Biochem. J. (1981) **197**, 661–668
Printed in Great Britain

Characterization of the inhibition of fibrin assembly by fibrinogen fragment D

Julia E. WILLIAMS,* Roy R. HANTGAN,* Jan HERMANS* and Jan McDONAGH†
*Departments of *Biochemistry and †Pathology, University of North Carolina, Chapel Hill, NC 27514, U.S.A.*

(*Received 19 March 1981/Accepted 8 April 1981*)

Fragment D (M_r 100000) prepared from a terminal plasmin digest of fibrinogen was isolated and used to study its effect on fibrin formation. Increasing amounts of fragment D added to a solution of fibrinogen and thrombin decrease the rigidity of the resultant gel (10% of control at 2 mol of fragment D/mol of fibrinogen). Half-maximal inhibition is achieved at 1 mol of fragment D/mol of fibrinogen for non-cross-linked clots and at ½mol of fragment D/mol of fibrinogen for cross-linked clots. 'Clottability' decreases concomitantly with the rigidity. Only small amounts of fragment D (less than 10% for non-cross-linked gels) are incorporated into the gel. Light-scattering shows an increase in the final fibre thickness at fragment D concentrations up to 2 mol of fragment D/mol of fibrinogen, from 60 molecules/cross-section for the control to 120 molecules/cross-section. Higher fragment D concentrations lead to a decrease in the final fibre thickness. The limit fibre thickness is 8 nm, with a length of 80 nm, which is equivalent to a fibrin trimer. On the basis of results of synthetic-substrate and fibrinopeptide-release assays, it is clear that thrombin inactivation is not responsible for this effect. These data suggest that fragment D may inhibit fibrin formation by blocking the bimolecular polymerization of activated fibrin monomer molecules to form protofibrils, although additional effects on subsequent assembly steps may also be involved.

Fibrinogen is the soluble plasma protein that, as a result of proteolytic cleavage by the enzyme thrombin, can be converted into a solid fibrin gel, a major component of the blood clot. Fibrinogen contains three pairs of polypeptides, which are organized into several functional domains. Doolittle (1977) has reviewed the electron-microscopic, amino-acid-sequence and physical-chemical data on fibrinogen and proposed a model for its three-dimensional structure in which all six *N*-termini are joined by disulphide links into a central domain termed the '*N*-terminal disulphide knot' ('N-DSK'). Helix prediction schemes (Doolittle, 1977) indicate that the α-, β- and γ-chains form regions of coiled coil that connect this central domain to two identical outer globular domains. Although recent electron-microscopic data indicate that these outer domains are multinodular (Weisel *et al.*, 1981), the trinodular picture of fibrinogen serves as a useful working model that is consistent with most of the available data. In the blood-coagulation mechanism, fibrin assembly is a multistep process that begins with the activation of fibrinogen by thrombin. Thrombin first cleaves the two A-fibrinopeptides from the Aα-chains of fibrinogen (Bettelheim, 1956; Blombäck, 1958; Blombäck *et al.*, 1978) and then more slowly the B-peptides from the Bβ-chains (Blombäck, 1958). Once fibrinopeptide A has been removed, fibrin monomer is formed and the self-assembly process is initiated. Fibrin monomer spontaneously polymerizes to form protofibrils in which the monomers are arranged end-to-end in a half-staggered-overlap pattern (Ferry, 1952; Casassa, 1955; Hall & Slayter, 1959). Protofibrils rapidly combine to form longer thicker fibres that, through branchpoint formation, organize a three-dimensional network or fibrin gel. The fibre size in the network is kinetically determined, and the network is held together by non-covalent interactions. The final step in fibrin assembly is the stabilization of the gel through the formation of ε-(γ-glutamyl)–lysyl bonds to yield cross-linked γ-chain dimers and α-polymers. The cross-linking reaction is catalysed by the transglutaminase, factor XIIIa.

When coagulation is initiated in plasma, the fibrinolytic system is also activated, and plasminogen is converted into plasmin. Plasminogen activation may also occur in certain pathological states in the absence of coagulation. Plasmin is a serine protease that will degrade all forms of fibrinogen and

Abbreviation used: SDS, sodium dodecyl sulphate.

0306-3275/81/090661-08$01.50/1

fibrin. The terminal fibrinogen degradation products are a number of small peptides and two core fragments, D and E. Fragment D corresponds roughly to the outer globular domain of fibrinogen, whereas fragment E contains most of the amino acids of the central *N*-terminal-disulphide-knot domain. Intermediates in the degradation pathway are the larger fragments, X and Y (Marder & Budzynski, 1975). The degradation products of non-cross-linked fibrin are similar to those of fibrinogen, whereas cross-linked fibrin yields a dimer of fragment D and high-molecular-weight complexes (Francis *et al.*, 1980*a*,*b*). The plasmin degradation products inhibit coagulation *in vivo* and gelation of fibrin *in vitro*. Such inhibitory activity has been reported for the intermediate fragments, X and Y (Marder & Shulman, 1969; Kowalski, 1968), and also for the core fragments D and D-dimer (Marder & Shulman, 1969; Kowalski, 1968; Larrieu *et al.*, 1972; Belitser *et al.*, 1975; Haverkate *et al.*, 1979; Dray-Attali & Larrieu, 1977; Budzynski *et al.*, 1979).

In the present paper we describe studies on the physical properties of fragment D and quantification of its inhibitory activity. Although plasmin cleavage of fibrinogen can yield a heterogeneous family of digestion products (mol.wts. 80000–100000) owing to multiple sites of γ-chain cleavage (Marder & Budzynski, 1974), we have used the reported protective effect of calcium (Haverkate & Timan, 1977) to prepare and isolate a relatively homogeneous large species of fragment D ($M_r \sim 100000$). The inhibitory effect of this material on fibrin formation has been quantified primarily by measurements of rigidity and light-scattering, which are sensitive and non-disruptive techniques. The observation of a prolonged clotting time in the presence of fragment D could be attributed to any of the following four possibilities: (1) fragment D could inhibit thrombin activity, thus blocking gel formation by interfering with fibrinopeptide release; (2) fragment D could be incorporated directly into the gel, resulting in an abnormal clot structure; (3) fragment D could alter the pattern of assembly events, resulting in either delayed or abnormal fibre formation; (4) inhibition of the incorporation of fibrin monomer into the growing fibrin fibres could cause the observed effects. The experiments described in the present paper have been undertaken to determine which of these possible modes of inhibition operates when gelation occurs in the presence of the fibrinogen degradation product fragment D.

Experimental

Materials

Human fibrinogen obtained from Kabi AB (grade L) or prepared as described by Blombäck &

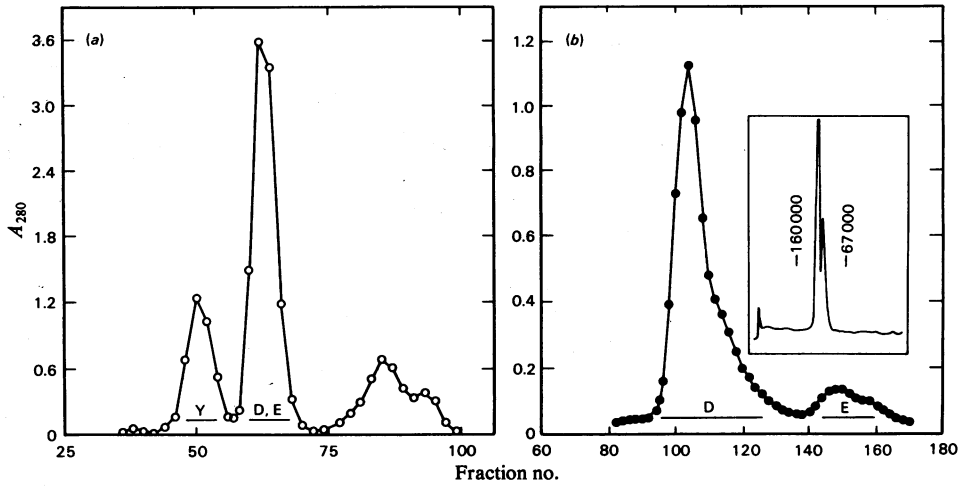

Fig. 1. *Purification of fragment D from a terminal plasmin digest of human fibrinogen*

(*a*) Ultrogel 34 chromatography of a digest of fibrinogen. A 5 ml sample containing 100 mg of material was put on a 2.6 cm × 100 cm column and eluted at 30 ml/h. The fraction volume was 4.3 ml. Material eluted after the main peak had a molecular weight below 30000 and corresponded to fragments A, B, and C. (*b*) DEAE-cellulose chromatography of the mixture of fragments D and E from (*a*). The DEAE-cellulose column (2.5 cm × 30 cm) was equilibrated with 0.01 M-$NaCO_3/Na_2CO_3$ buffer, pH 8.9. The flow rate was 60 ml/h and the fraction volume was 5 ml. A linear pH/salt gradient with 0.01 M-$NaHCO_3/Na_2CO_3$/0.3 M-NaCl, pH 8.0, as the limit buffer was used to elute the protein peaks. The inset shows scans at 570 nm from SDS/5%-polyacrylamide-gel electrophoresis of pool D. Relative locations of molecular-weight standards are indicated (γ-globulin, mol.wt. 160000; bovine serum albumin, mol.wt. 67000).

Blombäck (1956) (fraction I-4) was dissolved in 0.3 M-NaCl, centrifuged for 10 min at 30000 *g*, dialysed for 18 h to remove free calcium, and then divided into portions and stored at −70°C. Clottability was more than 90 and 97% respectively. Fibrinogen concentrations were determined from A_{280} by using a specific absorption coefficient of $5.27 \times 10^5\ M^{-1} \cdot cm^{-1}$ (Mihalyi, 1968). Plasmin (Kabi) was reconstituted to 50 units/ml with 50% (w/v) glycerol and stored at −20°C. Thrombin was either of bovine origin (Parke-Davis) or highly purified 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. Fibrin-monomer–Sepharose, prepared as described by Heene & Matthias (1973), was provided by Dr. N. A. Carrell.

Other reagents were obtained as follows: Trasylol, Mobay Chemical; DEAE-cellulose (DE-52), Whatman; Ultrogel 34, LKB; Sephadex G-25, Pharmacia; lactoperoxidase, 70–100 units/mg of protein, Sigma; carrier-free $Na^{125}I$, Amersham/Searle; rabbit antisera to human fibrinogen fragment D, Calbiochem–Behring.

All other reagents used were reagent-grade. Except where noted, buffer used in all physical experiments was 0.1 M-NaCl/0.05 M-Tris, pH 7.4 (Tris/NaCl).

Methods

Isolation of fragment D. Fibrinogen was digested with plasmin (25 casein units/g of fibrinogen) in the presence of 2.5 mM-calcium (Haverkate & Timan, 1977) for 13–20 h at room temperature. Digestion was stopped by addition of 75 k.i.u. (kallikrein inhibitory units) of Trasylol/ml. The digest was put on a gel-filtration column (Ultrogel 34), which was equilibrated with 0.3 M-NaCl/0.05 M-Tris, pH 7.4, to separate fragment Y from fragments D and E (Fig. 1). Pool 2, containing fragment D (and E), was dialysed extensively against 0.01 M-bicarbonate buffer, pH 8.9. This material was chromatographed on DEAE-cellulose essentially as described by Doolittle *et al.* (1977). A linear gradient, from 0.01 M-carbonate, pH 8.9, to 0.01 M-carbonate/0.3 M-NaCl, pH 8.0, was used (Fig. 1). In some cases, fragment D was concentrated on a short anion-exchange column (2.5 cm × 3.0 cm), with direct elution with the limit buffer and then further concentrated by dialysis against poly(ethylene glycol) 6000. Fragment D was dialysed against Tris/NaCl directly after chromatography or concentration and was used on the same day that it was isolated. The concentration of fragment D was determined by its A_{280}, a specific absorption coefficient of $2.08 \times 10^5\ M^{-1} \cdot cm^{-1}$ (Marder *et al.*, 1972) being used. As polyacrylamide-gel electrophoresis reduced 5% gels) has been extensively employed to characterize the molecular weights of the plasmin degradation products of fibrinogen (Pizzo *et al.*, 1972; Haverkate & Timan, 1977), this technique was employed to establish the identity of the chromatographed fragments. Non-reduced 5%-polyacrylamide gels were prepared as described by McDonagh *et al.* (1972). Before they were stained with Coomassie Brilliant Blue, the gels were fixed with a solution of 10% (v/v) acetic acid/10% (v/v) propan-2-ol. The major species of fragment D used in all experiments had a mol.wt. of 100000 ± 10000 (Fig. 1, inset).

Iodination of fragment D. Purified fragment D was labelled with ^{125}I by the lactoperoxidase procedure (Kudryk & Blombäck, 1979). Labelled fragment D was separated from unbound iodine on a column (1.0 cm × 20 cm) of Sephadex G-25 and eluted with 0.15 M-NaCl/0.05 M-Tris, pH 7.4, containing 1% ovalbumin. Radioactivity was measured in a Beckman Gamma 4000 spectrometer. Antibodies to fragment D were used to determine the antigenicity of the labelled material, with *Staphylococcus aureus* cells as a second precipitant. The iodinated fragment D precipitated to the extent of 90% with 20% (w/v) trichloroacetic acid, and 79% with anti-(fragment D) antibodies. Fibrin-monomer–Sepharose was packed in a 1 ml syringe. A sample of labelled fragment D was applied to the column, and the amount of bound label was found to be 63% of the applied material. SDS/polyacrylamide (reducing) gels of labelled and unlabelled fragment D were identical.

Binding of fragment D to fibrin and clottability of fibrinogen

Various amounts of unlabelled D were added to a constant amount of labelled D and fibrinogen (final concn. 0.4 mg/ml). The A_{280} was measured before the addition of thrombin. After 2 h the clots were removed by winding them around glass rods. The radioactivity of each clot and supernatant was measured, and the absorbance of the supernatant was recorded.

Thrombin assays. A thrombin assay in the presence of fragment D was performed by Dr. R. Lundblad. The substrate used was 100 μM-benzoylarginine *p*-nitroanilide in 0.05 M-Tris, pH 8.0. From 0 to 3.5 mg of fragment D were added to 200 units of human α-thrombin (specific activity 3000 units/mg), and the final A_{410}nm was monitored.

Fibrinopeptide release was measured by quantification of the reaction of peptide-bound arginine with phenanthrenequinone (Yamada & Itano, 1966). Fibrinogen concentration was constant at 3 mg/ml. Samples with or without 1.74 mg of fragment D/ml (2 mol of fragment D/mol of fibrinogen) were

incubated at 37°C for 0, 1, 3 or 60min with 0.36 units of thrombin/ml or for 60min with 9 units of thrombin/ml. The fibrinopeptides were separated and made to react with phenanthrenequinone, and fluorescence was measured at 395nm and compared with standard arginine solutions.

Rigidity measurements. The rigidity, or elastic modulus, of fibrin gels was measured as previously described (Carr *et al.*, 1976). The fibrinogen concentration was 0.15mg/ml and the fragment D concentration ranged from 0 to 0.25mg/ml. In some studies, buffer containing 5mM-$CaCl_2$ was used, and the fragment D concentration was varied from 0 to 0.12mg/ml. Gelation was achieved by the addition of thrombin to a final concentration of 1.25units/ml. Immediately after the addition of thrombin, the solution was mixed and poured into the Couette elastometer and allowed to clot before stress was applied. Stress was then applied at 5–10min intervals for less than 30s at a time. Measurements were made at 25°C.

Light-scattering. The apparatus and techniques for measurement were used as previously described (Carr *et al.*, 1977; Hantgan & Hermans, 1979). Buffer and fragment D solutions were degassed and filtered through presoaked 0.22μm-pore-size Millipore filters in 13mm-diameter Swinnex plastic filter holders (Millipore). The concentration of fragment D was determined spectrally after filtration. A small portion of a concentrated fibrinogen solution was added to achieve a final fibrinogen concentration of 0.076mg/ml. Fibrin assembly was initiated by the addition of a small portion of a concentrated thrombin solution to a final concentration of 3.8units/ml. For studies of the angular dependence of light scattered from inhibited clotting solutions, fibrinogen concentrations of 0.16mg/ml and a thrombin concentration of 3.3units/ml were employed. Ratios of 14 to 120mol of fragment D/mol of fibrinogen were investigated by angular-dependence studies, and from 0.5 to 50mol of fragment D/mol of fibrinogen for 90° intensity studies.

Results

Rigidity measurements

To assay for the inhibitory activity of fragment D on fibrin clots, the technique of clot rigidity was used. The data presented in Fig. 2 demonstrate a tenfold decrease in the elastic modulus of fibres formed at a ratio of 3mol of fragment D/mol of fibrogen. Half maximum inhibition occurs at about 1mol of fragment D/mol of fibrinogen, whereas clots formed under Factor-XIIIa-cross-linking conditions are even more sensitive to the presence of fragment D, with half-maximum inhibition at less than 0.5mol of fragment D/mol of fibrinogen. SDS/polyacrylamide-gel electrophoresis of the reduced proteins indicated

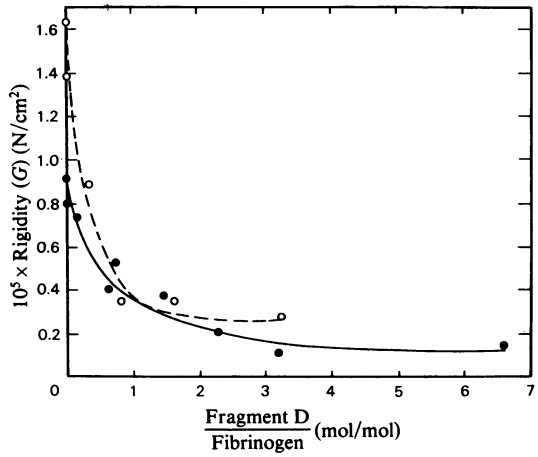

Fig. 2. *Clot rigidity at 80min for cross-linked and non-cross-linked gels as a function of fragment D concentration*

Fibrinogen concentration was constant at 0.15mg/ml. ●, Non-cross-linked clots; ○, clots stabilized by Factor XIIIa and calcium.

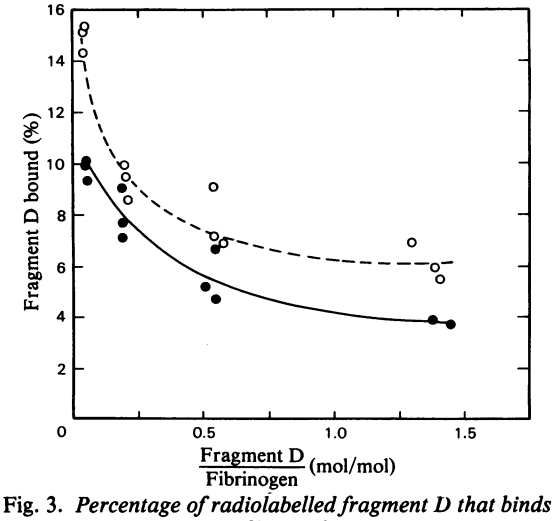

Fig. 3. *Percentage of radiolabelled fragment D that binds to fibrin gels*

Fibrinogen concentration was 0.4mg/ml. The radioactivity of both the clot and the supernatant was measured. ●, Non-cross-linked clots; ○, clots stabilized by Factor XIIIa and calcium.

that 100% of the γ-chains and at least 90% of the α-chains were cross-linked under these conditions.

Fragment D binding to fibrin and clottability

Fragment D radiolabelled with ^{125}I was employed to quantify the binding of this fragment to fibrin fibres. Fig. 3 expresses the results of these experiments as the percentage of fragment D present in

Table 1. *Quantification of the distribution of fragment D in fibrin gels*
(*a*) Shows results for non-cross-linked fibrin, and (*b*) those for fibrin stabilized by $CaCl_2$ and Factor XIIIa. Fibrinogen concentration was constant at 0.4 mg/ml. Abbreviation used: D, fragment D.

	Total D	Bound D	Bound D	
	Fibrinogen (mol/mol)	Clotted fibrinogen (mol/mol)	Total D	Clottable fibrinogen (%)
(*a*)	—	—	—	96.5 ± 0.5
	0.05	0.006	0.098	91.5 ± 0.3
	0.19	0.018	0.079	86.2 ± 0.3
	0.54	0.038	0.055	78.5 ± 1.8
	1.42*	0.111	0.038	50.0 ± 12.7
(*b*)	—	—	—	98.1 ± 0.3
	0.04	0.007	0.150	92.5 ± 1.1
	0.21	0.021	0.093	91.7 ± 1.3
	0.55	0.048	0.077	88.3 ± 1.0
	1.37	0.102	0.061	81.8 ± 0.7

* All results except this one represent the mean of three separate samples.

solution that binds to the resultant fibres, for clots with and without cross-linking by Factor XIIIa. A maximum of 10% (15% for cross-linked fibres) of the radiolabelled material binds at low ratios of fragment D to fibrinogen. As the molar ratio increases, the amount of fragment D bound decreases by a factor of about 2. Table 1 shows the increase in the total amount of fragment D bound to fibrin as the fragment D concentration increases. There is a linear increase in this parameter, with no indication of saturation in the accessible range of fragment D concentrations. Higher ratios of fragment D to fibrinogen inhibit gelation to the extent that the experiment cannot be performed.

Spectral determinations of the amount of clottable protein are listed in Table 1. These results show a decrease in clottability by a factor of nearly 2 over this same range of fragment D concentrations. There is a less pronounced change in clottability in the presence of calcium and Factor XIIIa.

Light-scattering

Measurements of the intensity of light scattered from solutions of macromolecules have frequently been used to determine the size and shape of the scattering species (Huglin, 1972). In particular, measurements of the angular dependence of the intensity of scattered light have been employed to measure the molecular weight and radius of gyration of fibrinogen (Hocking *et al.*, 1952), as well as the size and shape of intermediate polymers formed in an inhibited clotting system (Ferry, 1952). Casassa (1955) extended the theory and technique to measure the mass per unit length of long oligomers formed after thrombin activation of fibrinogen at high pH and ionic strength. Carr *et al.* (1977) measured the mass/length ratio of both coarse and fine gels from the angular-dependence of scattered-light intensity and found the values to agree well with those determined by solvent-perfusion measurements. They verified that, for dilute gels, the intensity of scattered light [$R(\theta)$] is inversely proportional to $\sin(\theta/2)$, in which θ is the angle between the primary and scattered beam. Thus measurement of the scattered-light intensity at a single angle of 90° can be used to determine the mass/length ratio of fibrin fibres. The scattering intensity is determined relative to the scattering of benzene, for which the absolute scattering has been accurately determined (Pike *et al.*, 1975). The following equations apply:

$$Kc/R(\theta) = (4n/\lambda_0\mu)\sin(\theta/2) \quad (1)$$

and

$$K = 2\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2/\lambda_0^4 N \quad (2)$$

where c is the concentration, K is an optical constant, n is the refractive index, λ_0 the wavelength *in vacuo*, N is Avogadro's number and μ is the mass/length ratio.

Measurements of light-scattering intensity were used to examine the characteristics of the fibres formed in the presence of fragment D (Fig. 4). The mass/length ratio, determined from the 90° scattering intensity, was found to increase from the control value of 60 molecules/cross-section to 120 molecules/cross-section at a fragment D concentration corresponding to 2 mol of fragment D/mol of fibrinogen. Higher fragment D concentrations lead to a decrease in final fibre thickness. Beyond 15 mol of fragment D/mol of fibrinogen, no clot is observed after 1 h, and the final fibre thickness corresponds to thin fibres with a width less than twice that of the protofibril. Cross-linked and non-cross-linked clots are qualitatively the same by this measurement. At

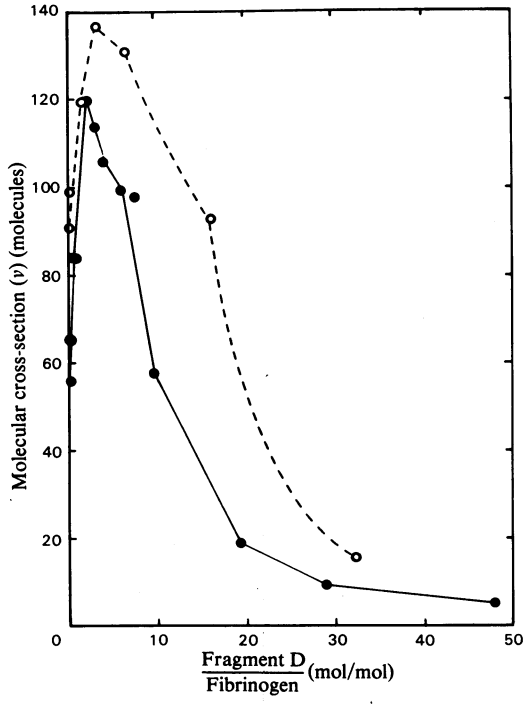

Fig. 4. *Average fibre thickness as measured by light-scattering with increasing fragment D concentrations*
Fibrinogen concentration: 0.076 mg/ml in 0.1 M-NaCl/0.05 M-Tris, pH 7.4. ●, Non-cross-linked clots; ○, clots stabilized by Factor XIIIa and calcium.

higher fibrinogen and fragment D concentrations, measurements of the angular dependence of the intensity of scattered light indicated that short oligomers resulted from thrombin activation. (This technique yielded molecular weights of 330000 ± 10000 and 105000 ± 18000 for fibrinogen and fragment D respectively.) The data in Table 2 demonstrate that raising the fragment D concentration causes a decrease in the molecular weight and length of the scattering species. The length of these oligomers decreases from 270 to 85 nm over the range of fragment D concentrations investigated. At lower fragment D/fibrinogen ratios, gelation results and the length of the fibres cannot be determined by this light-scattering technique.

The measured oligomer dimensions are compared in Table 2 with those calculated for protofibrils, formed by a half-staggered overlap of activated fibrinogen molecules (Ferry, 1952; Hantgan *et al.*, 1980). Molecular dimensions of fibrinogen determined by electron microscopy (Fowler & Erickson, 1979) were used in calculations. The results obtained indicate that, at 14.4 mol of fragment D/mol of fibrinogen, protofibrils consisting of nine ± one monomers are formed, whereas at ratios of 120 mol of fragment D/mol of fibrinogen, a polymer length corresponding to that of a trimer of activated fibrinogen molecules results. Viscosity measurements also show that protofibril length decreases with increasing concentrations of fragment D.

Table 2. *Protofibrils formed in the presence of fragment D*

Fragment D/ fibrinogen (mol/mol)	Molecular weight of protofibrils	Length of protofibrils (nm)
14.4	2.86×10^6	270
120.0	1.70×10^6	85
Ideal characteristics of protofibrils		
No. of monomers	Molecular weight	Length (nm)
3	1.02×10^6	90
9	3.06×10^6	225

Thrombin activity

Thrombin-activity assays, which monitor the hydrolysis of the synthetic substrate benzoylarginine *p*-nitroanilide, indicated that thrombin retains 90% of its activity at ratios of 15 mol of fragment D/mol of thrombin and 75% of its initial activity at a 20-fold excess of fragment D. The fibrinopeptide release assay showed no difference in the rate or of the final number of fibrinopeptides released from fibrinogen in the presence of 100-fold molar excess of fragment D to thrombin. This lack of significant thrombin inhibition is important to an interpretation of the physical-chemical studies of the mode of inhibition of fragment D.

Discussion

Previous studies of the anticoagulant activity of fragment D have emphasized the decreased fibrinogen clottability (Kowalski, 1968) and the prolonged thrombin clotting time in the presence of fragment D (Marder & Shulman, 1969; Kowalski, 1968; Larrieu *et al.*, 1972; Belitser *et al.*, 1975; Haverkate *et al.*, 1979). In the present paper we report further characterization of the inhibitory properties of fragment D based on measurements of the physical properties of the fibrin formed in the presence of fragment D.

Antithrombin activity

Until now, purified fragment D has not been assayed directly for its effect on the enzymic activity of thrombin; however, the following observations have been made. Alkjaersig *et al.* (1962) noted that

the benzoylarginine methylesterase assay for thrombin was unaffected by a mixture of fibrinogen proteolysis products, whereas Latallo *et al.* (1964) showed that late degradation products (mainly fragments D and E) had little or no effect on total fibrinopeptide release as measured by trichloroacetic acid-soluble peptides. These results are consistent with the data presented here, in which the use of a chromogenic substrate for thrombin showed the lack of significant inhibition at less than 20 mol of fragment D/mol of thrombin. We have also shown that fragment D does not affect either the rate or total release of fibrinopeptides. Also, Belitser *et al.* (1975) found that polymerization of preformed fibrin monomer was strongly inhibited by a late plasmin digest of fibrinogen formed in the presence of calcium, and thereby demonstrated that inhibition of thrombin, if any, is not a requirement for the inhibition of assembly. In summary, inhibition of polymerization by fragment D cannot be due to inhibition of thrombin activity.

Clot rigidity

The elastic modulus of fibrin fibres depends on the square of the fibrinogen concentration, the fibre thickness, the number of network branchpoints and the bulk rigidity of the fibres, and can be altered by changes in calcium concentration, ionic strength, and Factor XIIIa cross-linking (Shen *et al.*, 1975; Nelb *et al.*, 1976). It has been previously reported that plasmin degradation of fibrin(ogen) leads to a decreased clot rigidity (Shen *et al.*, 1977); the results presented here demonstrate that the purified plasmin degradation product, fragment D, can inhibit the development of the elastic modulus of fibrin fibres. As the binding of fragment D to fibrin shows no evidence of saturation, we can conclude that a large number of potential binding sites for fragment D are present on fibrin; but this inhibitory effect occurs under conditions where only small amounts of fragment D actually bind to the fibres (<10% of the fragment D in solution). Therefore we can rule out the explanation that the bound fragment D interferes with gelation by altering the structure of the fibrin formed. For example, at 1.4 mol of fragment D/mol of fibrinogen, less than 4% of the fragment D in solution binds to fibrin; there is a fourfold decrease in the elastic modulus, but there is also a twofold decrease in the amount of clottable protein. As the elastic modulus is proportional to the square of the fibrinogen concentration, this decrease in clot rigidity should be directly attributed to a decrease in the amount of clottable protein rather than to alterations in fibrin structure. That is, the binding, clot-rigidity and clottability data all indicate that the protein which clots in the presence of fragment D forms normal fibrin, and that the remainder of the protein is apparently blocked from entering the gel by the presence of fragment D.

Fibre formation

The results presented here demonstrate that fragment D, at concentrations of more than 2 mol of fragment D/mol of fibrinogen, inhibits fibre formation. Above 14 mol of fragment D/mol of fibrinogen, gelation is fully blocked. The data in Table 2 demonstrate that the molecular weight and length of the resulting short oligomers correspond to protofibrils composed of three to nine monomers, arranged in the half-staggered overlap structure. These observations suggest that fragment D may interfere with fibrin assembly by directly blocking the first assembly step, namely the bimolecular polymerization of activated fibrin monomer molecules to form protofibrils. Inhibition of this first assembly event would be observed at all subsequent stages of the gelation process. Therefore the observations of decreased clottability, decreased elastic modulus and the presence of short oligomers in solution, which characterize fibrin formation in the presence of fragment D, can all be explained by a mechanism in which fragment D binds to the growing protofibrils, thus stopping the incorporation of fibrin monomer into these two stranded polymers, which are obligatory intermediates in the fibrin-assembly pathway.

It has already been shown that fragment D contains a polymerization site, because it interacts with fibrin-monomer–Sepharose (Kudryk *et al.*, 1973; Matthias *et al.*, 1973; York & Blombäck, 1976; Heene *et al.*, 1979; Olexa & Budzynski, 1980) and thrombin activated *N*-terminal disulphide knot covalently linked to Sepharose (Kudryk *et al.*, 1974). The D-domain in fibrin monomer can form stable contacts with both the E- and D-domains of fibrin fibres (Hermans, 1979). Fragment D may also compete for this site, consequently blocking protofibril growth by forming a 'dead-end' complex that cannot undergo further bimolecular addition of the next fibrin monomer molecule. It is clear, however, that small amounts of fragment D, less than 1 mol/mol of fibrinogen, are sufficient to produce substantially weaker clots.

We thank Dr. R. Doolittle and Dr. B. Kudryk for providing protocols of fragment D separation and radiolabelling, Dr. J. Fenton for kindly giving α-thrombin, Dr. N. Carrell for fibrin-monomer–Sepharose, and Dr. R. Lundblad for performing thrombin assays. This work was supported by National Institutes of Health grants nos. HL20319, HL22505 and HL21139. J. M. is an Established Investigator of the American Heart Association.

References

Alkjaersig, N., Fletcher, A. P. & Sherry, S. (1962) *J. Clin. Invest.* **41**, 917–934

Belitser, V. A., Varetska, T. V., Tolstykh, V. M., Tsaryuk, L. A. & Pozdnyakova, T. M. (1975) *Thromb. Res.* **7**, 797–806
Bettelheim, F. R. (1956) *Biochim. Biophys. Acta* **19**, 121–130
Blombäck, B. (1958) *Ark. Kemi* **12**, 321–335
Blombäck, B. & Blombäck, M. (1956) *Ark. Kemi* **10**, 415–443
Blombäck, B., Hessel, B., Hogg, D. & Therkildsen, L. (1978) *Nature (London)* **275**, 501–505
Budzynski, A. Z., Olexa, S. A. & Brizuela, B. S. (1979) *Biochim. Biophys. Acta* **584**, 284–287
Carr, M. E., Shen, L. L. & Hermans, J. (1976) *Anal. Biochem.* **72**, 202–211
Carr, M. E., Shen, L. L. & Hermans, J. (1977) *Biopolymers* **16**, 1–15
Casassa, E. F. (1955) *J. Chem. Phys.* **23**, 596–597
Doolittle, R. F. (1977) *Horiz. Biochem. Biophys.* **3**, 164–191
Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J. & Takagi, T. (1977) *Biochemistry* **16**, 1710–1715
Dray-Attali, L. & Larrieu, M. J. (1977) *Thromb. Res.* **10**, 575–586
Fenton, J. W., Landis, B. H., Walz, D. A. & Finlayson, J. S. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W. & Mann, K. G., eds.), pp. 43–70, Ann Arbor Science Publishers, Ann Arbor, MI
Ferry, J. D. (1952) *Proc. Natl. Acad. Sci. U.S.A.* **38**, 566–569
Fowler, W. E. & Erickson, H. P. (1979) *J. Mol. Biol.* **134**, 241–249
Francis, C. W., Marder, V. J. & Barlow, G. H. (1980*a*) *J. Clin. Invest.* **66**, 1033–1043
Francis, C. W., Marder, V. J. & Martin, S. E. (1980*b*) *Blood* **56**, 456–464
Hall, C. E. & Slayter, H. S. (1959) *J. Biophys. Biochem. Cytol.* **5**, 11–16
Hantgan, R. R. & Hermans, J. (1979) *J. Biol. Chem.* **254**, 11272–11281
Hantgan, R., Fowler, W., Erickson, H. & Hermans, J. (1980) *Thromb. Haemostasis* **44**, 119–124
Haverkate, F. & Timan, G. (1977) *Thromb. Res.* **10**, 803–812
Haverkate, F., Timan, G. & Nieuwenhuizen, W. (1979) *Eur. J. Clin. Invest.* **9**, 253–255
Heene, D. L. & Matthias, F. R. (1973) *Thromb. Res.* **2**, 137–154
Heene, D. L., Matthias, F. R., Wegrzynowicz, Z. & Hocke, G. (1979) *Thromb. Haemostasis* **41**, 677–686
Hermans, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1189–1193
Hocking, C. S., Laskowski, M., Jr. & Scheraga, H. A. (1952) *J. Am. Chem. Soc.* **74**, 775–778
Huglin, M. B. (ed.) (1972) *Light Scattering from Polymer Solutions*, Academic Press, New York
Kowalski, E. (1968) *Semin. Hematol.* **5**, 45–59
Kudryk, B. & Blombäck, M. (1979) *Thromb. Haemostasis* **42**, 120
Kudryk, B., Reuterby, J. & Blombäck, B. (1973) *Thromb. Res.* **2**, 297–304
Kudryk, B., Collen, D., Woods, K. R. & Blombäck, B. (1974) *J. Biol. Chem.* **249**, 3322–3325
Larrieu, M. J., Riggollot, C. & Marder, V. J. (1972) *Br. J. Haematol.* **22**, 719–733
Latallo, Z. S., Budzynski, A. Z., Lipinski, B. & Kowalski, E. (1964) *Nature (London)* **203**, 1184–1185
Marder, V. J. & Budzynski, A. Z. (1974) *Prog. Haemostasis Thromb.* **2**, 141–174
Marder, V. J. & Budzynski, A. Z. (1975) *Thromb. Diath. Haemorrh.* **33**, 199–207
Marder, V. J. & Shulman, N. R. (1969) *J. Biol. Chem.* **244**, 2120–2124
Marder, V. J., Budzynski, A. Z. & James, H. L. (1972) *J. Biol. Chem.* **247**, 4775–4781
Matthias, F. R., Heene, D. L. & Konradi, E. (1973) *Thromb. Res.* **3**, 657–664
McDonagh, J., Messel, H., McDonagh, R. P., Murano, G. & Blombäck, B. (1972) *Biochim. Biophys. Acta* **257**, 135–142
Mihalyi, E. (1968) *Biochemistry* **7**, 208–223
Nelb, G. W., Gerth, C., Ferry, J. D. & Lorand, L. (1976) *Biophys. Chem.* **5**, 377–387
Olexa, S. A. & Budzynski, A. Z. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1374–1378
Pike, E. R., Romeroy, W. R. M. & Vaughan, J. M. (1975) *J. Chem. Phys.* **62**, 3188–3192
Pizzo, S. V., Schwartz, M. L., Hill, R. L. & McKee, P. A. (1972) *J. Biol. Chem.* **247**, 636–645
Shen, L. L., Hermans, J., McDonagh, J., McDonagh, R. P. & Carr, M. (1975) *Thromb. Res.* **6**, 255–265
Shen, L. L., McDonagh, R. P., McDonagh, J. & Hermans, J. (1977) *J. Biol. Chem.* **252**, 6184–6189
Weisel, J. W., Phillips, G. N. & Cohen, C. (1981) *Nature (London)* **289**, 263–267
Yamada, S. & Itano, H. A. (1966) *Biochim. Biophys. Acta* **130**, 538–540
York, L. L. & Blombäck, B. (1976) *Thromb. Res.* **8**, 607–618