Characterization of Soluble Polymerized Fibrin Formed in the Presence of Excess Fibrinogen Fragment D†

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ABSTRACT: Polymerization of fibrin is inhibited in the presence of excess fibrinogen fragment D. This study was performed in order to test the proposal that these inhibited solutions contain short linear polymers of fibrin (protofibrils) whose further polymerization is prevented as a result of attachment of a molecule of fragment D at each end. Negative-stain electron micrographs, intrinsic viscosities, angular dependence of light scattering intensity, and kinetics of the increase of the scattered intensity with polymerization all were found to support the above model of the inhibited polymer and to reflect the presence of a broad distribution of the lengths of the inhibited fibrin polymers. Furthermore, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of polymers stabilized with γ-dimer cross-links introduced by factor XIIIa demonstrates cross-linking of fragment D to fibrin oligomers. Cross-linked polymers have been separated from excess fragment D by gel exclusion chromatography in 1 M urea. (In the absence of urea, the purified polymers very slowly associate to fibers.) The observation of the relative stability of short isolated inhibited protofibrils and the decrease or absence of inhibition of fibrin gelation when fragment D was added to solutions in which fibrin had been given time to polymerize to long protofibrils demonstrate that the inhibitory effect of fragment D occurs as a result of inhibition of the first fibrin polymerization step.

In vitro studies have shown that assembly of fibrin occurs by a series of consecutive steps, namely, proteolytic activation of fibrinogen by thrombin, end to end polymerization of fibrin monomers to long protofibrils, and lateral association of protofibrils leading to a network of fibrin fibers and the resultant gel. Protofibrils and fibers may be stabilized by covalent cross-links introduced by factor XIIIa. In vivo, the fibrin network is sooner or later removed as a result of proteolysis by the enzyme plasmin, formed from an inactive precursor, plasminogen. Plasmin also cleaves fibrinogen into various fragments, some of which inhibit fibrin assembly. Thus, fibrinogen fragment D has been shown to decrease the clottability of fibrinogen solutions (Kowalski, 1968), to prolong thrombin clotting times (Marder & Shulman, 1969; Kowalski, 1968; Larrieu et al., 1972; Belitser et al., 1975; Haverkate et al., 1979), to decrease the rigidity, and to alter the light scattering of fibrin gels (Williams et al., 1981). The anticoagulant activity of fragment D could conceivably result from interference with any of the steps of fibrin assembly.

In a previous paper from this laboratory (Williams et al., 1981), fragment D has been shown not to inhibit thrombin. The results presented below indicate that fragment D also does not inhibit formation of fibers from protofibrils of sufficient length. In the earlier work, a mechanism of inhibition has been suggested, according to which each end of a growing protofibril can be blocked by reacting with a molecule of fragment D, instead of with a molecule of fibrin monomer or another unblocked protofibril end. It was proposed that fragment D attaches to the protofibril via a portion of the same polymerization contacts that are used for attachment of another fibrin monomer (Hermans & McDonagh, 1982; Hantgan et al., 1983). The structure of several such blocked protofibrils is schematized in Figure 1 (left-hand side). If formed in the absence of factor XIIIa, these polymers can redissociate (e.g., in denaturing solvents). In the presence of factor XIIIa, covalent γ-dimer cross-links are introduced that link the molecules where indicated by pairs of thin lines. According to the model, each blocked or unblocked protofibril in which all γ-dimer cross-links have been formed can still dissociate into two parts, as shown schematically in the right-hand side of Figure 1.

The results of five very different experiments reported below confirm the details of this scheme. Results of electron microscopy and viscosity and light-scattering measurements confirm the presence of short rodlike structures in inhibited solutions; stopped-flow light-scattering measurements show that polymerization in an inhibited solution follows a simple extension of the kinetic scheme followed in the absence of inhibitor; electrophoresis of cross-linked inhibited protofibrils on agarose gels in sodium dodecyl sulfate (SDS)–urea[1] solution shows the presence of the predicted species. Finally, we have succeeded in removing the excess fragment D from cross-linked inhibited polymer.

Materials and Methods

Materials

Human fibrinogen obtained from AB Kabi (grade L) or prepared according to Blombäck & Blombäck (1956) (fraction I-4) was dissolved in 0.3 M NaCl, centrifuged for 10 min at 30000*g*, dialyzed for 18 h to remove free calcium, and then divided into aliquots and stored at −70 °C. Clottability was greater than 90% and 97%, respectively. Fibrinogen concentrations were determined from the absorbance at 280 nm by using a specific extinction coefficient of 1.6 mL/(mg·cm) (Carr & Hermans, 1978). Plasmin (Kabi) was reconstituted to 50 units/mL with 50% glycerol and stored at −20 °C. Thrombin either was of bovine origin (Parke-Davis) or was highly pu-

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol); UV, ultraviolet.

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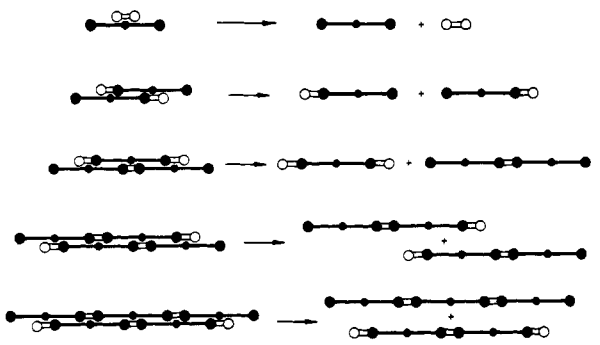

FIGURE 1: Proposed model of several cross-linked, inhibited protofibrils of different sizes (Williams et al., 1981; Hermans & McDonagh, 1982). Fibrin monomer is represented as three nodules (circles) connected by two rods (Hall & Slayter, 1959), fragment D is shown as a single nodule, and γ cross-links are shown as pairs of thin lines. The models on the left relate to aqueous conditions, while those on the right describe the two strands into which each protofibril on the left separates in a strongly denaturing solvent.

rified human thrombin (lot 88-B, specific activity = 2265 units/mg) which was a gift from Dr. J. W. Fenton (Fenton et al., 1977). Except where noted, the thrombin concentration used was 50 units/mg of fibrinogen. At this concentration, the rate of fibrin assembly was not limited by the rate if fibrinopeptide release (Hantgan & Hermans, 1979).

Fragment D was prepared and isolated as previously described (Williams et al., 1981). The procedure employed plasmin digestion of fibrinogen in the presence of 2.5 mM calcium; the resultant fragment is the form designated by Haverkate and Timan as Dcate, which has an intact C-terminal portion of the γ chain (Haverkate & Timan, 1977). The purified protein was concentrated by dialysis against PEG 20000 or against Sephadex (for viscosity studies). Protein concentration was determined by its absorbance at 280 nm, by using a specific extinction coefficient of 2.08 mL/(mg·cm). The major species of fragment D used in all experiments had a molecular weight of 100000 ± 10000, as determined by SDS–polyacrylamide gel electrophoresis and light scattering. Unless noted otherwise, all buffers contained 10 units/mL Trasylol (Mobay Chemicals) to inhibit residual plasmin in the purified fragment D.

Before use, purified factor XIII was activated at 37 °C for 30 min under vacuum with 1.5 NIH units/mL thrombin. The thrombin was then inactivated by adding 15 units/mL hirudin (Sigma). The inhibitory tetrapeptide Gly-Pro-Arg-Pro was generously provided by Dr. Russell Doolittle.

Methods

Isolation of Cross-Linked, Inhibited Fibrin Oligomers. A 5-mL sample of inhibited, cross-linked fibrin oligomers was prepared by polymerization of 1.4 mg/mL fibrinogen and 9.5 mg/mL fragment D with 0.1 unit/mL thrombin for 60 min in 0.1 M NaCl, 0.05 M Tris, and 10 units/mL Trasylol, pH 7.4. Calcium was then added to a concentration of 5×10^{-3} M and factor XIIIa to a concentration 0.4 time that of normal human plasma. Cross-linking was allowed to proceed for 30 min, and then *N*-ethylmaleimide (1.2×10^{-3} M) and D-phenylalanylprolylarginyl chloromethyl ketone (from Calbiochem; 1.2×10^{-6} M) were added to inhibit factor XIIIa and thrombin, respectively. Concentrated urea was added to a final concentration of 1 M and the sample applied to a 1.6 × 75 cm column of Sepharose 4B. Elution was carried out with 1 M urea, 0.1 M NaCl, 0.05 M Tris, and 10 units/mL Trasylol, pH 7.4, at 15 mL/h.

Two well-resolved peaks were obtained, and samples were removed for reduction and polyacrylamide gel electrophoresis. Reduced samples of the earliest eluting fraction exhibited peaks at molecular weights of 107000 (γ–γ), 96000 (γ_{fibrin}–γ_D), and 57000 (β_{fibrin}). The latter fraction was composed of peaks at 43500 (β_D), 35500 (γ_D), and 11000 (α_D) daltons. Thus, peak 1 was identified as cross-linked, fragment D blocked fibrin oligomers and peak 2 as the excess fragment D. Absence of a band for the fibrin α chain in the first fraction may be attributed to proteolytic cleavage and is not unexpected in solutions that have been manipulated at room temperature for a period of many hours. Although the loss of the α chain could be due to cross-linking, the absence of high molecular weight bands at positions corresponding to α polymers argues against this possibility.

Electron Microscopy. Solutions contained 0.01 mg/mL fibrinogen, 0.01 mg/mL fragment D, and 1 unit/mL thrombin in 0.5 M NaCl–0.05 M Tris-HCl, pH 7.4. Aliquots were removed at 0, 5, 15, 30, and 60 min after thrombin addition. Alternatively, a solution of isolated inhibited fibrin oligomers was diluted 20 times with buffer not containing urea. Samples for electron microscopy were obtained from these solutions and were negatively stained with 2% uranyl acetate by a procedure previously employed to study the rate of fibrin assembly (Hantgan et al., 1979; Fowler & Erickson, 1979). Specimens were examined in a Hitachi H500 electron microscope at 30000× normal magnification.

Viscosity. A Cannon-Ubbelohde dilution viscometer (size 100, Cannon Instrument Co.) was used at 25 ± 0.05 °C. Solutions were degassed and filtered directly into the viscometer.

The intrinsic viscosity is obtained from

$$[\eta] = \lim_{c \to 0} \eta_{sp}/c \tag{1}$$

$$\eta_{sp} = t_s/t_b - 1 \tag{2}$$

where t_s and t_b are the flow times measured for the sample and blank, respectively. Theory relates the hydrodynamic behavior of rigid rod-shaped molecules to the ratio of the rod's length and diameter, $p = a/b$, and the ratio of the effective molecular volume to the molecular mass:

$$[\eta] = \Lambda(p)v/m \tag{3}$$

The dimensionless function Λ was derived by Simha (1940) and by Kuhn & Kuhn (1945); the solution for long, cylindrical rods is given by Sadron (1953).

The intrinsic viscosities of solutions of fibrinogen and of fragment D were determined in 0.1 M NaCl–0.05 M Tris as 27.0 and 8.0 mL/g, respectively.

For measurement of the intrinsic viscosity of the inhibited polymer, we measured the viscosities of solutions containing fragment D at 0.7 and 2.0 mg/mL and fibrinogen at 0.5 mg/mL after activation by the addition of thrombin to a final concentration of 25 units/mL. These measurements were unsuccessful at low salt: successive flow times for the solution increased indefinitely. We suggest that in these experiments the great tendencies for formation of fiber and clot at low salt and in a velocity gradient combined to cause formation of small amounts of gel that increased the flow time, even in these inhibited solutions. Reproducible results were, however, obtained at high salt (0.5 M NaCl–0.05 M Tris, pH 7.4).

Angular Dependence of Light Scattering. The apparatus and techniques for measurement were used as previously described (Carr et al., 1977; Hantgan & Hermans, 1979; Williams et al., 1981; Rocco et al., 1984). The intensity of the scattered light was measured at angles from 30° to 130°. Solutions were made dust free by filtration through presoaked 0.22-μm Millipore filters. Protein concentrations in the filtered

solutions were determined from the UV absorption.

The majority of solutions was prepared as follows. Concentrated fibrinogen (I-4) solution was added to a filtered solution containing fragment D. Final fibrinogen concentration was 0.16 mg/mL in low-salt (0.1 M NaCl–0.05 M Tris, pH 7.4) or 0.5 mg/mL in high-salt (0.5 M NaCl–0.05 M Tris, pH 7.4) experiments. Fibrin assembly was initiated by the addition of a small aliquot of a concentrated thrombin solution to a final concentration of at least 20 units/mg of fibrinogen.

The results were analyzed with the usual equations (Huglin, 1972):

$$1/M_w = KcP(\theta)/R(\theta) \quad (4)$$

where $R(\theta)$ is the net scattering, i.e., the scattering of the solution or gel minus the scattering of the same solution before addition of fibrinogen and assembly, c is the protein concentration, M_w is the weight-average molecular weight, and

$$K = 4\pi^2 n^2 (dn/dc)^2/(N\lambda^4) \quad (5)$$

for a vertically polarized incident beam, with n the refractive index, N Avogadro's number, λ the wavelength in vacuo, and θ the scattering angle. The function $P(\theta)$ describes the angular dependence of the scattering intensity. For a molecule the size of fibrinogen, $P(\theta)$ is essentially equal to 1.0. For larger particles, $P(\theta)$ approaches 1.0 as the scattering angle approaches zero and is <1 at all other angles.

Photon correlation spectra were measured for a number of these samples. For solutions of fibrinogen, of fragment D, and of isolated inhibited protofibrils in 1 M urea, these results could be interpreted to give acceptable translational diffusion coefficients [cf. Knoll (1983), Palmer et al., (1979), and Palmer & Fritz (1979)]. Interpretation of results for inhibited solutions in dilute buffer required a large correction of the correlation spectrum for the contribution by excess fragment D (Knoll, 1983).

Stopped-Flow Light Scattering. Fibrinogen (0.25 mg/mL final concentration) with or without fragment D (0.6 mg/mL final concentration) was rapidly mixed with thrombin (38 units/mL final concentration) by using a device described by Hantgan & Hermans (1979) and pushed into a flow-through fluorescence cell. Light-scattering intensity at 90° was measured as a function of time.

Activation–Inhibition Experiments. These experiments were performed at a fibrinogen concentration between 0.01 and 0.02 mg/mL, with a final thrombin concentration of 2.5 units/mL. The rate of assembly is independent of the thrombin concentration under these conditions (Hantgan & Hermans, 1979). A small aliquot of concentrated fragment D or Gly-Pro-Arg-Pro was added either prior to thrombin activation (the final concentration of fragment D was 0.18–0.22 mg/mL) or at times ranging from 5 to 45 s after activation. A control experiment demonstrated that addition of an aliquot of buffer at 45 s did not alter the kinetics of the assembly process as observed by 90° light scattering.

Gel Electrophoresis of Fibrin Oligomers. In order to measure the size distribution of polymers in inhibited mixtures, electrophoresis in a 2% agarose gel was performed on cross-linked samples in the presence of 1% sodium dodecyl sulfate (SDS) according to Moroi et al. (1973).

(*A*) *Sample Preparation.* Sample preparation consisted of three phases: polymerization, covalent cross-linking, and solubilization, carried out successively. Fibrinogen was at ~1 mg/mL, and the molar ratio of fragment D to fibrinogen was near 15. Concentrations of salt and thrombin were variables. Polymerization proceeded for 1–2 h; cross-linking with factor XIIIa, at ~0.3 time the concentration in normal human plasma, proceeded for 1 h and was stopped with the addition of 0.1 mM *N*-ethylmaleimide. In the noninhibited control, activation was with a very low concentration of thrombin (0.01 NIH unit/mL) and proceeded concurrently with cross-linking for 1 h. Each sample was added to an equal volume of 10 M urea–5% SDS. Samples formed in high salt were first dialyzed into low-salt electrophoresis buffer.

(*B*) *Preparation of 2% Agarose Gels.* Agarose (Bio-Rad, low molecular weight) was dissolved in warm buffer (0.1 M NaH_2PO_4–1% SDS, pH 7.1), transferred to 130 mm × 5 mm glass tubes, covered at one end with Parafilm, immersed in a 50 °C water bath, and left to gradually cool.

(*C*) *Fixing and Staining.* After electrophoresis, the gels were fixed in 15% trichloroacetic acid prior to being stained and destained with Coomassie brilliant blue. Gels were scanned at 560 nm on a Gilford System 2600 spectrophotometer equipped with a gel scanning attachment.

(*D*) *Reduced Gels.* Samples were also electrophoresed on 5% polyacrylamide gels under reducing conditions to determine the extent of cross-linking according to McDonagh et al. (1972).

Table I: Protofibril Sizes Determined from Intrinsic Viscosity Data in 0.5 M NaCl

mol of fragment D/mol of fibrinogen	$[\eta]$ (mL^3/g)	length (nm)	i
11	117	190	7–8
40	70.6	130	4–5

Results

Electron Microscopy. An electron micrograph, obtained 15 min after thrombin addition to a solution of fibrinogen in 0.5 M NaCl,[2] which contained a 3-fold molar excess of fragment D, demonstrated that the inhibited reaction mixture contained a broad distribution of molecular sizes, ranging from individual fragment D and fibrinogen molecules via short oligomers to long, thin fibers (Figure 2A). Micrographs obtained at the same time of reaction, in the absence of fragment D, exhibited a predominance of long, thin fibers and an absence of short polymers.

Intrinsic Viscosity. Table I lists η_{sp}/c at concentrations of fragment D sufficient to fully inhibit gelation. Since the initial fibrinogen concentration is low (0.5 mg/mL), these values should be very close to those obtained at infinite dilution and may be treated as the intrinsic viscosity, $[\eta]$. (Measurements at lower concentrations were not sufficiently accurate.)

The intrinsic viscosity of fibrin oligomers is found to decrease as the fragment D concentration is increased, as shown in Table I. As the intrinsic viscosity of a solution of polymers is a sensitive function of polymer length, eq 1 and 3 can be used to obtain an estimate of the average oligomer length in these inhibited solutions. For this purpose, we modeled fibrinogen as a cylinder 45 nm long and 3.4 nm wide, the volume of which is equal to the anhydrous volume of the fibrinogen molecule, approximately 400 nm^3. Protofibrils made up of i fibrin monomers were modeled as cylinders of length 22.5 $(i + 1)$ nm and width 6.8 nm. The lengths corresponding to the measured intrinsic viscosities are cited in Table I. The length decreases from approximately 190 to 130 nm as the fragment D concentration is increased 4-fold. (Polymer length is fairly insensitive to the choice of polymer width; doubling

[2] This experiment was performed in 0.5 M NaCl since at increased salt concentration gelation can be prevented at a considerably lower ratio of fragment D to fibrinogen.

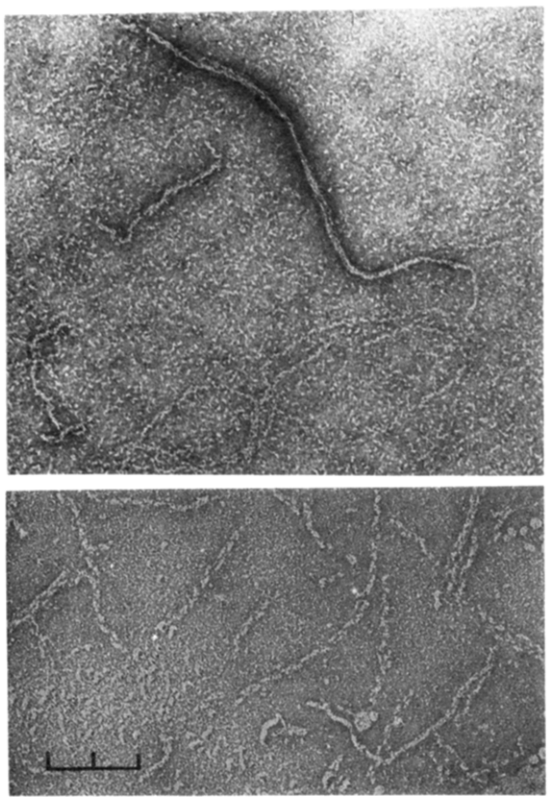

FIGURE 2: Electron micrographs of samples negatively stained with uranyl acetate. (A) 15 min after thrombin activation of fibrinogen (experimental conditions: 0.01 mg/mL fibrinogen and 0.01 mg/mL fragment D in 0.5 M NaCl–0.05 M Tris, pH 7.4). (B) Cross-linked, inhibited fibrin oligomers isolated by gel filtration chromatography in 1 M urea and then diluted 20-fold to a concentration of 0.01 mg/mL into 0.1 M NaCl–0.05 M Tris, pH 7.4. The bar measures 200 nm.

this parameter causes a 1.3-fold decrease of the estimated polymer length.)

Light Scattering. Molecular weights of 330 000 and 105 000 were obtained for fibrinogen and fragment D, respectively. Plots of $Kc/R(\theta)$ vs. $\sin^2(\theta/2)$ for these proteins were linear and exhibited little or no angular dependence, in agreement with the small dimensions of these molecules.

Figure 3 shows typical plots of the reciprocal intensity functions vs. $\sin^2(\theta/2)$ obtained with protofibrils inhibited by fragment D. In general, the reciprocal scattering function can be described by the following equation:

$$Kc/R(\theta) = [\sum_i w(i)M(i)P(i,\theta)]^{-1} \qquad (6)$$

in which $w(i)$ is the weight fraction, $M(i)$ the molecular weight, and $P(i,\theta)$ the particle scattering function of the ith oligomer; particle scattering functions of rodlike fibrin protofibrils have been established previously (Hantgan & Hermans, 1979). Thus, if the distribution of oligomer sizes and the particle scattering function of each oligomer are known, the reciprocal scattering function can be evaluated. Alternatively, a set of weight fractions for a small discrete set of oligomer sizes can be adjusted to give optimal agreement between the calculated and experimental scattering functions.

We have followed a somewhat simpler procedure, in which such a fit is performed by adjustment of the parameter(s) in an expression describing polymer size distribution, in this case, the parameter p, (the extent of reaction), defined as the fraction of polymerization sites which have reacted with other polymerization sites in a most probable distribution:

$$w(i) = i(1 - p)^2 p^{i-1} \qquad (7)$$

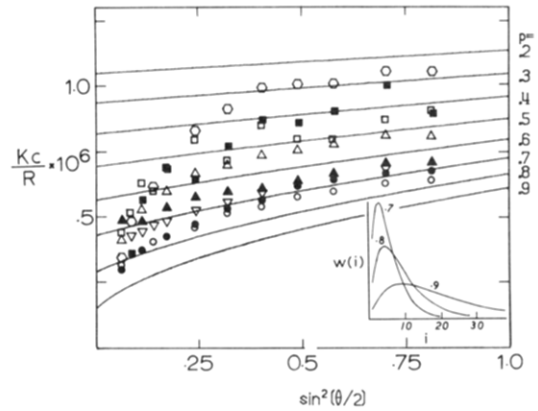

FIGURE 3: Angular dependence of (incremental) light scattered by protofibrils inhibited by fragment D and curves calculated for the most probable distributions. The solutions contained 14 (○), 19 (▽), 21 (●), 32 (▲), 45 (△), 52 (□), 68 (■), and 120 (○) mol or fragment D/mol of fibrinogen. The reciprocal scattering function (ordinate) is plotted vs. the square of the sine of half the scattering angle. The solid lines are drawn according to eq 6 (see text), with the weight fraction of each oligomer determined from eq 7 for various values of p; the inset shows examples of these distributions. (Experimental conditions: 0.1 M NaCl–0.05 M Tris, pH 7.4; fibrinogen concentration 0.16 mg/mL; thrombin at 3.3 NIH units/mL.)

This distribution happens to result from a bimolecular polymerization reaction in which all sites have the same reactivity and has been used to describe noninhibited fibrin polymerization (Hantgan & Hermans, 1979). In order to test the hypothesis that fragment D causes a decrease in the extent of reaction (p) and a shift in the distribution of oligomer sizes to smaller values of i, we have calculated the reciprocal scattering function according to eq 6 and 7 for different values of p and compared the experimental data obtained over the range of 14–120 mol of fragment D/mol of fibrinogen to this family of theoretical curves (Figure 3). At all ratios of fragment D to fibrinogen, significant deviations from the calculated curves are observed at low scattering angles. Further analysis shows that the deviations probably correspond to the presence of quite long polymers in addition to the species that correspond to the distribution, eq 7 (Knoll, 1983). The long polymer represents from 5 to 15% of the total fibrin, or less if it consists of thin fibers made up of several protofibrils.

Stopped-Flow Light Scattering. The inset of Figure 4 shows an oscilloscope trace of the light-scattering intensity as a function of time for a solution of fibrinogen and thrombin before, during, and after mixing, in the presence of an 11-fold excess of fragment D in 0.5 M NaCl.[2] The rate of change of the intensity of scattered light is similar to values obtained in the absence of fragment D, with a half-time of 3.1 s for a control experiment and 4.4 s in the presence of 11.3 mol of fragment D/mol of fibrinogen [cf. Hantgan & Hermans (1979)]. The net intensity difference obtained in the presence of fragment D is 30% less than when no fragment D is present. In another experiment, carried out at a ratio of 8.2 mol of fragment D/mol of fibrinogen, the half-time was 4.2 s and the intensity 25% less than the control experiment.

Kinetic Analysis. Fibrin monomers are known to polymerize in a half-staggered overlap pattern to form protofibrils, without any nucleation step. Fibrin polymerization proceeds as a bimolecular association of bifunctional monomers and oligomers:

$$\cdot f_i \cdot + \cdot f_j \cdot \rightarrow \cdot f_{i+j} \cdot \qquad i, j \geq 1 \qquad (8)$$

where the center dot stands for a binding site. A single rate constant, k_1, independent of oligomer size, i and j, suffices to fit the results of stopped-flow light-scattering experiments

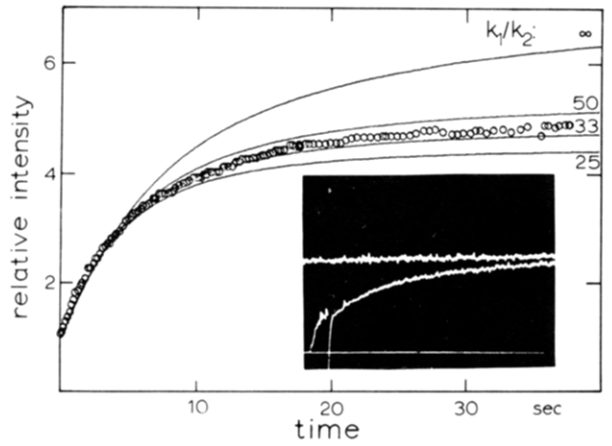

FIGURE 4: Experimental and calculated changes in light-scattering intensity as polymerization progresses in the presence of fragment D. The inset shows three consecutive oscilloscope traces: the lower trace is for solvent before mixing; the middle trace records the mixing event with the downward spike indicating the end of the mixing phase. The results have been replotted in the graph, together with curves calculated for the polymerization–inhibition model with $k_1 = 5 \times 10^5$ M^{-1} s^{-1} and a varying k_2/k_1 ratio as indicated by each curve. (Experimental conditions: 0.60 mg/mL fragment D, 0.25 mg/mL fibrinogen, and 38 units/mL thrombin in 0.5 M NaCl–0.05 M Tris-HCl, pH 7.4.)

(Hantgan & Hermans, 1979).

The model of the inhibited protofibril (Figure 1) suggests obvious extension of the kinetic scheme. In the presence of (monofunctional) fragment D, a growing oligomer can add either another fibrin monomer or a fragment D molecule. Once fragment D has been added, growth at that end of the protofibril is blocked. The other end is free to polymerize until it is also blocked. In the simplest case, one would have to consider a single additional reaction, that of a protofibril with a molecule of fragment D:

$$\cdot f_i \cdot + \cdot D \rightarrow \cdot f_i D \tag{9}$$

Once both ends are blocked, a protofibril cannot grow longer but may be cross-linked by factor XIIIa and associate laterally with other fibers if it is long enough.

As in earlier work, the problem is analyzed in terms of polymerization sites, of which each fibrin monomer (f) has two and each D fragment (D) has one. One fibrin polymerization site may react with another according to

$$2f \rightarrow f_p \tag{10}$$

or it may react with a fragment D polymerization site

$$f + D \rightarrow f^* \tag{11}$$

Integrating the rate equations which correspond to the above two kinetic equations

$$df/dt = -k_1[f]^2 - k_2[f][D] \tag{12}$$

$$df^*/dt = k_2[f][D] \tag{13}$$

and appropriately assuming a constant value of [D], as the fraction of bound fragment D is small, one obtains

$$[f] = [f]_0 k_2[D] \exp(-k_2[D]t)/\{k_2[D] + k_1[f]_0[1 - \exp(-k_2[D]t)]\} \tag{14}$$

$$[f^*] = (k_2[D]/k_1) \ln \{[k_2[D] + k_1[f]_0 - k_1[f]_0 \exp(-k_2[D]t)]/(k_2[D])\} \tag{15}$$

with

$$2[f_p] = [f]_0 - [f^*] - [f] \tag{16}$$

$[f]_0$ being twice the initial concentration of fibrinogen (Hantgan et al., 1983). From these equations, one may calculate the extent of reaction (p) as a function of time for given rate constants and initial concentrations:

$$p = 2[f_p]/[f]_0 \tag{17}$$

and again from the value of p, the most probable distribution of polymer lengths (Flory, 1953) and hence the scattering function of the oligomers [cf. Hantgan & Hermans (1979)] can be calculated. The presence of molecules of fragment D at one or both ends of a protofibril requires obvious minor modification of the scattering function of each oligomer.

In Figure 4 is shown a comparison of the stopped-flow results with a family of curves calculated for four values of the ratio k_1/k_2. The data are consistent with a value of about 40 for this ratio. The value of k_1 best fitting the results is about half that estimated from experiments done in the absence of inhibitor (Hantgan & Hermans, 1979). The difference, although not very great, is probably real.

If continued indefinitely, a reaction proceeding according to eq 12–17 terminates with the disappearance of unreacted fibrin polymerization sites; as follows from eq 14–17, the fraction of polymerized sites will then be given by

$$p_\infty = 1 + y \ln [y/(1 + y)] \tag{18}$$

$$y = k_2[D]/(k_1[f]_0) \tag{19}$$

In Figure 5 is shown the corresponding variation of p_∞ with y, together with the molar ratio of fragment D to fibrinogen derived from comparison of the calculated and observed angular dependences (Figure 3) at higher angles. The solid lines have been calculated from eq 18 and 19 for three different values of the ratio of the polymerization and inhibition rate constants, k_1/k_2. These results indicate a value for k_1/k_2 of ~60, i.e., about the same at the lower as at the higher ionic strength.

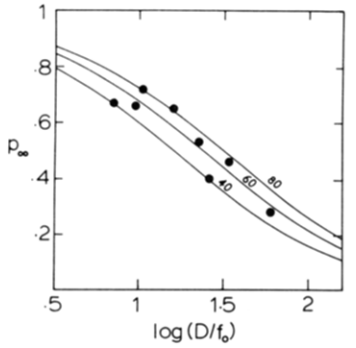

FIGURE 5: Comparison of the degrees of polymerization, p_∞, observed for inhibited protofibrils at long times (Figure 3) with those calculated from eq 18 and 19 for values of the k_1/k_2 ratio equal to 40, 60, and 80 (solid lines).

Activation–Inhibition Experiments. At a fibrinogen concentration of 0.01 mg/mL in 0.1 M NaCl, the time required for protofibril growth is increased to 45 s. Under these conditions, at a molar ratio of fragment D to fibrinogen of 52, the lag time for fiber growth increases by nearly 50%, the half-time for lateral association doubles, and final fiber thickness is 4 times less. However, when the same amount of fragment D is added 45 s after activation by thrombin, no significant change from the control in the rate or extent of the subsequent assembly reaction is observed (Figure 6). As a control, it was observed that addition of an aliquot of buffer at this same time after activation did not alter the progress of fibrin formation in the light-scattering assay.

Addition of fragment D 5 s after activation leaves the lag time unaltered whereas the half-time is nearly doubled; the final fiber thickness is decreased by a factor of 2. Addition

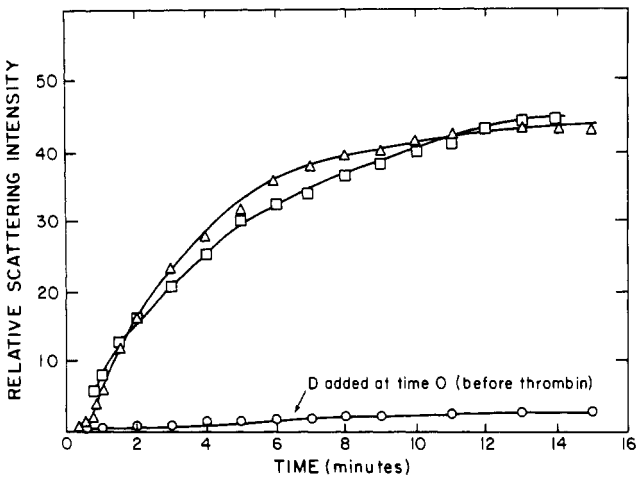

FIGURE 6: Time dependence of the scattering intensity at 90° of fibrinogen activated with thrombin: (△) no fragment D was present (control); (○) the solution contained 52 mol of fragment D/mol of fibrinogen; (□) fragment D was added 45 s after activation to the same final concentration. (Experimental conditions: fibrinogen at 0.01 mg/mL in 0.1 M NaCl–0.05 M Tris-HCl, pH 7.4.)

of the inhibitor at 25 s yields a similarly prolonged lateral association rate but a normal final fiber thickness. It is to be concluded that fragment D does not inhibit formation of fibers from fibrin protofibrils of sufficient length.

Activation–inhibition experiments performed at 0.02 mg/mL fibrinogen and a 14 000-fold molar excess of Gly-Pro-Arg-Pro (Laudano & Doolittle, 1980) yielded results similar to those described for fragment D at a 50-fold excess. Addition of the tetrapeptide prior to thrombin activation resulted in substantial inhibition of fiber growth; i.e., for 60 min, the scattering intensity was comparable to that observed for a solution of protofibrils, and no gel was noted 20 h later. Addition of the same amount of Gly-Pro-Arg-Pro 40 s after activation resulted in fiber growth at a rate similar to that for a control with no inhibitor present; the final intensity was approximately 76% of the control. With an experiment carried out at higher fibrinogen concentration, 0.1 mg/mL, at a 3700-fold molar excess of Gly-Pro-Arg-Pro and a rate-limiting thrombin concentration, gelation was delayed but not blocked as fully. In this experiment, the inhibited reaction mixture exhibited an intensity 1% of the control at t = 5 min but reached 94% of the control by 10 min. At 0.6 mg/mL fibrinogen and a 13 000-fold excess of Gly-Pro-Arg-Pro, the inhibition was more pronounced; i.e., the scattering intensity reached after 180 min a value only 1.6 times higher than that for fibrinogen alone, but still a gel was observed to have formed at t = 20 h.

Gel Electrophoresis of Protofibrils. As was shown by Moroi et al. (1973), more than 10 fibrin oligomers of different lengths can be separated by electrophoresis in agarose gels, in the presence of urea and SDS, of a solution of fibrinogen that has been partly activated with thrombin and in which the polymerized material has been stabilized with γ cross-links introduced by factor XIIIa [see also Nelb et al. (1980)]. A scan of a stained gel prepared by their method is shown in Figure 7; this curve shows at least 15 maxima, each for a higher oligomer. In Figure 8 is shown a plot of the logarithm of peak index vs. distance moved relative to that of the monomer band (which consists of unreacted fibrinogen). The plot is approximately linear, as found by previous authors.

In Figure 9 is shown a similar scan for a solution of fibrinogen that had been fully activated with thrombin, but in which gelation had been completely inhibited by the presence

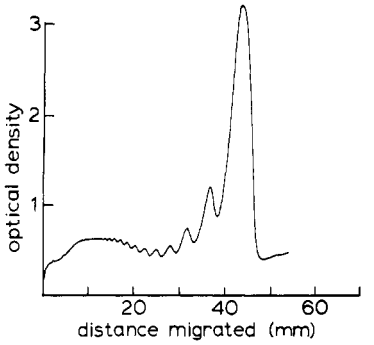

FIGURE 7: Scan of a stained agarose gel after electrophoresis in 1% SDS of cross-linked fibrin oligomers formed from fibrinogen during the course of activation by a very low concentration of thrombin.

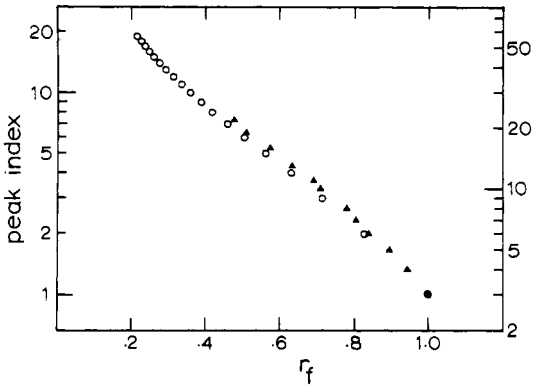

FIGURE 8: Semilogarithmic plot of peak index vs. relative distance traveled, r_f. Results for the noninhibited mixture refer to the scale on the left (open circles; data from Figure 7). Results for the inhibited mixture refer to the scale on the right (closed triangles; data from Figure 9). Points for monomeric fibrin(ogen) for the two sets of results were forced to coincide at r_f = 1.

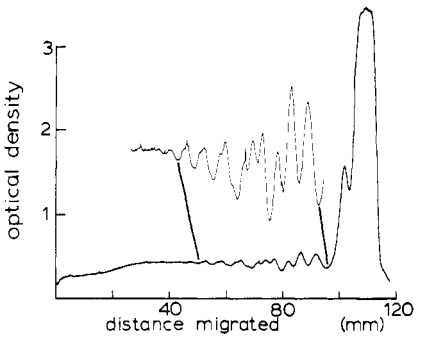

FIGURE 9: Scan of a stained agarose gel after electrophoresis in 1% SDS of cross-linked, inhibited fibrin oligomers formed from fibrinogen some time after complete activation by thrombin in the presence of fragment D at a 15-fold molar ratio. The inset shows a portion of the same scan to a different scale.

of a large excess of fragment D. Besides the intense band corresponding to the excess fragment D and a shoulder corresponding to the dimer of fragment D and perhaps some fragment Y, there are several weak bands which are not present in a control experiment without fibrinogen. The weak bands are more closely spaced, and fewer bands can be distinguished than in Figure 7. From a calibration experiment with fragment D and unactivated fibrinogen, it was determined that the first minor peak corresponded to monomer. This allowed calculation of the relative distance moved for the other minor bands. Thus, it was found that the first six minor bands were 3 times as closely spaced as the bands for fibrin monomer, dimer, and trimer in the control (Figure 7). Since the molecular weight of fragment D is slightly less than one-third that of the fibrin monomer, the first six bands presumably corre-

spond to the monomer and dimer of fibrin and complexes of these with one and with two molecules of fragment D, respectively. The peak positions for the minor bands in Figure 9 have been plotted in Figure 8; peak indices were computed by taking the index of the fibrin monomer equal to 3 and adding 1 for each additional band or shoulder.

These results offer strong support for the model of the structure of the cross-linked fibrin protofibril inhibited by fragment D, according to which every protofibril is blocked at each end by a molecule of fragment D. The model predicts that a protofibril separates into two cross-linked strands in denaturing solvents as used in the electrophoresis experiments [cf. Bale et al. (1982)]. Apparently, a separated strand can contain two, one, or zero cross-linked molecules of fragment D. This is a natural outcome of the model, according to which a blocked protofibril containing an even number, $2k$, of fibrin monomers separates into two strands each containing k monomers and a single molecule of fragment D, while a blocked protofibril containing an odd number, $2k + 1$, of monomers separates into one strand containing just $k + 1$ monomers and another strand containing k monomers and both molecules of fragment D. These relationships are illustrated in Figure 1.

Ideally, one would like to quantitate the relative amount of protein in each band and in the diffuse staining part of the gel where higher oligomers have migrated. For this to be possible, one would have to show that the staining–destaining procedure had yielded a constant background throughout the length of the gel and a stain content proportional to protein concentration, with the same proportionality constant for each fraction. In our opinion, such a quantitation is better not attempted.

Isolation of Fibrin Oligomers. Fibrin oligomers can be isolated by gel filtration chromatography in 1 M urea (Palmer & Fritz, 1979). We employed a similar procedure to remove the excess fragment D from a solution of cross-linked, inhibited polymers. Characterization of these polymers in 1 M urea yielded scattering intensity vs. angle data similar to those in Figure 3; i.e., a distribution of oligomer sizes was present which included some very high molecular weight polymers or fibers. We calculated a diffusion coefficient of 5.5×10^{-8} cm^2 s^{-1} at 20.0 °C from dynamic light-scattering data at 90°.

This (average) value is that calculated for a protofibril containing ~18 monomers [Knoll, 1983; cf. also Palmer & Fritz (1979)]. Electron micrographs of this sample show images of fibrin oligomers in a broad range of lengths compatible with this average length (Figure 2B).

Urea was removed from the solution of inhibited protofibrils by dialysis against dilute buffer. When the urea had been nearly completely removed, a slowly increasing turbidity evidenced that the protofibrils were forming fibers.

Discussion

As we have reported the results of a number of different kinds of experiments, we have discussed how each serves to characterize the formation and structure of the inhibited fibrin polymer. While each result taken individually may be open to several different interpretations, the aggregate of results establishes that the model that we have proposed earlier (Figure 1; Williams et al., 1981; Hermans & McDonagh, 1982) is indeed the correct one. Thus, the inhibited protofibrils are similar to those that form as intermediates during normal fibrin polymerization and that in the absence of inhibitor become the constituents of a network of fibrin fibers. The inhibitor action of fragment D is due to the blocking of both ends of the growing protofibrils, each by a molecule of fragment D. The slight instability of isolated inhibited protofibrils in dilute buffer, i.e., in the absence of excess fragment D, may indicate an additional minor inhibitory effect of fragment D on the formation of fibers from protofibrils.

As this work progressed, we concluded that interpretation of nearly every experiment was made more difficult by the same single cause, namely, the very strong tendency of the longer protofibrils to associate laterally to form fibers (or a network of fibers). Even in the presence of a large excess of inhibitor, a few long polymer molecules form, and these associate to form fibers that sometimes appear to dominate the electron micrographs, may clog capillary viscometers, and may scatter light quite out of proportion to their concentration, especially at low angles.

As has been the case with normal fibrin polymerization (Hantgan & Hermans, 1979), it has been possible to interpret the results in terms of a unidirectional kinetic scheme. This contains just two reactions, the first for polymerization and the second for blocking of a polymerization site by the inhibitor. The rate of the polymerization step is considerably higher than that of the inhibition step, 50-fold for inhibition by fragment D and 10^4-fold for inhibition by the tetrapeptide Gly-Pro-Arg-Pro. Rates for the reversal of these steps, and hence the equilibrium constants, have not been determined. However, according to the model of the protofibril and its mode of inhibition (Figure 1; Hermans & McDonagh, 1982), one expects a ranking of the equilibrium constants like that of the measured rate constants.

Solutions of protofibrils inhibited by fragment D appear to be stable for several days. When the excess fragment D is removed (by gel filtration), cross-linked protofibrils are still relatively stable under physiological conditions, although a slow formation of fibers sets in and the solutions do eventually gel. In contrast, inhibited protofibrils have heretofore been obtained only in inhibitory solvents (at high pH, in a hexamethylene glycol–water mixture and in aqueous urea) (Ferry et al., 1952; Casassa, 1955; Carr et al., 1977; Marguerie et al., 1973; Fritz & Palmer, 1979). We suggest that solutions of inhibited protofibrils prepared by activation of fibrinogen by thrombin in the presence of excess fragment D and, of course, a catalytic amount of factor XIIIa, and subsequent removal of the unpolymerized fragment D by gel filtration, may facilitate studies of interactions of polymerized fibrin with other macromolecules and with cells, as a result of an absence not only of thrombin but also of fiber formation and gelation during experiments.

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Kinetics of the Interaction of Hemin Liposomes with Heme Binding Proteins†

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ABSTRACT: As a model for the transport of hemin across biological membranes, sonicated phosphatidylcholine liposomes with incorporated hemin were characterized. The interaction of the hemin liposomes with the heme binding proteins albumin, apomyoglobin, and hemopexin was examined as a function of liposome charge and cholesterol content. In all cases, there was an almost complete transfer of hemin from liposome to protein; a rapid phase and a slow phase were observed for the transfer. For negatively charged liposomes (with 11% dicetyl phosphate), the rapid and slow phases showed observed rates of transfer of ca. 2 and 0.01 s^{-1}, respectively, for all three proteins. The presence of cholesterol in the liposomes decreased the observed rates by a factor of 2, and positively charged liposomes (with 11% stearylamine) showed about one-fifth the observed rates of negatively charged liposomes. The observed rates were independent of protein concentration, indicating that the rate-determining step is hemin efflux from the lipid bilayer. The hemin interaction with the phospholipid bilayer is suggested to be primarily hydrophobic with some electrostatic character. The two phases are suggested to arise from two different populations of hemin within the liposomes and are interpreted as arising from two different orientations of hemin within the bilayer.

The final step of the biosynthesis of heme[1] is catalyzed by the enzyme ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) located on the matrix side of the inner mitochondrial membrane (Granick & Beale, 1978). Following this, heme must be transported across the inner and outer mitochondrial membranes to be combined with apoproteins to assemble the heme proteins and enzymes located elsewhere in the cell. Similarly, since the heme precursor protoporphyrinogen is

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[1] Abbreviations: PC, phosphatidylcholine; CtP, dicetyl phosphate; StA, stearylamine; PS, phosphatidylserine; chol, cholesterol; Hm, hemin; P, protein; P_i, inorganic phosphate; Me_2SO, dimethyl sulfoxide; CF, carboxyfluorescein; L, liposomes. "Heme" is used to denote iron protoporphyrin IX, irrespective of oxidation state, whereas "hemin" refers specifically to the 3+ oxidation state.

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