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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

enzyme from the snake venom of *Agkistrodon rhodostoma*. We have also determined the reversibility of the effect of calcium for both the thrombin and the snake venom enzyme clots.

Materials and Methods[1]

Bovine fibrinogen, "normal" (not treated with EDTA),[2] of stock concentration 85.3 mg/mL and human thrombin (4480 NIH units/mL) were the same preparations described in the previous paper (Donovan & Mihalyi, 1985). The clotting enzyme from the venom of the Malayan pit viper *Agkistrodon rhodostoma* (trade names: Ancrod, Arvin, Venacil) was a commercial preparation obtained from Abbott Laboratories, North Chicago, IL, lot 33-657-DB, nominal activity 100 NIH clotting units/mL. When used, the preparation contained 83 NIH units/mL (esterase activity on TAME substrate) and had a specific activity of 1980 units/mg.

Differential scanning calorimetric experiments were carried out with the Du Pont 990 thermal analyzer. The kinetic studies of the clotting reaction were performed essentially as described in the preceding paper (Donovan & Mihalyi, 1985). Some thermal denaturations of fibrinogen were carried out with the pressure DSC cell at 250 psig of nitrogen, which allowed heating to over 150 °C without sample pan failure. Heating rates of 2 and 5 °C/min were used with this cell and 10 °C/min for clotting experiments with the regular DSC cell. The more lengthy procedure required to use the pressure cell precluded its use in the kinetic experiments of clotting.

Calculations. The calorimetric enthalpy (ΔH_{cal}) of the thermal unfolding reaction, here also called ΔH_d, was calculated from the area of the endotherm. This enthalpy is the sum of the enthalpies of whatever number or kind of components unfold within the envelope of the endotherm. It is experimentally determined, and expressed in units of calories per gram of material placed in the calorimeter. Multiplying ΔH_d by the molecular weight of the species or nodule denatured gives ΔH_{cal} in units of calories per mole.

The van't Hoff enthalpy, ΔH_{vH}, is the enthalpy calculated from the van't Hoff equation, on the assumption that each point of the endotherm (the transition curve) represents an equilibrium between native and denatured forms. Since unfolding is a time-dependent reaction, fullfilment of this assumption depends on the rate at which the temperature change is effected during the experiment. At slow heating rates, a quasi-equilibrium may be established; the slower the heating rate, the closer will be the measured apparent equilibrium to the true one. However, technical factors pertaining to the instrument, as well as the practical limitations of carrying out a series of experiments to evaluate the kinetics of a clotting reaction, preclude the use of very slow heating rates. Mainly because of the constraints presented by the kinetics of clotting, we have chosen the somewhat high heating rate of 10 °C/min for the kinetic experiments. This appears to be permissible in view of the good agreement of our data (Donovan & Mihalyi, 1974) obtained at a heating rate of 10 °C/min with the data of Privalov & Medved' (1982) obtained at a heating rate of 1 °C/min.

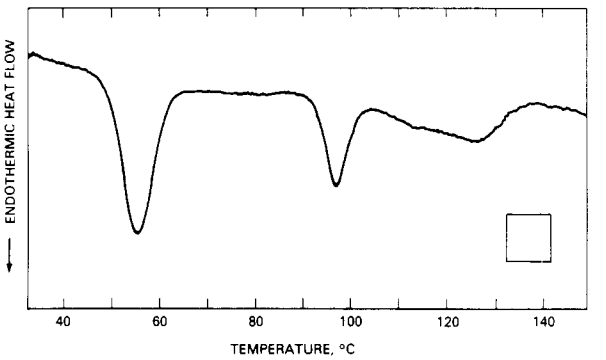

FIGURE 1: Differential scanning calorimetric recording of the denaturation of fibrinogen in 0.3 M NaCl, no buffer, pH 6. Heating rate was 5 °C/min. A total of 18.8 mg of sample of an 85.3 mg/mL fibrinogen solution was heated in a hermetic sample pan in the pressure DSC cell at 250 psig. The area of the calibration square is equivalent to 1.2 mcal.

A number of forms of the van't Hoff equation are used to evaluate ΔH_{vH} from thermograms. We have used the form of the equation given by Sturtevant (1974) for a two-state (one-step) transition:

$$\Delta H_{vH} \simeq 7T_m^2/\Delta T_{1/2}$$

where T_m is the temperature on the absolute scale of the maximum excess heat capacity, and $\Delta T_{1/2}$ is the width of the endotherm at half-height ("half-width"), in degrees. We have used the maximum of the transition endotherm (T_d) for T_m, since the error introduced by this assumption is very small. Changes in T_m in the course of the reaction of fibrinogen with thrombin are small on the absolute temperature scale. Accordingly, changes in the half-width of the transition endotherm are approximately inversely proportional to changes in ΔH_{vH} during the reaction.

When a molecule is composed of a number of identical unfolding units that are unfolded independently, then the ratio $\Delta H_{cal}/\Delta H_{vH}$ is equal to the number of these units. If the unfolding units are not identical but have closely spaced transition temperatures, the resulting composite transition curve will be broadened, and the apparent ΔH_{vH} will be too small. This will result in an overestimate of the number of unfolding units.

Results

Analysis of the Complete Unfolding of Fibrinogen and Fibrin. In the present studies, the pressure cell permitted DSC measurements to 150 °C. Figure 1 shows a thermogram obtained with this cell at pH 6 for fibrinogen that had not been treated with EDTA. An overlapping (double) endotherm is clearly visible in the thermogram between 105 and 140 °C. This high-temperature endotherm could not be resolved with confidence into two components, so the total enthalpy of denaturation of both components was calculated. Molecular weights used for the calculations where as follows: D, 8.6×10^4; E, 5.0×10^4. In Table I, thermodynamic quantities derived from this thermogram are compared with those reported by Privalov & Medved' (1982) for fibrinogen at pH 8.5 and at pH 3.5.

Comparison of the Clotting of Fibrinogen with Ancrod to That with Thrombin. Ancrod removes only the two fibrinopeptides A from the fibrinogen molecule (Ewart et al., 1977) and produces a clot with slightly different properties from that

[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; psig, pounds per square inch gauge.

Table I: Estimates of Enthalpies and Cooperativities

species[a]	ΔH_{cal}[b] (kcal/mol)[b]	ΔH_{vH} (kcal/mol)	$\Delta H_{cal}/\Delta H_{vH}$	source
	Fibrinogen and Its Fragments			
D (fibrinogen)	340 (5)	106 (3)	3.2	this paper, pH 6
D_H frag (LT1)	263	110	2.4	Privalov and Medved', pH 3.5
D_H frag (LT1)	350	143	2.4	Privalov and Medved', pH 8.5
E (fibrinogen)	245 (6)	204 (3)	1.2	this paper, pH 6
E frag (HT1)	203	161	1.3	Privalov and Medved', pH 3.5
E frag (HT1)	265	210	1.3	Privalov and Medved', pH 8.5
105–140 °C	500 (2)			this paper, pH 6
	EDTA-Reversal Species[c]			
D′ (51.5 °C)	125 (2)	123 (2)	1.0	this paper, pH 7.2
D″ (58 °C)	249 (2)	85 (2)	2.9	this paper, pH 7.2

[a] D_H is the "heavy D" fragment (M_r 9.5 × 10^4), and LT1 and HT1 are the major endotherms of the D and E nodules, respectively (Privalov & Medved', 1982). [b] Per mole of fragment or nodule, except for the unresolved high-temperature peaks at 105–140 °C, for which the value is given per mole of fibrinogen. Standard deviations (σ_{n-1}) average 5% of the values given. Numbers in parentheses are numbers of replicates. [c] Assumed M_r values: D′, 2.8 × 10^4; D″, 5.6 × 10^4.

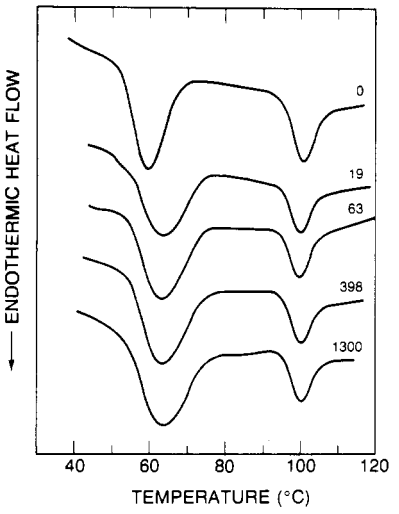

FIGURE 2: Clotting of fibrinogen with Ancrod at 5 × 10^{-3} M total calcium ion concentration. (Upper curve) Stock fibrinogen in 0.3 M NaCl before addition of buffer, calcium, and Ancrod. Other curves are labeled with the time (min) after addition of Ancrod (6.2 units/mL final concentration). For these samples, the solvent is 0.3 M NaCl and 0.05 M Tris-HCl, pH 6.8. Clotting time was 206 s. DSC heating rate was 10 °C/min. Concentration of stock fibrinogen was 85.3 mg/mL; those of other samples were 60.0 mg/mL. All sample volumes are equal within 5%.

obtained with thrombin (Shen et al., 1977; Spellman et al., 1977). The main difference appears to be a decrease of the rate of lateral association, which results in thinner fibers than with thrombin. Since the experiments in our previous paper suggested that the changes in thermal stability of fibrin are due to association, it seemed desirable to compare the clots obtained with the two enzymes.

Thermograms obtained at various time intervals during clotting of fibrinogen with Ancrod are shown in Figure 2. An

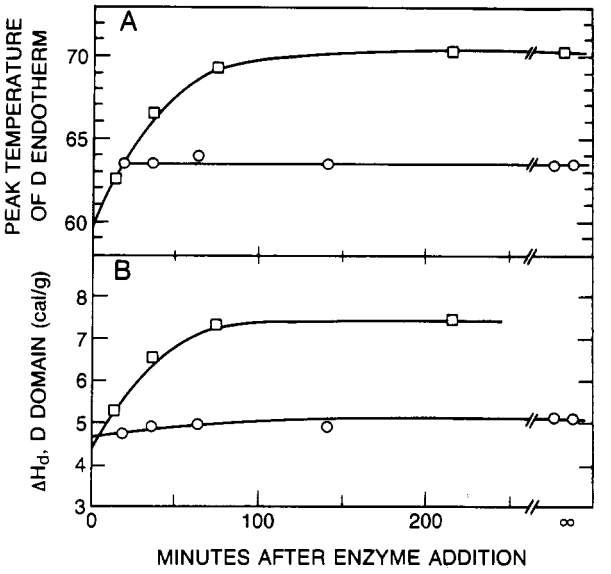

FIGURE 3: Clotting of bovine fibrinogen with Ancrod compared to clotting with thrombin. Conditions: fibrinogen, approximately 60 mg/mL in 0.3 M NaCl and 0.05 M Tris-HCl, pH 6.8. (A) Change in peak temperature of the D endotherm: (○) Ancrod, 6.2 units/mL, with normal fibrinogen, at 5 × 10^{-3} M calcium; (□) thrombin, 2.1 units/mL with EDTA-treated fibrinogen, at 10^{-3} M calcium. (B) Change in enthalpy of the denaturation of the D nodule observed in the same experiments.

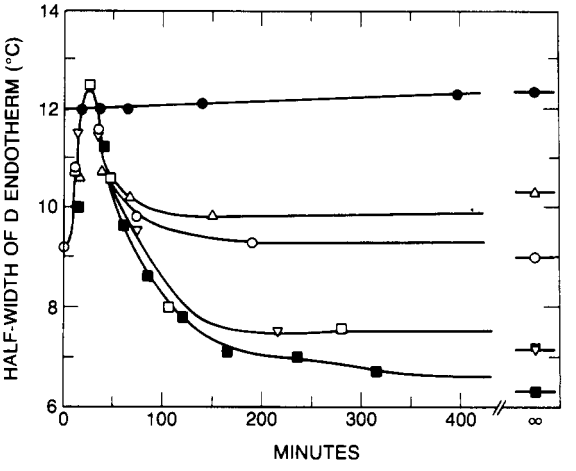

FIGURE 4: Changes in the half-width of the D endotherm of fibrinogen after clotting with thrombin or Ancrod. Thrombin: (△) no added calcium; (○) 5 × 10^{-4} Mm calcium; (▽) 10^{-3} M calcium; (□) 10^{-2} M calcium; (■) 2 × 10^{-3} M EDTA. Ancrod: (●) 5 × 10^{-3} M calcium. All solvents are 0.3 M NaCl and 0.05 M Tris-HCl, pH 6.8, except for the EDTA experiment, in which phosphate, 0.025 M, pH 7, replaced the Tris-HCl.

immediate broadening and a moderate shift to higher temperature of the D endotherm is apparent. The enthalpy of the D transition increases gradually by 7% over the course of the reaction. The peak temperature of the E endotherm shifts abouts 0.5 °C to lower temperature, but the enthalpy is unchanged, within experimental error, throughout the experiment. The changes in enthalpy and peak temperature of the D endotherm are compared in Figure 3 to those observed when thrombin is used for clotting at a comparable calcium ion concentration. The large increase in enthalpy and in peak temperature of the D endotherm do not occur when Ancrod is employed for clotting. The immediate increase in peak temperature is the result of the effect of high calcium ion concentration on the transition of fibrinogen itself, as shown in the previous paper. Clotting does not seem to add anything to this shift. Thus, removal of the B fibrinopeptides may be

Table II: Changes in Thermodynamic Properties of the D Nodule on Clotting with Thrombin and Ancrod

reaction time (min)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$\Delta H_{cal}/\Delta H_{vH}$
thrombin clots[a]			
0	370	86	4.3
25	530	65	8.2
∞	660	116	5.7
Ancrod clots[b]			
0–∞	430	66	6.5

[a] Thrombin, 2.1 NIH units/mL, normal fibrinogen, total calcium concentration 10^{-2}–10^{-3} M. [b] Ancrod, 6.2 NIH units/mL, normal fibrinogen, total calcium concentration 5×10^{-3} M.

required so that the large changes in enthalpy and peak temperature can occur.

Changes in the Number of Unfolding Units during Clotting with Thrombin or Ancrod. Figure 4 shows the changes of the half-width of the D endotherm during clotting of fibrinogen by thrombin in the presence of various concentrations of calcium or in the presence of EDTA. Also shown in Figure 4 are data for clotting with Ancrod at high calcium concentration. It is apparent that with the thrombin clots the endotherm first broadens, then becomes narrower, and, at high calcium concentrations, even narrower than before thrombin addition. Whereas the maximum broadening is about the same for all the thrombin curves, the final endotherm width decreases with increasing calcium concentration. Surprisingly, in the presence of EDTA, the endotherm becomes even narrower than in the presence of the highest calcium ion concentration used. In contrast, with Ancrod the endotherm broadens to approximately the maximum breadth observed, and this broadening persists through the whole experiment.

It was mentioned under Calculations that the width of the transition peak at the midpoint is inversely proportional to ΔH_{vH} and that the ratio $H_{cal}/\Delta H_{vH}$ gives the apparent number of unfolding units, n, that contribute to the transition endotherm (assuming the units are identical). In Table II are assembled calculations of ΔH_{cal}, ΔH_{vH}, and n during clotting of fibrinogen with thrombin and with Ancrod. Since these calculations are made from calorimetric experiments made at a heating rate of 10 °C/min, observed values of $\Delta T_{1/2}$ will be too large and calculated values of ΔH_{vH} too small. However, the relative magnitudes of the quantities listed are of interest. It is apparent that with thrombin, n almost doubles and then decreases substantially by reaction completion. With Ancrod, both ΔH_{cal} and ΔH_{vH} are approximately constant throughout the reaction. This results in an essentially constant value of n, which is, however, about 50% greater than n determined for the D nodules of native fibrinogen.

Reversibility of the Effect of Calcium Ion on the Unfolding Endotherm of the D Nodule. In Figure 5 is shown the course of two clotting experiments with thrombin and Ancrod. The upper curves show the relative areas of the D endotherm as a function of time with calcium ion present. At intervals during the experiment, pieces of the clot were placed in solvent containing 10^{-2} M EDTA, at sufficient concentration to bind all calcium ion present. These pieces of clot were then sealed in calorimeter pans or rinsed first in solvent without EDTA before sealing. When these samples were run at the times shown, the increase in area of the D endotherm was nullified; the area observed was essentially the same as for the original fibrinogen. This was true for both types of clot. Finally, for the thrombin clot, one piece of clot was soaked first in solvent containing EDTA and then in solvent containing calcium ion, before it was run in the calorimeter. The increase in area was restored; if anything, the specific area was greater.[3]

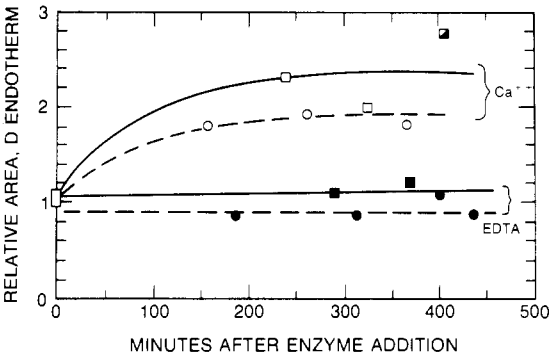

FIGURE 5: Effect on enthalpy of denaturation of the D nodule of normal fibrinogen of addition of EDTA at varying times after addition of thrombin. (Open symbols) 10^{-3} M total calcium [thrombin (□)] or 4.4×10^{-3} M total calcium [Ancrod (○)]. (Closed symbols) Samples of the clot were transferred into 10^{-2} M EDTA about 50 min before the time shown. Sample of thrombin clot transferred back from EDTA to solvent with calcium before run (right-diagonal-solid box). All solvents also contain 0.3 NaCl and 0.05 M Tris-HCl, pH 7.2. Endotherms of the D nodule are shown in Figure 6. Range of enthalpies observed for native fibrinogen samples (open rectangle).

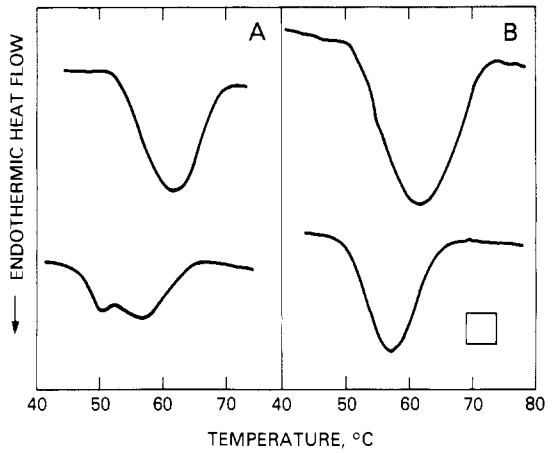

FIGURE 6: Reversal of the effect of calcium ion by EDTA: D endotherms from the experiment of Figure 5. (A) Clotting of normal fibrinogen with thrombin at 10^{-3} M total calcium: (upper curve) 5.3 mg of clot after 238 min; (lower curve) 6.7 mg of clot after 190 min with calcium present and then 45 min in 0.01 M EDTA. (B) Clotting of normal fibrinogen with Ancrod at 4.4×10^{-3} M total calcium: (upper curve) 9.3 mg of clot after 261 min; (lower curve) 11.3 mg of clot after 240 min with calcium ion and then 55 min in 0.01 M EDTA. The area of the calibration square is equivalent to 0.60 mcal.

When clotting by thrombin or Ancrod is completed, as determined by constancy of D endotherm peak temperature and enthalpy, and EDTA is added to samples of the clot to bind calcium ion, not only is the area of the D endotherm restored to nearly its original value, but in the case of Ancrod, the half-width of the D endotherm returns to a value characterisitc of fibrinogen (Figure 6B). For thrombin clotting a very different kind of reversal is observed (Figure 6A). The D endotherm is incompletely split into what appears to be two endotherms at approximately 51.5 and 58 °C. Both these temperatures are lower than the temperature observed for the D endotherm of native fibrinogen. Rough estimates suggest that the 51 °C peak has half the area of the 58 °C peak. The half-widths of the incompletely resolved peaks appear to be 6 °C for the former and 9 °C for the latter. Assuming two species are present with molecular weights proportional to

[3] Enthalpy measurements in this experiment are subject to error due to changes in water content of the gel when it is transferred from one solvent to another and to how well the wet gel is "blotted" free of adhering solvent before it is sealed in the calorimeter pan and weighed.

endotherm areas, we calculate the enthalpies and cooperativities shown in Table I. These data suggest that one of the unfolding units in the D nodule is different after reversal of the calcium effect in a thrombin clot.

Discussion

With elucidation of the three-dimensional structure of a large number protein molecules it has become apparent that the geometric arrangement of the polypeptide chains is not homogeneous but presents apparent fissure planes, or looser structures, that delineate distinct portions of the molecules. The term "domains" was coined by Wetlaufer (1973) for these substructures. The concept of the two-state transition for protein denaturation (all-or-none process) was modified also, since denaturation in many instances was shown to be a multistep process, encompassing, in succession, distinct segments of the molecule (Baldwin, 1975). Both the geometrical domains and independently unfolding segments have been observed for small globular molecules. It is no surprise that they are more obvious with large complex molecules, a prototype of which is fibrinogen. The geometrical doomains and the unfolding units may be identical, but this is not an absolute requirement. The delineation of domains depends also on the resolution of fine details of the structure. Domains in the gross structure as seen in electron micrographs may be resolved by X-ray crystallography into distinct subdomains. Although the detailed X-ray crystallographic structure of fibrinogen has not yet been determined, that there is finer structure within the gross molecular envelopes of the D and E nodules is becoming increasingly evident. This both from the study of the morphology (Slayter, 1983; Williams, 1981, 1983; Cohen et al., 1983; Erickson & Fowler, 1983) and from the study of the unfolding units of the molecule.

The thermograms obtained at pH 3.5 (Privalov & Medved', 1982) suggested that fibrinogen may contain additional unfolding units besides the conventional D and E nodules. Thermal transitions of these units at neutral pH may be contained within the main endotherms and only become separated from them by the lowering of the pH, or the transitions of the additional units may occur outside the temperature range of the experiments at neutral pH. A modest shift in temperature of the main endotherms of fibrinogen was observed when the pH was changed from 8.5 to 3.5 (Privalov & Medved', 1982). Their results show that decreasing the pH to 3.5 not only shifted the LT1 and HT1 endotherms of the D and E nodules to lower temperature by 5–10 °C but also decreased the enthalpies of unfolding an average of 30%. This is understandably a reflection of the loosening of the molecular structure with increasing electrostatic stress, revealed in changes in optical rotation (Mihalyi, 1965). Assuming that the same percentage decrease in enthalpy holds true for Privalov and Medved's second high-temperature peak (HT2 in their notation) and neglecting the small LT2 endotherm, the total enthalpy of unfolding of the fibrinogen molecule at pH 8.5, from their data, is 1440 kcal/mol. The sum of the enthalpies of all the transitions observed in the present experiments at pH 6 (Table I) is 1425 ± 70 kcal/mol (two D nodules, one E nodule, and one unresolved high-temperature transition). This comparison suggests that in the temperature range covered by Figure 1 we are observing all the transitions of the fibrinogen molecule. Unfortunately, the resolution and identification of the high-temperature transitions were not possible. We assume that they correspond to the LT2 and HT2 endotherms of Privalov and Medved', which, at pH 3.5, are shifted drastically to lower temperature, by approximately 63 and 40 °C, respectively. This suggests that the structures giving rise to the LT2 and HT2 transitions are present at neutrality and are not the result of a "splitting" of the larger nodules under electrostatic stress.

In the previous paper, it was shown that the high-temperature endotherm at about 125 °C did not change in either position or area during clotting of fibrinogen in the absence or presence of calcium. In the pressure cell, changes were not observed in the high-temperature endotherms of fibrin. This does not exclude interactions in the native clot, because at temperatures above 105 °C, for the structures giving rise to these endotherms, interactions with the rest of the molecule have been destroyed.

We have now complemented the data of Privalov & Medved' (1982), by detecting unfolding units in the high-temperature range at neutral pH. Further work is necessary to prove that these are identical with the unfolding units LT2 and HT2 described by Privalov & Medved', which, at pH 3.5, are denatured at temperatures close to those of the "classical" D and E nodules (LT1 and HT1 in their notation). Definitive identification of the molecular regions involved in these transitions is also wanting. It is clear, however, that they complete the spectrum of unfolding units within the fibrinogen molecule. Unfortunately, the differential scanning calorimetric method is not applicable to the study of the interactions between all parts of the molecule. As the units are unfolded one by one, the interactions between those already unfolded and the remaining ones are lost. Therefore, it is not surprising that all of the changes connected with clotting are observed in the unit unfolding at the lowest temperature, the D nodule.

Some further insight into the clotting process was provided by the comparison of the thrombin and Ancrod clots. This revealed that practically all the changes in the D endotherm, i.e., the shift of the transition to higher temperatures and the increase of the enthalpy of unfolding, seem to be dependent on the removal of fibrinopeptides B. Thus, although clotting occurs when only the A fibrinopeptides are cleaved off and the clot has characteristics only slightly altered from those of thrombin clots (Shen et al., 1977), the large changes in the stability of the fibrin monomers may take place only when the B peptides are also removed. Perhaps their removal either creates new contact sites between the D and E nodules or removes a steric or electrostatic constraint hindering formation of such a contact.

The changes in the number of unfolding units during clotting could be explained by intermediates that form as the monomers arrange themselves into the final clot. When the molecule is subjected to external or internal forces, such as association at new contact surfaces, or increased electrostatic forces due to ionization of certain groups, or binding of highly charged ions, the unfolding transition may be shifted to higher or lower temperatures, and the enthalpy of unfolding may change. This may result in unfolding units that have closely spaced unfolding temperatures (a broadening of the transition curve). Estimation of the number of unfolding units will be erroneously high in this case. It may be recalled at this point that the D endotherm, in the early stages of clotting of fibrinogen with thrombin in the presence of moderate amounts of calcium, is actually split into two components. One is presumably the original free species and the other the one bound into the fibrin fibers. As clotting proceeds, material is progressively transferred from one into the other form. This is clearly visible in the thermograms of the preceding paper (Donovan & Mihalyi, 1985). Presumably, the apparent increase in the number of unfolding units during clotting is a reflection of this process. Each one of the original unfolding units may exist in two forms,

one free and the other bound to the fibrin network. A temporary doubling of the number of apparent unfolding units (Table II) may be a reasonable observation under these circumstances.

Privalov & Medved' (1982) found that the transition envelope of isolated heavy D fragments can be deconvoluted into three closely spaced transitions. Our experiments of reversing the calcium effect in thrombin clots suggest that there must be at least two different kinds of unfolding units in the D nodule.

Finally, the reversal experiments also furnish additional proof that stabilization of the D nodules is not related to cross-linking of the clot; otherwise, no reversal would be observed on removal of calcium.

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Purification and Sequencing of the Active Site Tryptic Peptide from Penicillin-Binding Protein 1b of *Escherichia coli*†

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ABSTRACT: This paper reports the sequence of the active site peptide of penicillin-binding protein 1b from *Escherichia coli*. Purified penicillin-binding protein 1b was labeled with [^{14}C]penicillin G, digested with trypsin, and partially purified by gel filtration. Upon further purification by high-pressure liquid chromatography, two radioactive peaks were observed, and the major peak, representing over 75% of the applied radioactivity, was submitted to amino acid analysis and sequencing. The sequence Ser-Ile-Gly-Ser-Leu-Ala-Lys was obtained. The active site nucleophile was identified by digesting the purified peptide with aminopeptidase M and separating the radioactive products on high-pressure liquid chromatography. Amino acid analysis confirmed that the serine residue in the middle of the sequence was covalently bonded to the [^{14}C]penicilloyl moiety. A comparison of this sequence to active site sequences of other penicillin-binding proteins and β-lactamases is presented.

The cytoplasmic membrane of *Escherichia coli* contains seven proteins that form a covalent bond with β-lactam antibiotics (Blumberg & Stromiger, 1974; Spratt & Pardee, 1975). These proteins, termed PBPs,[1] can be detected by incubation of *E. coli* membranes with [^{14}C]penicillin G, followed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. PBPs are involved in the last stages of cell wall biosynthesis and the cross-linking of the peptidoglycan layer. Penicillin exerts its lethal effect by binding to these PBPs and

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[1] Abbreviations: PBP, penicillin-binding protein; $NaDodSO_4$, sodium dodecyl sulfate; CPase, D-alanine carboxypeptidase; TPCK trypsin, trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin.

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