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Conformation of Fibrinogen: Calorimetric Evidence for a Three-Nodular Structure

(fibrin/blood clotting/differential scanning calorimetry/protein subunits/thermal denaturation)

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ABSTRACT **Solutions of fibrinogen show two endothermal (denaturing) transitions, at 61° and at 100°, when heated in a differential scanning calorimeter. Similar transitions are observed for a mixture of the fragments D and E obtained by limited proteolysis of fibrinogen. Isolated fragment E shows only a single transition, at 97°. The independent thermal denaturation of these portions of fibrinogen supports the three-nodular model proposed for fibrinogen. The D and E subunits retain their characteristic denaturation behavior when fibrinogen is clotted by thrombin addition, but over a period of about one hundred times the clotting time, the denaturation temperature of the D subunit increases by 9° and its enthalpy of denaturation by one-third. Since this change takes place in the absence of Factor XIII activity, and its rate is proportional to thrombin concentration, it is presumed to be mediated by a proteolytic cleavage distinct from those which liberate the A and B fibrinopeptides.**

The three-dimensional structure of the fibrinogen molecule has long been a controversial issue, still unresolved (1, 2). The most popular and enduring three-nodular model of Hall and Slayter (3) has been challenged recently. Some of the models proposed to replace it are variations of the Hall and Slayter model (4, 5), but the Köppel model (6) constitutes a radical departure. Studies of the proteolytic dissection of fibrinogen (7–8) provide strong support for the three-nodular model, since limited proteolysis produces three large fragments which comprise 65% of the weight of fibrinogen, and peptides which make up the remainder of the molecule (10). Depolarization of fluorescence measurements (11) indicate a looseness in organization of the native molecule compatible with the three-nodular model, but the interpretation of these fluorescence experiments rests on a number of assumptions.

The thermal denaturation of fibrinogen was investigated in search of additional support for a model composed of three compact subunits§ linked by relatively long stretches of peptide chains, i.e., lacking cooperativity between subunits. The high-molecular-weight fragments§, D and E, isolated after proteolytic cleavage of fibrinogen are known to be heat-denatured at widely separated temperatures (8). The present differential scanning calorimetric (DSC) experiments show that the subunits are heat-denatured independently, both in the native fibrinogen molecule and in the fibrin clot.

Abbreviations: DSC, differential scanning calorimet-er/ry/ric; T_d, denaturation temperature; ΔH_d, enthalpy of denaturation.

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§ We choose to refer to the nodular moieties as *subunits* when present in the native fibrinogen or fibrin and *fragments* when isolated by proteolytic digestion.

MATERIALS AND METHODS

Bovine fibrinogen (Fraction I from bovine plasma), lot no. G 10505, obtained from Reheis Chemical Co., Chicago, Ill., was purified by Laki's procedure (12). This yielded a preparation of approximately 94% clotability, considered sufficiently pure for the present purposes. The protein precipitated by ammonium sulfate was dissolved in a small volume of 0.3 M NaCl and was dialyzed exhaustively against 0.3 M NaCl, 0.025 M Na phosphate (pH 7.18) to give a very viscous but homogeneous solution. Its viscosity decreased markedly when it was warmed to room temperature. Fibrinogen concentration of this stock solution, determined optically assuming $E^{1\%}_{1\text{ cm}}$ (280 nm) = 15.06 (13), was 8.76%. Factor XIII activity was absent from this preparation, since clots formed in experiments were soluble in 6 M urea 24 hr after clotting.

Fragments D and E were prepared in the following way. Fibrinogen was digested with trypsin to the extent of 56 moles of base (pH-stat) consumed per mole of fibrinogen at pH 8.0 and 25° (14), and the digest was fractionated on a 100-cm Sephadex G-200 column. The main peak (63.5% of the weight of protein put on the column) was dialyzed against distilled water and lyophilized. The powder was dissolved in 0.05 M Na phosphate buffer (pH 7.18). An aliquot of the solution (15 mg of protein per ml) was preserved as the mixture of D and E fragments. Scans at 280 nm of polyacrylamide gel electrophoresis runs in the presence of sodium dodecyl sulfate showed that the preparation contained 74.5% fragment D and 25.5% fragment E by weight. Fragment E was prepared by heating the D + E mixture at 93° for 5 min. The supernatant contained only fragment E, according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in nearly 100% yield. The solutions were lyophilized; the powders were dissolved in water to yield pH 7.2 (0.2–0.3 M phosphate) solutions of 10.1% (D + E mixture) and 4.7% (fragment E). Specific absorbances calculated on the basis of Kjeldahl nitrogen determinations were: $E^{1\%}_{1\text{ cm}}$ (280 nm) = 19.9 for fragment D and 9.0 for fragment E. The amounts of D and E in fibrino-

‖ Reference to a company or product name 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.

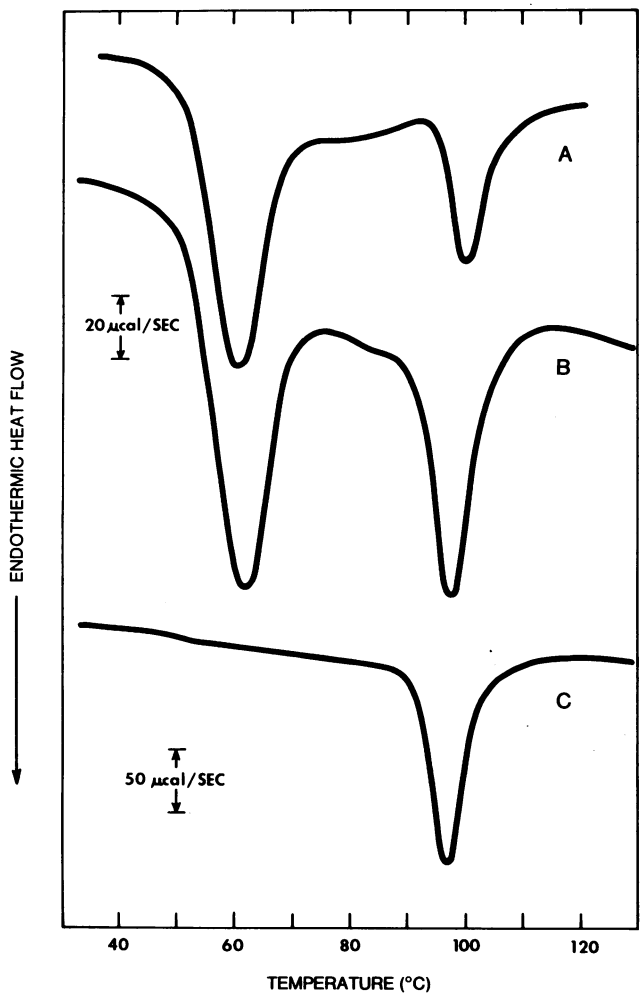

FIG. 1. Thermal denaturation of the D and E subunits of bovine fibrinogen. (A) Native fibrinogen, 25.7-μl sample of 87.6 mg/ml; (B) mixture of D and E fragments, 24.9-μl sample of total concentration 101 mg/ml; (C) E fragment, 22.2-μl sample of 47.0 mg/ml. Upper calibration mark is for curves A and B; lower calibration mark is for curve C. Heating rate was 10°/min. The slope of the baseline of these thermograms varies primarily because different weights of water were used as reference material in the DSC.

gen (stoichiometry: 2D + 1E) were calculated to be 50.6% and 14.7%, respectively, on the basis of molecular weights of 86,000 for D (9, 10) and 50,000 for E (9). Enthalpies of denaturation of these subunits in fibrinogen were calculated on this basis.

Fragment Y (one D subunit covalently linked to one E subunit) was prepared from a digest arrested at 46 moles of base consumed per mole of fibrinogen. It was isolated by a procedure similar to that used for the preparation of the mixture of D and E fragments, and dissolved in 0.3 M NaCl, 0.025 M phosphate (pH 7.18).

Bovine thrombin (EC 3.4.21.5), grade III (150 NIH units/mg), lot 61C-80239, was obtained from Sigma Chemical Co. It was dissolved in distilled water to give a solution of 30 NIH units/ml.

Thermal measurements were made with a DuPont model 990 differential scanning calorimeter used and calibrated as described (15, 16). For clotting experiments, fibrinogen and thrombin solutions were mixed in a small beaker at room temperature. Aliquots were then quickly transferred to hermetic DSC pans and allowed to clot in the pans. Clotting time was determined from observation of the portion of solution remaining in the beaker.

TABLE 1. *Denaturation temperatures and enthalpies of denaturation of the D and E subunits of fibrinogen*

	D		E	
Sample	T_d* (°C)	ΔH_d (cal/g)	T_d* (°C)	ΔH_d (cal/g)
Fibrinogen	61.0	4.24 ± 0.11†	100.2	6.46 ± 0.31†
E fragment			97.0	6.88 ± 0.9‡
D + E mixture	62.0	3.68 ± 0.4‡	97.7	6.36 ± 0.9‡

* At a heating rate of 10°/min.
† Standard deviation of the mean (10 measurements).
‡ Standard deviation of a single measurement.

Choice of a temperature characteristic of irreversible denaturation of a protein is essentially arbitrary. For this study, the denaturation temperature, T_d, was defined as the temperature of maximum rate of heat flow (peak temperature) when the DSC was operated at a heating rate of 10°/min. Enthalpies of denaturation (ΔH_d) were determined from the area between the denaturation curve and a baseline drawn under the denaturation peak.

RESULTS

When fibrinogen solutions are heated in the DSC, two endotherms are observed, near 60° and 100°. Fig. 1 compares the thermogram of fibrinogen (curve *A*) with those of a mixture of the D and E fragments (curve *B*) and the E fragment only (curve *C*). It is clear that the E fragment has no endotherm in the 60° range, and that the thermogram of the mixture of the D and E fragments closely resembles that of fibrinogen. (The difference in magnitude of the endotherms is caused by difference in sample weights and in the relative amounts of fragments D and E in the mixture compared to fibrinogen.)

Table 1 shows that the enthalpies of denaturation of the D and E fragments differ substantially, and, within experimental error, are the same whether they are combined in the fibrinogen molecule, separated by proteolytic hydrolysis of the interconnecting peptide chains, or (in the case of E) isolated as pure species. Likewise, the temperatures characteristic of their heat-denaturation are essentially the same whether the subunits are combined in the native fibrinogen or separated by proteolysis. Neither T_d nor ΔH_d observed in these experiments is a function of protein concentration over the range 20–90 mg/ml.

The Y fragment has two thermal transitions, at 60.7° and 96.6° (not shown). On the basis that the Y fragment contains one D and one E subunit with molecular weights given above, the ratio of the enthalpy of denaturation of the D subunit to that of the E subunit is determined to be 0.58. The ratio calculated from the data in Table 1 is 0.65. Although the agreement is not as good as expected, it is clear that the Y fragment must contain one D and one E subunit that are heat-denatured independently.

The D and E subunits are also denatured independently in the fibrin clot (Fig. 2). At times equal to or less than five times the clotting time, D has essentially the same thermal denaturation properties as it has in fibrinogen. (The T_d of the

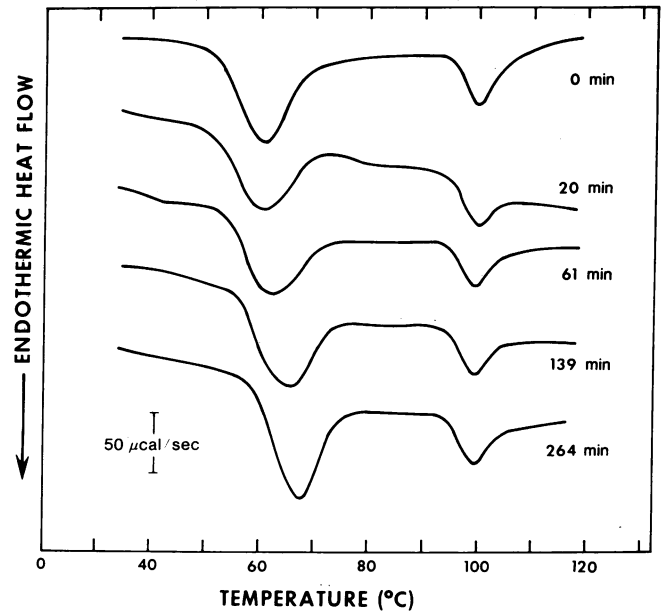

FIG. 2. Changes in the DSC thermogram upon clotting of fibrinogen with thrombin. From top to bottom, fibrinogen before addition of thrombin and at specified times after addition of thrombin, at 24.5°. Fibrinogen concentration, 88 mg/ml; thrombin activity, 0.6 NIH units/ml; clotting time, 5 min. Volumes of samples, from top to bottom, were 22.6, 17.7, 17.5, 18.6, and 18.6 μl. Heating rate, 10°/min.

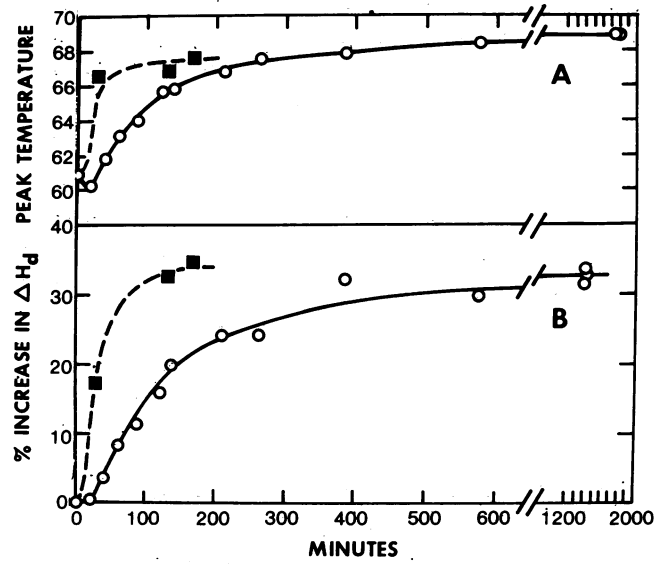

FIG. 3. Time dependence of the position (A) and size (B) of the DSC denaturation peak for the D subunit of fibrinogen upon clotting. Zero-time values are for fibrinogen. O, Points from experiment of Fig. 2 (0.6 NIH units of thrombin per ml). ■, An experiment with 5.1 NIH units/ml (clotting time, about 100 sec).

D subunit appears to be shifted very slightly, perhaps by 0.7°, to lower temperature immediately upon clotting.) Over a period of time of the order of 100 times the clotting time, T_d of the D subunit increases about 9°, and ΔH_d of D increases by about one-third. Fig. 3 shows that both of these processes appear to take place at approximately the same rate. After the short induction period of about five times the clotting time (during which separate experiments show that the turbidity change on clotting is essentially complete), the kinetics for these changes appear to be first order. The rate at which these changes in the denaturation properties of the D subunit take place is a function of thrombin concentration. However, altering the concentration of thrombin does not alter the extent of the change in either T_d or ΔH_d of D. Neither the T_d nor ΔH_d of the E subunit appears to change significantly after clotting. A clot heated in a test tube does not dissolve, but instead becomes more turbid as the 50°–60° temperature range is attained. Accordingly, thermal effects from the dissociation of the fibrin polymers appear to be absent. It has been reported that uncrosslinked fibrin does not dissolve when heated in solutions of low salt concentration (17).

DISCUSSION

The principal findings of these experiments, that portions of the structure of fibrinogen undergo thermal denaturation independently, and that these portions correspond to the D and E fragments obtained on limited proteolysis of native fibrinogen (8), are results that could be expected on the basis of the Hall and Slayter model (3) of fibrinogen. They are also consistent with other nodular models that have been proposed (4, 5). However, there does not appear to be any way to reconcile these results with the model of fibrinogen proposed by Köppel (6), since that model appears to require cooperativity among all portions of its cage-like structure. The near-identity of the denaturation temperatures and enthalpies of denaturation of fibrinogen and of the D and E fragments means that these fragments exist independently as subunits in native fibrinogen in essentially the same conformations that they have as proteolytically released fragments.

When fibrinogen is clotted with thrombin, the D and E subunits retain sufficient independence that their thermal denaturation is essentially unaltered *immediately after clotting*. Thus, at least at first, the conformation of these subunits in fibrin must be very similar to their conformation in fibrinogen, and removal of the fibrinopeptides does not significantly alter the conformation or stability of the subunits. Mechanisms of clotting involving a conformational change transmitted throughout the fibrinogen molecule on removal of fibrinopeptides (ref. 18, and ref. 2, p. 95) therefore appear untenable. At longer times after clotting, the thermal stability of the D subunit increases, while that of the E subunit appears unchanged. It cannot be concluded from this last observation that no significant interaction takes place between D and E subunits in the clot, since the thermal denaturation of the E subunit is only observed when the D subunit has already been denatured. On the contrary, there is electron microscopic (19) and chemical (20) evidence for association of D subunits with E subunits in the initial formation of overlapping fibrin dimers after the A and B fibrinopeptides have been split from the E subunit by thrombin. Whether the interactions leading to increases in T_d and ΔH_d of the D subunits are D–D interactions of D–E interactions, or both, the strength of the interactions between D and E subunits is not sufficient to cause both to denature simultaneously, as trypsin and soybean trypsin inhibitor do when associated (16).

The increase in T_d and ΔH_d of the D subunit does not appear to begin until after about five times the clotting time measured in the usual way—by visual inspection of gel formation. Release of A and B fibrinopeptides is mostly completed in this time period (21). Since the rate of this slow change in the

thermal properties of the D subunit is proportional to thrombin concentration, it 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. It is possible that this change in the thermal properties of the D subunit follows the proteolytic release of a tripeptide from the α-chain (22), but the relative rates of these processes have not been determined accurately. Moreover, it is not yet certain that the change in the thermal properties of the D subunit is actually mediated by thrombin rather than by a contaminant of thrombin. It seems possible that thrombin mediates two interactions—a weaker one that aligns overlapping dimers by electrostatic interactions between D and E subunits, made possible by the splitting off of the A and B fibrinopeptides, and a stronger interaction between D subunits that occurs after further proteolytic action of thrombin (22–24), and that serves to orient the D subunit pairs preparatory to covalent crosslinking by Factor XIII. The ordering of these reactions in time could be determined by the relative rates of hydrolysis of the corresponding peptide bonds. Slow changes in fibrin gels in the absence of Factor XIII activity have been observed by measurements of gel elasticity (25–27). It has been suggested that a slow reorganization of the fibrin network is the rate-limiting step for crosslinking by Factor XIII (26).

The polymerization of fibrin at pH 6.9 has been shown to be exothermic by 45 kcal/mole (28, 29). Accordingly, the enthalpy of denaturation of the fibrin clot might be larger than the enthalpy of fibrinogen by this amount. Since ΔH_d for two D subunits is 730 kcal/mole of fibrin, an increase of 45 kcal/mole would be a 6% change. These DSC results (Fig. 3, and other experiments not presented) do not show an increase in ΔH_d of the D subunit at times up to five times the clotting time, when the polymerization reaction would be essentially complete (25), although an increase in ΔH_d of 6% should easily be detectable. However, these calculations assume that the intermediate denatured states of fibrin and fibrinogen (D subunits denatured, E subunit native) are equivalent. Their nonequivalence may explain this discrepancy.

A previous study of the heat-denaturation of fibrinogen (30) indicated that the coagulation temperature was in the neighborhood of 55°. The transition temperature of fibrin also has been reported to be approximately 55° (17). It is evident from the present study that this temperature corresponds to denaturation of the D subunit only, and that denaturation at this temperature leaves the E subunit intact. This difference in heat stabilities of the subunits was used to prepare the E fragment from the mixture of D and E fragments.

The enthalpies of denaturation observed for the D and E fragments and subunits (Table 1) are characteristic of native globular proteins. The 35% of the fibrinogen molecule not present in the D and E subunits does not appear to possess a significant enthalpy of denaturation either in native fibrinogen or in the smaller peptides and fragments obtained on limited proteolysis, since the DSC measurements show no other endotherms.

Proteins that show more than one distinct stage of denaturation are not common. Stable intermediates that have been documented were all obtained by variation of pH or denaturant concentration (31). At present, fibrinogen appears to be the only protein having a stable intermediate (with denatured D subunits and a native E subunit) or *thermal* denaturation. It appears to fit Tanford's criterion of a protein consisting "of several moieties loosely held together in the native state, which can denature independently" (ref. 31, p. 236).

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