 mol of calcium bound per mole of fibrinogen. To remove the residual calcium, the fibrinogen was dialyzed against 10 mM Na_2EDTA[2] adjusted to pH 8.1 in 0.3 M NaCl for 24 h and then against four to five changes (12 h each) of 0.3 M NaCl and 0.025 M Tris-HCl buffer, pH 7.2, to remove EDTA (Shen et al., 1975). All dialyses were performed at 4 °C. A specially purified NaCl preparation, Aristar grade from BDH Chemicals, Ltd., Poole, England, containing 0.1 ppm calcium was used throughout the preparation of calcium-free fibrinogen. The resulting preparations contained 0.04–0.05 mol of calcium/mol of fibrinogen and will be referred to as "EDTA-treated" fibrinogen. Clottability was the same as that of the normal fibrinogen.

In our previous experiments fibrinogen solutions of high concentration, of the order of 7–8%, were used. Since those experiments were carried out, the sensitivity of the DSC instrument has been increased so that lower concentrations of fibrinogen could have been used in the present experiments. However, for the calcium binding studies that will be reported elsewhere (E. Mihalyi, unpublished results), the higher concentrations were still required. It seemed advantageous, therefore, to maintain a uniformly high fibrinogen concentration throughout these studies. For the present experiments, in which changes in intermolecular interactions of the D nodules in the fibrin clot are followed as a function of time, the high protein concentrations used are not a significant liability. The D nodules in fibrin are in a semicrystalline fiber. The same number of nearest-neighbor intermolecular interactions are present, regardless of fibrin concentration. These high protein concentrations were achieved by rolling up the ammonium sulfate precipitated material obtained in the last step of the purification procedure and loading it directly into a slightly wetted dialysis bag (Spectra/Por 1, Spectrum Medical Industries Inc., Los Angeles). Dialysis gave a very viscous, almost gellike, solution that became fluid upon being warmed to room temperature and could be centrifuged to yield a clear homogeneous solution. Fibrinogen concentration was estimated by optical density measurement, with an $E^{1\%}_{1cm}$ of 15.06 (Mihalyi, 1968).

Human fibrinogen, grade L, lot 59940 of higher than 90% clottability, was obtained from AB Kabi, Stockholm. Calcium-free and concentrated solutions were obtained in the same way as described above, loading the lyophilized powder as a thick slurry into the dialysis bag and putting it through the same dialysis routine. Another human fibrinogen preparation from Calbiochem Inc., lot 600628, was sold as bovine but proved to be human fibrinogen, as documented recently (Mihalyi & williams, 1984). This was purified by Laki's procedure and then treated as described above, yielding preparations of better than 95% clottability.

Human thrombin was a highly pure preparation, lot 192 obtained from Dr. John W. Fenton, II. This originally had an α-thrombin content of 95.1% and an activity of 6112 NIH units/mL. Activity was redetermined by us recently both by esterase assay in the pH stat with TAME as substrate and by clotting assay on fibrinogen. α-Thrombin is the activation product of prothrombin possessing full esterase and clotting activity. β-Thrombin is its first autolytic product that has full esterase but practically no clotting activity, and γ-thrombin, a further autolytic product, is almost entirely inactive in both respects (Fenton et al., 1977). The first assay gave 5900 NIH units/mL and is a measure of both α- and β-thrombin; the second gave 4480 NIH units/mL and corresponds to the α-thrombin present. Apparently, the total activity diminished somewhat, while the β-thrombin content reached approximately 24%.

Methods. Thermal measurements were made with a Du Pont Model 990 differential scanning calorimeter modified to increase sensitivity and signal-to-noise ratio (Donovan, 1979). Du Pont coated hermetic sample pans were treated with Desicote (TM, Beckman Instruments) to keep water solutions away from the sealing surfaces. Thermal grease (Dow Corning 340 heat-sink compound or equivalent) was used to improve thermal contact between sample pans and thermocouple detectors.

Kinetic experiments were carried out as follows. Calcium chloride solutions or other reagents were added to fibrinogen solutions of 0.2–0.3-mL volume. Thrombin was added and the solution mixed thoroughly. Aproximately 20-μL samples were removed quickly with a glass capillary pipette and placed in sample pans. Up to eight pans were filled if the clotting time (the earliest time that the gel could be pulled into a thread) allowed. Usually, a thrombin concentration was chosen so that the clotting time was about 200 s. The sample pans were sealed in a press, left at room temperature ($\sim$23 °C), and then run in the calorimeter at times appropriate for the reaction observed. A heating rate of 10 °C/min was used, so that early kinetic observations could be made. Time was measured from addition of thrombin to the time the sample reached 60 °C in the calorimeter. The first sample usually could not be hearted to 60 °C (approximate temperature of denaturation of the D nodule) much before 8 min had elapsed after thrombin addition. The calorimeter was cooled rapidly between samples. Enthalpies of denaturation (ΔH_d) and denaturation temperatures (T_d) were determined as described (Donovan & Mihalyi, 1974; Donovan & Ross, 1973).

Calcium analyses were carried out by atomic absorption spectrophotometry under standard conditions with a Model 303 Perkin-Elmer atomic absorption spectrophotometer. Lanthanum was added to fibrinogen hydrolyzed in 6 N HCl at 100 or 120 °C. Solvents and calcium standards were run concurrently.

Free calcium ion concentrations were calculated from total calcium ion and fibrinogen concentrations with the dissociation constant for the three high-affinity binding sites of fibrinogen determined by Marguerie et al. (1977) and Nieuwenhuizen et al. (1981a by equilibrium dialysis. The dissociation con-

[1] Reference to a company and/or product name is only for purposes of information and does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DSC, differential scanning calorimetry; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester; SDS, sodium dodecyl sulfate; T_d, temperature of the peak in the denaturation endotherm; ΔH_d, enthalpy of denaturation.

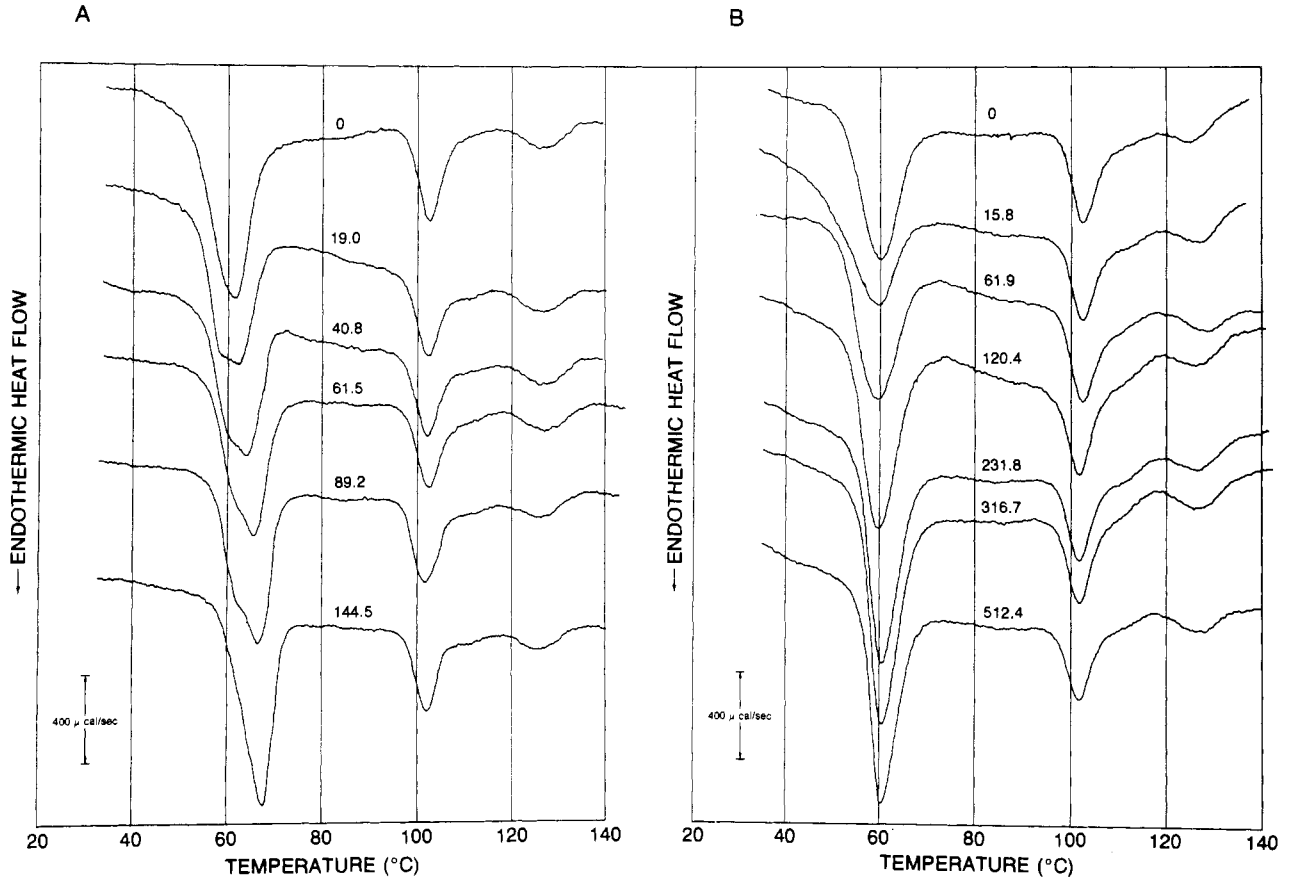

FIGURE 1: Differential scanning calorimetric thermograms of normal bovine fibrinogen, 87 mg/mL, before (O) and at successive times (min) after addition of 2.7 units of thrombin/mL. Buffer was 0.3 M NaCl and 0.025 M phosphate, pH 7.0. (A) Fibrinogen with 3.0 calcium ions bound per mole; free calcium ion concentration was 2.0×10^{-4} M (calcium electrode). Weight of fibrinogen from top to bottom was 1.55, 1.17, 1.18, 1.22, 1.18, and 1.18 mg. (B) As in (A), except that no extra calcium ion has been added and that 1.6×10^{-3} M EDTA is present in all samples but the first. Weight of fibrinogen was 1.65, 1.57, 1.68, 1.56, 1.57, 1.63, and 1.31 mg.

stant is pH dependent. The average value of the constant for bovine fibrinogen between pH 6.0 and 7.5, 3×10^{-6} M, appeared to be the most suitable value for our experimental conditions. For human fibrinogen, we used the reported dissociation constant of 1.9×10^{-5} M.

Free calcium was determined directly with an F 2110 Ca calcium electrode (940-271 Ca Selectrode tube) from Radiometer, Copenhagen, in connection with a Ag/AgCl micro reference electrode, MI-401, from Microelectrodes, Inc., Londonderry, NH. The calcium electrode was standardized with 10^{-6}–10^{-1} M $CaCl_2$ in Aristar-grade 0.3 M NaCl. Within experimental error, observed calcium concentrations agreed with those calculated from the binding constants reported by Marguerie et al. (1977) and Nieuwenhuizen et al. (1981a).

In some experiments, phosphate buffer was used. At the calcium and phosphate ion concentrations generally present, no visible precipitation occurred. Measurements with the calcium electrode on protein-free solvent containing phosphate showed that activity of calcium was reduced to 0.93 of that without phosphate. When this solvent was titrated with calcium, a visible precipitate of calcium phosphate appeared at 3.5×10^{-3} M free calcium ion concentration. The free calcium then dropped to a stable 4.1×10^{-4} M. The same phenomenon was also observed when the fibrinogen solution was titrated with calcium. The same final concentration of free calcium ion was reached after precipitation occurred.

Fibrin clots were tested for cross-linking in two ways: (1) The clot was reduced in 8 M urea and run in SDS–polyacrylamide gels according to the method of Pizzo et al. (1973). The gels were scanned directly at 280 nm (Mihalyi et al., 1976). (2) Samples of clot were suspended in 8 M urea (final concentration 6 M), allowed to stand at room temperature overnight, and then centrifuged. The optical density of the supernatant liquid was measured at 280 nm.

Results

Changes in Thermal Stability during Clotting. A distinct difference was observed between the appearance of the thermograms on clotting fibrinogen in experiments with calcium ion or without calcium ion in the reaction mixture. These differences are illustrated in Figure 1, where the first panel (A) shows a clotting reaction with calcium present and the second one (B) that with calcium chelated by EDTA. The denaturation endotherm near 60 °C is that of the D nodule, and the endotherm near 100 °C is that of the E nodule (Donovan & Mihalyi, 1974). There is also an endotherm near 125 °C, not observed in our previous work, because the pans previously used failed at about 120 °C. The portion of the molecule responsible for this transition has not yet been identified, but a comparison with the calorimetric results of Privalov & Medved' (1982) at pH 3.5 suggests that this small high-temperature endotherm is probably due to denaturation of the portion of the coiled-coil connector adjoining the D nodule and containing a disulfide ring [TSD, in the notation of Medved' et al. (1983)].

The successive scans in Figure 1A, obtained as the clotting reaction proceeded, show first a broadening of the D endotherm, followed by sharpening in the later stages to an even narrower endotherm than the starting one. At some intermediate stages, the endotherm appeared to be split, with two

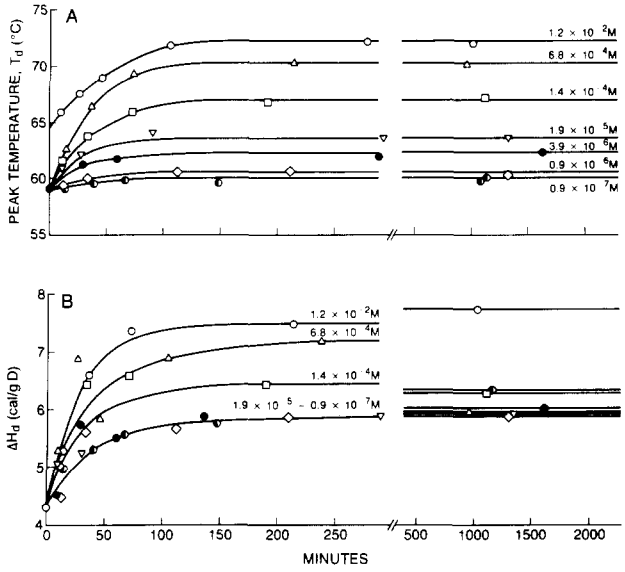

FIGURE 2: (A) Shift in temperature of the endotherm of the D nodules of bovine fibrinogen as a function of time elapsed after addition of thrombin. Fibrinogen concentration was 63.5 mg/mL in 0.3 M NaCl and 0.05 M Tris-HCl, pH 6.8. Thrombin concentration was 3.1 units/mL. Free calcium ion concentration was as shown. (B) Increase in enthalpy of denaturation of the D nodules under the conditions given in (A).

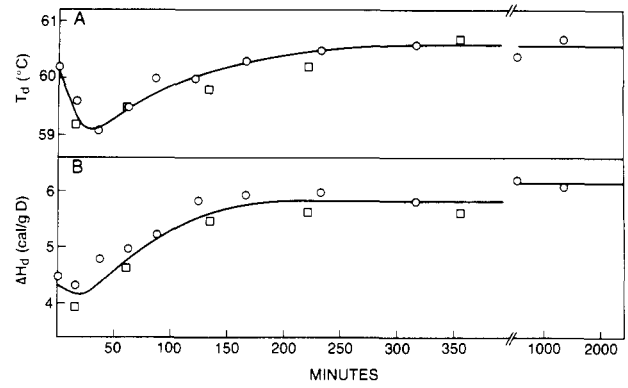

FIGURE 3: (A) Shift in temperature of the endotherm of the D nodules of bovine fibrinogen as a function of time after addition of thrombin, in the presence of 0.002 M EDTA. Concentration of fibrinogen and solvent conditions are the same as for Figure 1. Thrombin concentration was 1.8 units/mL. The different symbols represent two different experiments. (B) Change in enthalpy of denaturation of the D nodules of fibrinogen, in the same experiments.

peaks, as if two species were present. The area of the endotherm shifted progressively from the peak at lower temperature to the peak at higher temperature. Over the course of time, the peak temperature shifted in this experiment by about 7.4 °C from the position at zero time to that at 145 min after thrombin addition. The area of this endotherm (proportional to enthalpy of denaturation of the D nodule) also increased by 26% during this time. In other experiments where the rate of the change was slower, the first sample run in the calorimeter often showed a decrease in the transition temperature of about 1 °C for the D endotherm (see Figure 3). No substantial changes have been observed for the E endotherm in the absence of added activated factor XIII or in the endotherm near 125 °C, as a function of time.

In the experiment shown in Figure 1B, the reaction mixture had the same composition as that in Figure 1A except that instead of calcium 1.6×10^{-3} M Na_2EDTA was present. As shown by the successive scans, only a very small change in the peak temperature of the D endotherm was observed. The changes in width of the endotherm and the increase in specific area still occurred, however.

Similar experiments performed with normal fibrinogen without added calcium presented an intermediate behavior between the two cases shown in Figure 1. This became understandable when approximately 1 mol of residual calcium/mol of fibrinogen was found to be present in our normal fibrinogen preparations. The EDTA-treated fibrinogen behaved identically with fibrinogen run in the presence of EDTA. These findings prompted a systematic study of the effect of calcium.

Effect of Calcium Ion on Changes in Thermograms of Fibrinogen during Clotting with Thrombin. In Figure 2, experiments are presented of thermal measurements with EDTA-treated fibrinogen to which increasing amounts of calcium were added. Panel A shows the temperature of the peak of the endotherm of the D nodule (T_d), and panel B shows ΔH_d of unfolding of this nodule calculated from the area of this endotherm. In each experiment, the endotherm shifts to higher temperatures as the reaction progresses and then remains stable indefinitely. The shift appears to correspond to a reaction of the first order, and the rate is approximately the same at the various calcium concentrations. The plateau region reached in each experiment increases in height as the calcium concentration is increased. The time dependences of the changes in ΔH_d are similar but less regular than those of T_d. In contrast with T_d, ΔH_d is observed to increase even in the absence of added calcium ion, and within experimental error, the same increase in ΔH_d is observed in the range of calcium concentrations from 10^{-7} to about 2.5×10^{-4} M. The plateau region for ΔH_d in this range of calcium concentration appears to persist indefinitely and shows an approximately 40% increase in ΔH_d. Increasing the calcium concentration further, however, increases the plateau ΔH_d considerably, and this increase reaches 70–75% at 10^{-2} M calcium. This additional increase in ΔH_d is not stable but has a tendency over a very long period of time to descend approximately to the same level as that of the curves at low calcium concentrations. The rate of change of ΔH_d is not affected appreciably by changes in calcium concentration.

Figure 3 presents data obtained with normal fibrinogen in the presence of EDTA. Here, the T_d scale is expanded 5-fold with respect to that of Figure 2 to show more clearly the decrease in T_d in the initial phase of the reaction. The total change in T_d, in agreement with Figure 2A, is only a fraction of a degree. The change in ΔH_d is similar to changes observed in the presence of calcium (Figure 2B).

In Figure 4, the asymptotic values of the temperature shifts of the D endotherm of the fibrin gels are plotted against the free calcium concentration. The latter was measured down to 10^{-6} M with the calcium electrode. The measurements between 10^{-5} and 10^{-6} M are only approximate, but since the effect of calcium is very small in this region, the greater error is inconsequential. Calcium concentrations below this range were obtained from the calcium content of the last dialyzate of the EDTA–fibrinogen determined by atomic absorption spectrophotometry or by calculations based on the binding constants of Marguerie et al. (1977). The changes in T_d for both bovine and human fibrinogens were converted to the same scale in the following way. Let $T_{d,0}$ equal the denaturation temperature at zero time and $T_{d,\infty}$ the denaturation temperature at the end of the experiment. Then the change in T_d at a particular time is $\Delta T_d = T_d - T_{d,0}$. The T_d values at zero time were taken as either the T_d of fibrinogen before thrombin addition or the value of T_d determined by extrapolation to zero time, at low calcium concentrations, as shown in Figure 2A.

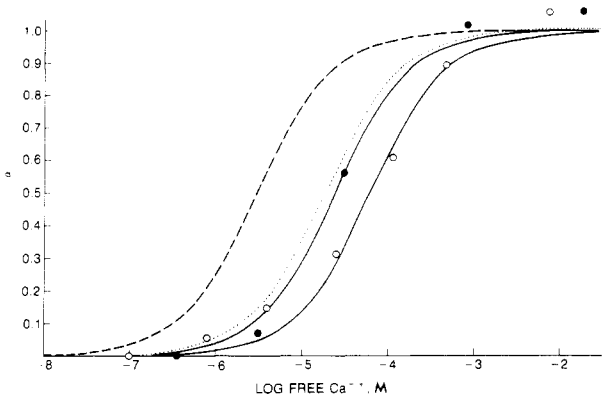

FIGURE 4: Temperature shift of the D endotherm of fibrin as a function of calculated free calcium ion concentration. The fractional attainment of the plateau value, $\alpha = \Delta T_{d,\infty}/11.5$ °C, is shown for EDTA-treated bovine fibrin (○) and EDTA-treated human fibrin (●). At the highest Ca^{2+} concentrations, $\Delta T_{d,\infty}$ for bovine fibrin is 12.0 °C; for human fibrin, 13.8 °C. Solution conditions were as follows: bovine fibrinogen, 63.5 mg/mL, in 0.3 M NaCl and 0.05 M Tris-HCl, pH 6.8; human fibrinogen, 110 mg/mL, in 0.3 M NaCl and 0.025 M Tris-HCl, pH 7.4. The binding of calcium (α = fraction of sites filled) to the high-affinity calcium binding sites of bovine (dashed line) and human (dotted line) fibrinogen is shown for comparison. Dissociation constants of 3.0×10^{-6} M for bovine (Marguerie et al., 1977) and 1.9×10^{-5} M for human (Nieuwenhuizen et al., 1981a) fibrinogens and three high-affinity sites for both species were assumed for calculations.

T_d of both bovine and human EDTA-treated fibrinogen was 59.0 ± 0.5 °C. At higher calcium concentrations, T_d extrapolated to zero time is clearly greater (see next section). Asymptotic values of ΔT_d, $\Delta T_{d,\infty} = T_{d,\infty} - T_{d,0}$, for both bovine and human samples, when plotted against − log [Ca^{2+}], reached a plateau of approximately 11.0 °C in the intermediate calcium binding region near $pCa^{2+} = 3$ and continued to increase slightly at higher Ca^{2+} concentrations. For comparison with the calcium binding experiments, fractional attainment of the plateau value of $\Delta T_{d,\infty}$ was defined as $\alpha = \Delta T_{d,\infty}/11.5$ °C. Figure 4 also shows the calculated binding of calcium to the high-affinity binding sites of bovine and human fibrinogen obtained with the binding constants given above, where α is the degree of occupancy of the high-affinity binding sites.

Effect of Calcium Ion on Thermal Stability of Fibrinogen. In Figure 2A it is apparent that the T_d values extrapolate to the same value at zero time, for calcium concentrations less than approximately 10^{-3} M. At higher calcium concentrations, zero-time values of T_d are considerably larger. These zero-time values of T_d should correspond to the T_d of fibrinogen. Zero-time measurements of T_d were determined for human fibrinogen without added thrombin, at various levels of calcium, in separate calorimetric experiments. The results are shown in Figure 5, together with extrapolated zero-time values of T_d for bovine fibrinogen. There is no change in T_d for fibrinogen in the calcium concentration range in which the large increase in T_d was observed on clotting. There are marked increases in T_d for bovine and human fibrinogens when the free calcium concentration is greater than about 3×10^{-4} M. A theoretical binding curve for a single group of $pCa^{2+} = 3$ is shown in the figure. Marguerie et al. (1977) report that the binding constant of the low-affinity calcium binding sites of bovine fibrinogen is 10^{-3} M. It appears that the change in T_d of the D nodules of both bovine and human fibrinogens at higher calcium ion concentrations is due to binding of calcium to low-affinity binding sites. No low-affinity binding sites were reported for human fibrinogen in the equilibrium dialysis studies of Nieuwenhuizen et al. (1979, 1981a).

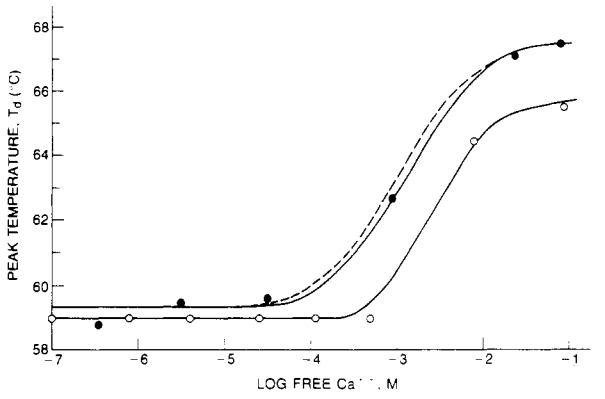

FIGURE 5: Denaturation temperature (T_d) of the D nodules of bovine (○) and human (●) fibrinogens as a function of free calcium concentration. The dashed curve is the binding of calcium ion by the low-affinity calcium binding sites of bovine fibrinogen (Marguerie et al., 1977), adjusted in height to span the temperature range of the human curve.

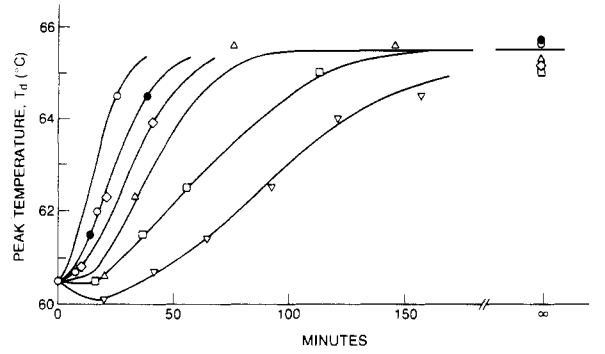

FIGURE 6: Shift of the peak temperature of the D endotherm of bovine fibrin as a function of time at various concentrations of thrombin. Concentration of fibrinogen (untreated with EDTA), 70 mg/mL, in 0.3 M NaCl and 0.05 M Tris-HCl, pH 7.2. Free calcium concentration was $\sim 5 \times 10^{-4}$ M. Thrombin concentrations (NIH units/mL) were (○) 3.26, (●) 2.18, (◇) 1.37, (△) 1.10, (□) 0.734, and (▽) 0.55. Infinite time (∞) is 800 min. Clotting times (thread-pull test) ranged from 1.3 to 10.8 min.

Effect of Thrombin Concentration on Rate of Change of Peak Temperature and Enthalpy Observed during Clotting of Fibrinogen. To demonstrate that the observed changes in T_d and ΔH_d are the result of the action of thrombin, the rate of the reaction was determined as a function of the amount of thrombin added. Fibrinogen that had not been treated with EDTA was used for this experiment. The shift in temperature of the D endotherm as a function of time with various amounts of added thrombin is shown in Figure 6. Within experimental error, all final temperature shifts are the same, independent of the amount of thrombin added. The lag period observed in turbidity studies of clotting (Hardy et al., 1983) is quite prominent here and clearly inversely proportional to the amount of thrombin added. We take here as the half-time of the reaction the time required for a temperature shift that is half of the total shift. When the logarithm of the reciprocal of the half-time is plotted against the logarithm of the concentration of thrombin, the data shown in Figure 7 are obtained. The line drawn through the points has a slope of unity. This demonstrates that the reaction is first order with respect to thrombin.

Role of Factor XIIIa. Our fibrinogen preparations contained factor XIII, i.e., the inactive form of the transglutaminase of plasma. Activation of this form requires calcium, thrombin, and reducing agents [for a review, see Folk & Finlayson (1977)]. Since all but the reducing agent were

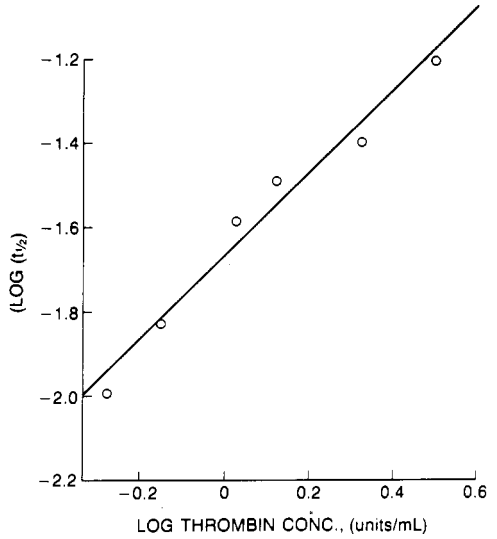

FIGURE 7: Order of the reaction with respect to thrombin. The logarithm of the reciprocal of the half-time for the progress of the changes observed by DSC ($t_{1/2}$) (min) is plotted against the logarithm of the thrombin concentration (NIH units/mL). The line is drawn with unit slope. Data are from the experiments of Figure 6.

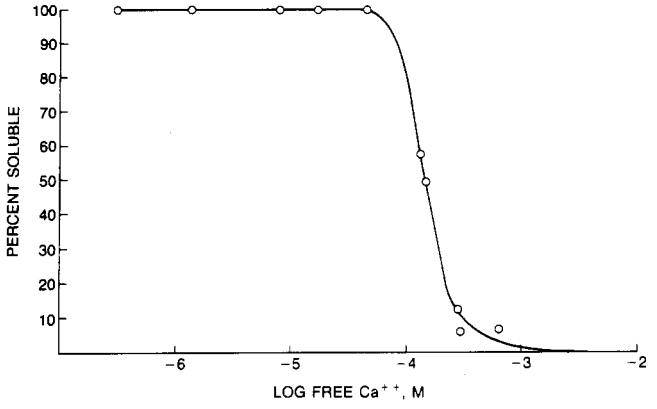

FIGURE 8: Solubility of fibrin clots in 6 M urea. Samples were clotted at the calcium concentrations indicated on the abscissa.

present in most of our experiments, activation was to be expected. In spite of this, we did not elect to eliminate factor XIII from our preparations because of the uncertain and laborious operations involved in doing this. Instead, we sought to determine whether cross-linking of the fibrin gel occurred and, when it did occur, to see what effect, if any, resulted from the specific inhibition of factor XIIIa.

First, cross-linking or its absence was ascertained by the solubility of the gels in 6–8 M urea or 5 M guanidine hydrochloride solutions. Figure 8 shows the solubility of clots in 6 M urea 30–45 min after addition of thrombin. Since cross-linking is an enzymatic reaction, increasing the interval between thrombin addition and suspension in the solvent should probably shift the curve somewhat to lower calcium concentrations. As shown, the transition between soluble and insoluble clots is a very sharp function of the calcium concentration. This transition roughly corresponds to the saturation of the high-affinity binding sites of fibrinogen. Below this calcium concentration, fibrinogen acts as an efficient chelator, and the free calcium concentration remains low, preventing activation of factor XIII. Above this calcium concentration, activation occurs. The solubility experiments were confirmed by examination of reduced fibrin in SDS–polyacrylamide gel electrophoresis. In these electrophoresis experiments, clots that were soluble showed neither γ-chain dimers nor α-chain polymers, whereas those that were completely insoluble showed no free γ-chains, only γ-chain dimers, and no α-chain polymers. These experiments were done in parallel with the DSC runs, but clots that went through the DSC thermal cycle showed the same results on gel electrophoresis if the heating did not exceed 110 °C. Heating at higher temperatures apparently produced a nonspecific cross-linking by some other mechanism. These results show that most of the effects of calcium occurred in the absence of cross-linking. However, in the experiments at calcium concentrations above 10^{-4} M, cross-linking definitely was present.

The most convincing evidence against cross-linking as the cause of the increased thermal stability was obtained in experiments carried out in the presence of 0.01 M iodoacetamide. This reagent had no effect on the clotting action of thrombin or formation of the gel, as evidenced by the absence of any effect on the clotting time. On the other hand, factor XIIIa, an SH enzyme, is completely blocked by iodoacetamide. In DSC experiments in the presence of 0.01 M iodoacetamide and 5×10^{-3} M calcium, there was no difference in the rate of the reaction or extent of the increase in T_d or ΔH_d of the D endotherm, compared to control experiments without iodoacetamide. Further, there was no effect on the changes in the D nodule observed by DSC on clotting when a large amount of factor XIIIa was added to fibrinogen in the presence of calcium. On the basis of all these data, we state with confidence that the changes in the thermal stability of the D nodule brought about by the presence of calcium during clotting are not caused or affected by the cross-linking of the molecules by factor XIIIa.

In conclusion, some remarks should be made about the endotherm of the E nodule in the clotting experiments. Little or no change is observed in T_d on clotting either in the presence or absence of calcium. However, when active factor XIII was added before thrombin addition, an increase in T_d of about 3 °C is observed for the E nodule. We have not attempted to elucidate further details of these E interactions.

Discussion

The experiments described in this paper confirm our previous demonstration of a substantial increase in stability of the fibrinogen molecule following clotting elicited by thrombin (Donovan & Mihalyi, 1974). This increase in the stability of fibrin, as compared to the separated molecules of fibrinogen, leads to a noncovalent stabilization of the fibrin clot that is probably accompanied by a significant change in conformation of the monomer. Further studies, which indicated that calcium ions play a significant role in these changes (Mihalyi & Donovan, 1978), are elaborated in the present paper. Because calcium binding is related to the conformational change, we review here our present knowledge of the binding of calcium to fibrinogen and its effect on the protein and its clotting.

Clear demonstration and quantitation of calcium binding to bovine fibrinogen was provide by Marguerie et al. (1977). It appears that bovine fibrinogen possesses two classes of sites: three high-affinity sites with a dissociation constant of 2×10^{-6} M and 14–20 sites of lower affinity with K_D of approximately 10^{-3} M, at pH 7.5. Human fibrinogen, on the other hand, has three sites of lower affinity than the corresponding ones in the bovine protein, with K_D equal to 1.9×10^{-5} M and no weak binding sites at all (Nieuwenhuizen et al., 1979, 1981a). Binding of calcium to the high-affinity sites of bovine fibrinogen did not affect conformation as judged from circular dichroism spectra but increased thermal stability and resistance to attack by plasmin (Marguerie, 1977). The protective effect against plasmin was localized to the C-terminal region of the γ-chain, i.e., to the D nodule of fibrinogen (Haverkate &

Timan, 1977; Purves et al., 1978; Nieuwenhuizen et al., 1981c). Electron microscopy revealed that each D nodule takes the form of two 4-nm diameter spheres in the presence of millimolar concentrations of calcium (Williams, 1981). Direct determination of the binding of calcium to isolated D nodules confirmed the existence of one site per nodule, with an affinity constant equal to that of the high-affinity sites of fibrinogen (Nieuwenhuizen et al., 1979, 1981a). The position of the third high-affinity binding site still seems controversial (Marguerie & Ardaillou, 1982; Nieuwenhuizen et al., 1983).

Acceleration of the overall clotting reaction by calcium was discovered long ago (Rosenfeld & Janszky, 1952; Ratnoff & Potts, 1954). Other studies showed that calcium increased rigidity of the clots (Ferry et al., 1951; Shen et al., 1974, 1975), but decreased clot opacity (Ferry & Morrison, 1947). The accelerating effect of calcium on clotting seems to be connected with the increased rate of polymerization of the fibrin monomer (Katz et al., 1953; Lorand & Konishi, 1964; Endres & Scheraga, 1972). Most of these studies preceded the demonstration of the high- and low-affinity binding sites, and the effects were not correlated with the binding per se. More recently, Marguerie et al. (1979) have shown that calcium ion at concentrations saturating the high-affinity binding sites increases the rate of thrombin action upon fibrinogen and also mediates, in the initial phase of the clotting reaction, the formation of a dimer between intact fibrinogen and fibrin monomer devoid of only fibrinopeptides A.

In the DSC experiments reported here, with the exception of the heavily cross-linked fibrin produced by a large amount of added factor XIIIa, the changes in the thermal unfolding of the domains of fibrinogen were localized exclusively to the D nodule. The reason for this may not be an intrinsic difference in this respect between the D and the E nodules but may be due to the large difference in their temperatures of unfolding; on heating, the D nodules are completely unfolded before the E nodules begin to unfold. Thus, if structural alterations in the E nodule were caused by D–E interactions, these alterations would be eliminated when the D nodule was heat denatured, and the E nodule might revert to its original conformation.

Alterations of the conformational transitions of fibrinogen produced by calcium are distinctly different from those observed for fibrin. Calcium-free fibrinogen and fibrinogen in the presence of up to about 3.5×10^{-4} M free calcium (with all the high-affinity binding sites saturated) do not show an observable difference in either T_d or ΔH_d, as shown in the scheme presented in Figure 9. Above this level of calcium, there is a marked increase in T_d, but no change in ΔH_d. Thus, the increase in T_d can be ascribed to the binding of calcium to the low-affinity binding sites. The shift in T_d is in accord with the increased resistance of fibrinogen to thermal denaturation in the presence of calcium, described by Godal (1960, 1969) and Marguerie (1977).

Fibrinogen also undergoes a conformational transition in the presence of EDTA, manifested in a decrease of the sedimentation coefficient, without change in molecular weight (Blömback et al., 1966; Endres & Scheraga, 1971). This change may be due to removal of the tightly bound calcium ions or to binding of EDTA. Indeed, it was shown recently that fibrinogen has strong binding sites for EDTA (Nieuwenhuizen et al., 1981b). Removal of EDTA by dialysis reversed the effect, but the reversal may have been caused by rebinding of calcium from calcium impurities in the NaCl used. The clottability of EDTA-treated fibrinogen, with EDTA removed by thorough dialysis, was also unchanged, as

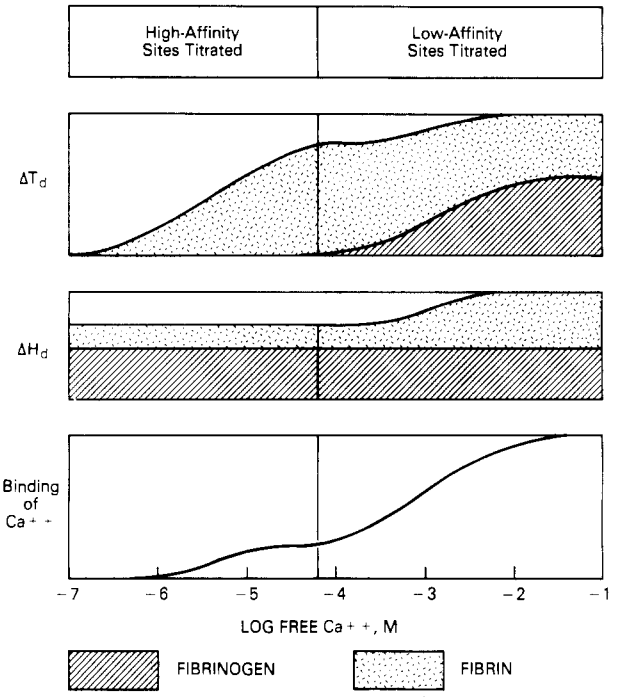

FIGURE 9: Scheme of the dependence of the thermodynamic changes on the concentration of free calcium ion. Calcium is bound only to the high-affinity binding sites on the left side of the figure and to both the high-affinity and low-affinity binding sites on the right side. The relative changes in denaturation temperature, T_d, and enthalpy of denaturation, ΔH_d, are shown in comparison with binding of calcium to the high- and low-affinity binding sites. For clarity, changes in thermal properties are shown to occur at the same calcium concentrations as occupancy of the calcium binding sites.

shown in this paper. However, it was reported that EDTA-treated fibrinogen formed clots of appreciably reduced rigidity (Shen et al., 1975). This, in contrast to the above findings, would suggest irreversible changes caused by the EDTA treatment. Further investigations are needed to resolve this discrepancy.

Formation of the fibrin gel entails conformational changes that are manifested in two ways: (1) by increase of the transition temperature and (2) by increase of ΔH_d, the enthalpy of unfolding. Some of these changes occur in the absence of calcium ion, but all are influenced by calcium. These are not trivial changes: ΔH_d may increase by 87%, an increase of 1190 kcal/mol, and T_d may increase by as much as 12 °C. These two changes in the thermodynamic parameters occur in parallel in most cases but are separated in others. Although an increase in T_d is always associated with an increase in ΔH_d, an increase in ΔH_d may occur without increase in T_d. Thus, there might be at least two different conformational transitions leading to formation of the gel. The pattern of these changes as a function of the free calcium ion concentration, which determines the degree of occupancy of the high- and low-affinity binding sites, shows that each set of thermodynamic parameters can be assigned to a particular kind of calcium binding. The data are distinctly different for bovine and human fibrinogens. The majority of the data were obtained with bovine fibrinogen, and these data will be discussed first.

In the absence of bound calcium, clotting of bovine fibrinogen produces an increase in ΔH_d of about 40%, but as shown in Figure 3, T_d remains practically unchanged, save for a small decrease that will be discussed later. As the calcium concentration is increased gradually, T_d increases also, as shown in Figure 2A and schematically in Figure 9. Up to a free

calcium concentration of 1.4×10^{-4} M, approximately, there is no further change in ΔH_d (Figure 2B). From the curves shown in Figure 4, it is obvious that the high-affinity calcium binding sites of fibrinogen are filled in this range of calcium ion concentrations. Shifted by about 1.5 pCa^{2+} units to the right, the curve of T_d as a function of $-\log [Ca^{2+}]$ increases parallel to the calcium binding curve. Either the binding of calcium is weakened by formation of the gel or new binding sites with a K_D of approximately 4×10^{-4} M are created by the clotting. With human fibrinogen, the binding curve to the high-affinity sites and the T_d vs. $-\log [Ca^{2+}]$ curves nearly coincide. Thus, fibrinogens from the two species form gels that have distinctly different physicochemical characteristics. However, this is not a unique phenomenon. Other physicochemical parameters also distinguish gels of protein from these two species. Thus far, we have seen the effect on the clotting process of the binding of calcium to the high-affinity binding sites. It must be remembered that unclotted (native) fibrinogen does not show a change in either ΔH_d or in T_d at these calcium concentrations.

In the range of calcium concentrations in which calcium binds to the high-affinity sites, there must be two conformational states of the fibrin molecules: one in the absence of calcium for which only the increase in ΔH_d is observed and a second one with the high-affinity binding sites saturated with calcium, for which the increase in ΔH_d is associated with an increase in T_d (Figure 9). A noted already, under some conditions the two forms appear to coexist (Figure 1A), resulting in a broadening of the endotherm for the D nodule and sometimes a clear splitting of the endotherm. Observation of the two forms also means that reequilibration of these forms must be slow compared to the rate of heating employed in the DSC experiments.

Another aspect of the binding of calcium ion to the high-affinity sites deserves comment. Although the increase in the T_d of the D nodules appears proportional to the amount of calcium ion present, the enthalpy of denaturation does not appear to be affected unless the calcium ion concentration is very high and the low-affinity sites bind calcium (Figures 2 and 9). Accordingly, the effect of calcium ion at concentrations below about 10^{-4} M must be largely entropic, in determining, for example, the position of water molecules, polypeptide chains, or orientations of domains, without affecting their enthalpies of interaction. This is a curious feature of the calcium binding to the high-affinity sites. A straightforward calculation of the entropy change produced by calcium binding, from the change in T_d on clotting, gives an entropy decrease of about 40 eu/mol of D nodules in the absence of a specific effect of Ca^{2+} on ΔH_d.

Increasing the calcium concentration above about 10^{-4} M causes the filling of the low-affinity sites (Figures 5 and 9). This has no effect on the ΔH_d of fibrinogen, as shown by the extrapolation to zero time of the curves of Figure 2B. Although we did not increase the calcium concentration enough to saturate the low-affinity binding sites, it appears that when 90% saturation is reached, T_d is increased modestly on clotting (about 2 °C above the value reached on saturation of the high-affinity sites), but ΔH_d nearly doubles. The increase of ΔH_d, however, is only transient, at least in the lower range of calcium concentration. The ΔH_d shifts back to the level reached in the binding range of the tight-binding sites, as shown in Figure 2B.

At the higher calcium concentrations used in these experiments, cross-linking by factor XIIIa definitely occurs, as shown by the insolubility of clots in 6 M urea, Figure 8. However, as demonstrated under Experimental Procedures, these cross-links, exclusively between γ-chains, had no effect on any of the thermodynamic quantities observed for the D nodules.

The increase of T_d in the course of the clotting reaction has a definite lag period. This is clearly visible in Figure 6. The lag is inversely proportional to the rate of the reaction. Because of this lag, first-order rate constants are difficult to calculate, and we chose to use reaction half-times as a measure of the rate. Similar lag periods are observed also when other techniques such as turbidity or light scattering are used to measure polymerization. The basis for the lag period lies in the multistep nature of the clotting process. In the initial phase of thrombin action, predominantly dimers are formed, either of incomplete fibrin monomers with only one of the fibrinopeptides A cleaved off or between a complete fibrin monomer with both peptides cleaved off and an intact fibrinogen molecule (Smith, 1980). Further action of thrombin on these dimers produces completely activated species that are able to associate first to form long protofibrils and then, by lateral association, fibrin fibers (Hermans & McDonagh, 1982). The development of turbidity (and, apparently, changes in T_d) is connected mainly with the last step, fiber formation. Lag periods were very seldom observed in the course of the changes in ΔH_d. This may mean that the change in ΔH_d is a result of the initial D–E contact. The time curves of T_d not only show a lag but also a slight shift to lower temperature in this initial period. This is especially marked when the total change in T_d is small, as in the presence of EDTA, shown in Figure 3A. Since this occurs in the period in which fibrinopeptides are cleaved off by thrombin most vigorously but polymerization has not yet started, it may represent a small conformational change resulting from the loss of the fibrinopeptides, or from the formation of the initial dimers.

The polymerization of fibrin monomer is exothermic, having a ΔH of −44 kcal/mol (Sturtevant et al., 1955; Laki & Kitzinger, 1956), and is probably due to formation of D–E contacts. The heat evolved in this reaction should largely be dissipated, even with the earliest clots, by the time the DSC run is performed and is not observed in the DSC. The new interactions formed on this exothermic association must be disrupted during the heat denaturation of the fibrin in the DSC, and the same amount of heat must be supplied for disruption. However, 44 kcal/mol is only a small fraction of the total increase in ΔH_d observed after clotting, which is, on the average, 540 kcal/mol of D nodule. Therefore, there must be substantial rearrangements of the molecules following the initial contacts between the D and E nodules.

Studies of the rate of change in T_d as a function of thrombin concentration demonstrate that the reaction is first order with respect to thrombin (Figure 7). The rate of change of ΔH_d follows the same course, but the experimental data points (not shown) are more scattered. Thus, the thermodynamic changes are directly connected to thrombin action.

In our previous paper (Donovan & Mihalyi, 1974), we stated that the change in the thermal properties of the D nodule "appears to be mediated by a proteolytic process that takes place at perhaps a 20-fold slower rate than release of A and B fibrinopeptides." This conclusion prompted serious efforts on our part to demonstrate a second reaction of thrombin with fibrinogen, liberating stoichiometric amounts of peptides other than the classical fibrinopeptides A and B, at the slower rate suggested above. No other peptides conforming to these requirements were found in experiments in which up-to-date HPLC techniques were used. However,

minute amounts of a variety of other peptides were detected. Some of these had possibly been adsorbed on the fibrinogen as prepared. Others were undoubtedly produced by the extremely slow reaction of thrombin on fibrinogen, reported by Kang & Triantaphyllopoulos (1977), that eventually cleaves all the plasmin-susceptible bonds. Thus, given the direct connection between thrombin action and the calorimetric changes demonstrated in this paper, this failure to find new peptides implicates the cleavage of either the A or the B peptide, or both, with the conformational changes. We shall not give an extensive discussion of kinetics at this point, because this paper is limited to demonstration of the conformational changes taking place during the clotting and their connection to the binding of calcium to fibrin(ogen). Nevertheless, it is important to state the causal relation between thrombin action and the conformational changes. A more accurate delineation of the relationships between the release of fibrinopeptides and the conformational changes obviously is desirable. The description of the experiments already performed and their analysis is too lengthy to be included here. These will be published in a separate paper. It may be stated, however, that the conformational changes appear to occur at about half the rate of release of the B fibrinopeptide. Although the post hoc ergo propter hoc reasoning is dangerous, this is in accord with the suggestion of the following paper (Mihalyi & Donovan, 1985) that the conformational changes do not take place without release of fibrinopeptide B.

The rate relationship between conformational change and fibrinopeptide release given above is considerably different from our estimate in the previous publication. The reason for this is that fibrinopeptide B is released at approximately one-fourth the rate of fibrinopeptide A, and clotting time, according to presently accepted views, is determined largely by the release of fibrinopeptide A (Blömback & Vestermark, 1958). In our previous publication we compared the half-time of the change detected calorimetrically with the clotting time, *assuming that clotting occurs at about half the reaction time.* This was an error, since at moderate fibrinogen concentrations clotting occurs at about 25% completion of the release of fibrinopeptide A (Blömback, 1958). In our experiments at high fibrinogen concentration, clotting seems to occur at even earlier stages of completion of the fibrinopeptide A release. If (1) clotting occurs at one-fourth completion of release of fibrinopeptide A (at about half of the half-time of release of A), (2) release of fibrinopeptide B is one-fourth the rate of release of A, and (3) the conformational change occurs at half the rate of release of B, then the half-time of the conformational change would be roughly $^1/_2 \times {}^1/_4 \times {}^1/_2$ or $^1/_{16}$ of the clotting time. This is in agreement with our previously published observations of the rate of the conformational change. However, as stated above, we incorrectly assumed that the factor of 20 applied to the rate of release of fibrinopeptides as well as to clotting. If thrombin were to cleave a third peptide bond at a 20-fold slower rate than the liberation of the A and B fibrinopeptides, the reaction would probably have little chance to occur under physiological conditions. In the time required, the thrombin could be inactivated by the potent antithrombin of plasma. Assuming that cleavage of fibrinopeptide B elicits the conformational change, this restriction on lifetime of thrombin action is lifted, and the conformational change could be an obligatory part of the physiological clotting process.

The kinetics of the release of the fibrinopeptides is complicated and controversial at present (Martinelli & Scheraga, 1980; Higgins et al., 1983). Furthermore, the polymerization reaction seems to be even more complicated. We therefore do not think it worthwhile, at present, to consider possible relationships between different stages of polymerization and the changes observed calorimetrically. To speculate on the origin of the enthalpy changes seems equally futile.

In summary, the data presented here show changes in the denaturation temperature and enthalpy of denaturation of the D nodules of fibrinogen that are produced by intermolecular interactions of these nodules within the fibrin clot. These interactions, which may include interactions with E nodules, are modified and strengthened by the binding of calcium at the high-affinity binding sites on the D nodules. The conformational alterations and positional changes accompanying these interactions may be obligatory steps in preparation for covalent cross-linking of the clot by the transglutaminase, factor XIII.

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Registry No. Ca, 7440-70-2; thrombin, 9002-04-4.

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Clotting of Fibrinogen. 2. Calorimetry of the Reversal of the Effect of Calcium on Clotting with Thrombin and with Ancrod

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ABSTRACT: When clotting is effected by thrombin in the presence of calcium, the endotherm for the D nodules of fibrinogen broadens significantly and then becomes narrow again, while increasing in size. Clotting effected by the snake venom enzyme Ancrod, which releases only the A fibrinopeptides from the E nodule, shows only the broadening of the D endotherm. Accordingly, significant interactions of the D nodules of fibrinogen become possible only when the B fibrinopeptides of the E nodule are released on clotting. When calcium present during clotting is removed from the fibrin clot with ethylenediaminetetraacetic acid, the endotherm for the D nodules of fibrin shows nearly complete reversal if clotting was effected with Ancrod but appears to be divided into two endotherms if clotting was effected with thrombin. At neutral pH, new endotherms were observed for fibrinogen in the temperature range 105–140 °C.

The three-nodular model of the fibrinogen molecule originally proposed by Hall & Slayter (1959) on the basis of electron microscopic studies now appears to require revision in that finer detail can be recognized within the nodules of the molecule (Slayter, 1983; Williams, 1981, 1983; Cohen et al., 1983; Erickson & Fowler, 1983). Similar conclusions were reached by Privalov & Medved' (1982) and Medved' et al (1982), on the basis of their differential scanning calorimetric studies.

In the preceding paper (Donovan & Mihalyi, 1985), we reported differential scanning calorimetric studies of the clotting of fibrinogen and, in particular, the effect of calcium on clotting. It seemed likely that our analysis of the experiments could be further refined under conditions that might reveal substructures of the molecule. We have now evaluated apparent changes in the number of domains in the D and E nodules in the course of clotting with thrombin and with the