Fibrin assembly after fibrinopeptide A release in model systems and human plasma studied with magnetic birefringence

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Magnetically induced birefringence was used to monitor fibrin polymerization after the release of the small negatively charged A fibrinopeptides from human fibrinogen by the action of the snake-venom-derived enzymes reptilase and ancrod. A range of conditions was investigated. Fibrin polymerization in solutions of purified fibrinogen shows a distinct break near the gelation point. On addition of Ca^{2+} or albumin the lag period is shortened, fibre thickness is increased and the break in assembly almost vanishes, probably because both of these additives promote lateral aggregation. There are minor differences in the kinetics, depending on the venom enzyme used. The kinetics of fibrin assembly in model systems containing either Ca^{2+} or albumin and in human plasma with a largely dormant coagulation cascade are very similar. Therefore in the latter condition there is no significant alteration in the assembly process due to interaction between fibrin or the venom enzymes and any of the plasma proteins. When the cascade is activated, the polymerization progress curves have a character that resembles a combination of the reactions observed when the venom enzymes and endogenously generated thrombin separately induce coagulation, except for a region near gelation where, paradoxically, polymerization appears to be slower on activation. The low-angle neutron-diffraction patterns from oriented gels made with thrombin or reptilase are identical. Therefore at low resolution the packing of the monomers within fibres is the same when fibrinopeptide A only or both fibrinopeptides A and B are removed.

INTRODUCTION

The final stages in blood-clot formation are a triptych of activated Factor X generation, thrombin formation and limited proteolysis of fibrinogen, leading to fibrin polymerization (for reviews see Jackson & Nemerson, 1980; Nemerson & Furie, 1980; Osterud, 1984). The steps involved in fibrin assembly summarized below have been elucidated by many workers using a variety of techniques; more details are available in reviews by Hermans & McDonagh (1982) and Doolittle (1984). Thrombin activates fibrinogen (M_r approx. 340 000) by catalysing the release of the small negatively charged fibrinopeptides A and B. The liberation of fibrinopeptide A is sufficient for fibrin fibre assembly and gelation. The release of fibrinopeptide B, which is slower than that of fibrinopeptide A, promotes, but is not essential for, the lateral association necessary for fibre development. During fibrin gel assembly protofibrils first form by linear polymerization; these aggregate sideways, giving rise to an interlinked fibre network. Gelation is a relatively early event, and thereafter the gel grows mainly by attachment to the existing network (Freyssinet *et al.*, 1983). Protofibrils, which can be several hundred nanometres long before aggregation (Hantgan *et al.*, 1980), are two-stranded polymers staggered by half (22.5 nm) a monomer length. Within fibres the protofibrils are packed in accurate longitudinal register, and there is evidence, from neutron diffraction of magnetically aligned gels, suggesting three-dimensional ordering (Torbet *et al.*, 1981).

The enzymes reptilase (Stocker & Barlow, 1976; Stocker, 1983) and ancrod (Nolan *et al.*, 1976), extracted respectively from the venoms of the vipers *Bathrops atrox* and *Agkistrodon rhodostoma*, when pure release only fibrinopeptide A and differ from thrombin in other important respects. For example, ancrod (Nolan *et al.*, 1976) does not activate Factor XIII (fibrinolytic precursor), whereas activation by reptilase does occur but the product has a different substrate-specificity from that of the thrombin-activated form (Okada *et al.*, 1985). There is no evidence suggesting that these enzymes have an effect on other clotting factors, and they are only weakly inactivated by plasma proteinase inhibitors (Pitney & Regoeczi, 1970; Egberg, 1974; Nolan *et al.*, 1976; Stocker & Barlow, 1976; Stocker, 1983). Reptilase and ancrod have attracted limited clinical interest as a means to produce controlled defibrination (Pizzo *et al.*, 1972). Therefore it is of interest to know how they influence clot formation in conditions as near physiological as possible.

Blood coagulation is an elaborate system, and therefore its understanding requires the study of clot formation not only in model systems but also in conditions in which the interactions and concentrations of all the components are close to physiological. Magnetically induced birefringence helps to open up this approach, as it can be used over a wide range of conditions (Torbet, 1986). Here it is used to study fibrin assembly after the addition of ancrod or reptilase to model systems and human plasma. The object is to find out how assembly occurs as a result of the action of these

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enzymes, and also to gain further insight into fibrin formation in physiological conditions by exploiting the differences between the properties of these enzymes and thrombin.

MATERIALS AND METHODS

Sample preparation

Fibrinogen was purified from human plasma as described in Kekwick *et al.* (1955). Unless otherwise stated, the buffer was 0.05 M-Tris/HCl containing 0.1 M-NaCl, 0.5 mM-EDTA and 0.01% (w/v) NaN_3 at pH 7.5. Human albumin came from Fluka. Reptilase came from Laboratoire Stago (Asnières, France) and ancrod (arvin) from Berk Pharmaceuticals (Shalford, Surrey, U.K.). All were used without further purification.

Citrated human plasma was obtained by collecting freshly drawn blood (9 vol.) into 3.8% (w/v) sodium citrate (1 vol.) followed by centrifugation at approx. 5000 g for 20 min at 15 °C. The plasma was either used within a few hours, or stored frozen at -70 °C and thawed at 37 °C before use. No systematic differences were found between fresh and frozen-and-thawed plasma. The final plasma concentration was lowered to 85% of that in whole plasma, on taking into account the dilution resulting from the addition of the snake-venom enzymes and Ca^{2+} . The results reported are from plasma donated by a single individual. The concentration of fibrinogen in plasma was assayed by using the Diagnostica Stago procedure. The time of gelation, which was assessed by eye in control samples outside the magnet, occurs soon after the end of the lag period, when $\Delta n/c = 0.7 \pm 0.2$ mg/ml. Because of the steep rise in the birefringence in this region and the small differences in history between samples and controls, the gelation point could not be located more accurately. The gelation point varied from about 1 to 60 min.

The samples for neutron diffraction were made at 19 °C in a magnetic field of 10 T, by using a solution of pure human fibrinogen (10 mg/ml) containing 6 mM- Ca^{2+} . The thrombin and reptilase concentrations both ranged from 0.1 to 0.05 unit/ml. The lag periods were in the range 10–20 min. The gels are stable, and so the H_2O in the buffer was replaced by $^2\text{H}_2\text{O}$ by diffusion over a period of several days. The sample thickness was 0.1 or 0.2 cm.

Birefringence measurements

The samples were contained in quartz cells with an optical path length of 0.1 cm and placed in a temperature-stabilized (± 0.1 °C) sample holder in a Bitter-type magnet, which had a small radial bore and could attain a maximum field of 13.5 T. The birefringence Δn (λ 632.8 nm) was measured by using a combined photoelastic-modulation and compensation technique as described in detail by Maret & Weill (1983). All measurements were made at 37 °C.

Theory

The magnetic orientation of fibrin is due to its diamagnetic anisotropy, and the magnetic birefringence developed when a solution of pure fibrinogen is converted into fibrin is simply proportional to polymer concentration in both purified systems (Freyssinet *et al.*,

1983) and whole plasma (Torbet, 1986). In the experiments reported in the present paper it was verified that the shapes of the birefringence curves are independent of field strength between 2 and 10 T. Also, the birefringence of albumin and the plasma used was much less than that of fibrin. Therefore, as with the purified system, the variation in the birefringence, Δn , with time gives a direct measure of the behaviour of fibrin as it polymerizes.

Neutron-diffraction measurements

The neutron-diffraction patterns were obtained on the small-angle-scattering camera D17 at the Institut Laue-Langevin (Grenoble). The scattered neutrons were detected with a two-dimensional (64 cm $\times$ 64 cm) BF_3 multidetector. The wavelengths used were 1.0 nm and 1.2 nm ($\Delta\lambda/\lambda$ was 10%, full width, half maximum) and the specimen-to-detector distance was either 1.4 or 2.8 m. A water spectrum, which is isotropic under these conditions, was used to correct for detector response.

RESULTS AND DISCUSSION

Fibrin assembly in model systems

When a rate-limiting amount of ancrod (Fig. 1a) or reptilase (Fig. 1c) is added to a solution of purified fibrinogen at physiological pH and ionic strength and placed in a constant magnetic field, the plot of induced birefringence against time or the polymerization progress curve shows that the lag period is followed by rapid fibre assembly, which abruptly gives way to a phase of slow prolonged growth close to the gelation point. The shape is not sigmoidal as was found with thrombin in similar conditions (Freyssinet *et al.*, 1983). The early part of the birefringence curves resulting from the action of the venom-derived enzymes or thrombin can be qualitatively explained by the same sequence of assembly events. During the lag period protofibrils form after the release of fibrinopeptide A, but, as they orient poorly (Freyssinet *et al.*, 1983), the induced birefringence is weak. When these double-stranded polymers reach a sufficient length they aggregate laterally, so generating an interlinked fibre network. As the growing fibres orient significantly the birefringence rises, gelation takes place and the stock of protofibrils is depleted. Now the assembly pathways diverge. With thrombin the increasing release of fibrinopeptide B favours lateral fibre growth (Hantgan & Hermans, 1979); assembly consequently appears to be continuous, as shown by the unbroken sigmoidal shape of the birefringence curves (Freyssinet *et al.*, 1983). In contrast, neither ancrod nor reptilase releases fibrinopeptide B, and so the affinity for lateral attachment is decreased, which gives rise to a break in the process of assembly (Figs. 1a and 1c). This explanation is supported by the experiments reported below showing that the break in the progress curves is very much diminished in conditions that promote lateral aggregation.

When Ca^{2+} (Figs. 1b and 1c) or albumin (Figs. 1a and 1c) is introduced into fibrinogen solutions the lag period is shortened and the birefringence rises more rapidly and attains a markedly increased maximum value. In a constant magnetic field the degree of orientation, and therefore the magnitude of the birefringence, increases as the magnetic anisotropy of the orienting units becomes

Fig. 1. Variation of the birefringence, Δn , normalized to fibrinogen concentration, c (1 mg/ml), as fibrin forms at 37 °C in a constant magnetic field of 6 T from a solution of purified human fibrinogen

The venom enzymes used are as indicated and their concentrations are shown in units/ml. (a) In the absence (----) and in the presence (—) of human albumin (70 mg/ml) in the solution. (b) With Ca^{2+} (2 mM) added to the buffer. (c) With buffer only (----) and with the addition of Ca^{2+} (2 mM) (—) or of albumin (70 mg/ml) (.....).

larger. When thrombin was used to activate purified fibrinogen it was shown that at high ionic strength, which causes thin fibres to form, the magnetic birefringence is weak, whereas at physiological ionic strength the resulting fibres are thicker and consequently the magnetic birefringence is much stronger (Freyssinet *et al.*, 1983). This is further increased by the addition of Ca^{2+} , which is known to augment fibre thickness (Hantgan *et al.*, 1980). It follows from a comparison of the different curves in Fig. 1 that the effect of albumin on ancrod-induced and reptilase-induced fibrin assembly is similar to that of Ca^{2+} (Shen *et al.*, 1977) insofar as both increase the average fibre thickness and decrease the lag period. This has also been found to be true in parallel

experiments with thrombin (Torbet, 1986). The curves have a more rounded shape in the presence of albumin than with Ca^{2+} (Fig. 1), which denotes a minor difference in the kinetics of assembly.

The effect of albumin cannot be attributed to an increase in the rate of fibrinopeptide release, as the changes are not reproduced by the addition of more enzyme (Fig. 1a). Wilf *et al.* (1985) have measured the rate of fibrinopeptide release and found it to be unaffected by albumin. Thus albumin must exert its influence by promoting aggregation, which may be due to its involvement in intermolecular interactions, or, alternatively, non-specific volume exclusion arising from increased fractional occupancy of the solution volume by the macromolecules could be important (Minton, 1983; Wilf *et al.*, 1985). This has been proposed as an explanation of the decrease in the lag period caused not only by albumin but also by γ -globulin, haemoglobin and ovalbumin (Wilf *et al.*, 1985). Non-specificity gains additional, if limited, support from the observation that the decrease in lag period and thickening of fibres in the presence of albumin occurs equally when only fibrinopeptide A (Figs. 1a and 1c) or when both fibrinopeptides (Torbet, 1986) are released by the action respectively of the enzymes ancrod or reptilase and thrombin.

Fibrin assembly in human plasma

Ca^{2+} ions are required for the reactions involving vitamin K-dependent clotting factors, and consequently thrombin production is arrested in citrate-treated plasma owing to chelation of the innate Ca^{2+} . However, on re-addition of Ca^{2+} thrombin is normally endogenously generated, so that fibrin formation takes place at a near linear rate from the end of the lag period (Torbet, 1986). The lag period after the re-addition of Ca^{2+} usually varied from approx. 7 to 30 min at 37 °C depending on the plasma preparation. Occasionally a sample had an unusually long (≥ 60 min) lag period and could therefore be used to investigate the effect of Ca^{2+} on ancrod-induced and reptilase-induced fibrin assembly while the coagulation cascade was largely dormant. In these conditions the birefringence-induced curves without (Figs. 2a and 2c) and with (Figs. 2b and 2d) the re-addition of Ca^{2+} are virtually identical for the same lag period. Their similarity in magnitude indicates that the presence of free Ca^{2+} does not lead to a significant increase in fibre diameter. The only effect of Ca^{2+} is to shorten the lag period. Particularly at the higher enzyme concentrations, the reptilase-induced curves are more rounded (Fig. 2), so that for the same lag period polymerization takes longer than with ancrod. The source of this minor difference is conjectural, but one potential cause is the additional cleavages performed on fibrin by ancrod (Nolan *et al.*, 1976; Shen *et al.*, 1977) but not by reptilase.

The polymerization progress curves produced by plasma (Fig. 2) are in both shape and magnitude very like those resulting when Ca^{2+} is present in purified fibrinogen solutions (Fig. 1), and somewhat less rounded than when albumin is added (Fig. 1). Thus fibrin assembly and fibre thickness are only marginally altered by the near-physiological concentration of all of the plasma proteins, some of which, such as Factor XIII (Greenberg & Schuman, 1982; Janus *et al.*, 1983) and plasminogen (Garman & Smith, 1982; Lucas *et al.*, 1983), interact with fibrinogen, fibrin or both. This is in

Fig. 2. Variation in the birefringence, Δn , normalized to fibrinogen concentration, c (2.1 mg/ml), as citrated human plasma was clotted at 37 °C in a constant magnetic field of 6 T after addition of the venom enzymes indicated at the concentrations shown

(a) and (c) are without re-addition of Ca^{2+} , and for (b) and (d) re-addition of Ca^{2+} (10 mM) and addition of enzyme were simultaneous. The $\cdots$ curve in (b) was obtained by adding thrombin (0.1 unit/ml) and shows that the linear phase of fibrin growth is very slow to appear: cf. the $\cdots$ curve in Fig. 3. The decrease in the final birefringence as the enzyme concentration is increased results from a decrease in orientation (confirmed with the optical microscope) due to the faster rate of polymerization. The lag period after the re-addition of Ca^{2+} without addition of enzyme was greater than 60 min for this plasma.

agreement with similar experiments performed with thrombin (Torbet, 1986), but those results are less clear because of the high rate of thrombin inactivation by plasma antithrombins.

Fig. 3 (— curves) shows the polymerization progress curves resulting from the combined action of reptilase and endogenous thrombin. Shape differences between these curves and those obtained without the re-addition of Ca^{2+} (Fig. 3, ---- curves) are evident even at short lag periods, and become more pronounced as the lag period is increased, probably because there is more time for both thrombin formation and action. When re-addition of Ca^{2+} only is performed, fibrin assembly takes place at an approximately linear rate from the end of the lag period to completion (Torbet, 1986), which respectively occur at about 27 and 50 min for the plasma used to obtain Fig. 3. Thus, although the curves obtained with reptilase in the presence of re-added Ca^{2+} can be broadly described as being made up of different proportions of the separate reactions, this does not provide an adequate description, because, with similar lag periods, polymerization actually takes place more slowly in the immediate post-gelation region in the plasma to which Ca^{2+} had been re-added (Fig. 3). This implies that the activation of the cascade temporarily inhibits either polymerization or fibrin monomer formation. There is no prior evidence to suggest that the activity of reptilase (Stocker & Barlow, 1976) or ancrod (Nolan *et al.*, 1976) is altered during coagulation, nor have they been reported to affect the activity of any coagulation factor. Also, their slow inhibition by α_2 -macroglobulin (Pitney & Regoeczi, 1970; Egberg,

Fig. 3. Variation of the birefringence, Δn , normalized to fibrinogen concentration, c (2.3 mg/ml), as citrated human plasma was clotted at 37 °C in a magnetic field of 6 T after addition of reptilase to the concentrations (units/ml) shown

— curves are with simultaneous addition of enzyme and re-addition of Ca^{2+} (10 mM). ---- curves are without re-addition of Ca^{2+} . The enzyme concentrations were adjusted, except for the 0.12 unit/ml sample, to obtain lag periods similar to those obtained with the re-addition of Ca^{2+} . The $\cdots$ curve was obtained with thrombin (0.1 unit/ml) and re-added Ca^{2+} (10 mM). After the re-addition of Ca^{2+} only, the progress curve was also nearly linear, and the lag period was 27 min and the completion time was about 50 min.

Fig. 4. Neutron-diffraction pattern from human fibrin produced by reptilase at 20 °C in a magnetic field of 10 T

The gel was 0.2 cm thick and in buffer in $^2\text{H}_2\text{O}$. The sample-to-detector distance and wavelength were respectively 140 cm and 1.0 nm. The meridional reflexions are the first and third orders of the fibre axial repeat, 22.5 nm. There is a weak equatorial spacing at approx. 9.0 nm, and another appears at 18.0 nm when the detector is moved to 280 cm so that the low-angle diffraction becomes better resolved. Contour levels are 3.0, 1.5, 1.0, 0.7 and 0.45. The horizontal bar corresponds to $3 \times 10^{-2} \text{ nm}^{-1}$. The direction of the field, H , and orientation of the fibres are shown.

1974) was not reported to be dependent on activation of the coagulation cascade. Thus no explanation, benefiting from corroborative data, can be proposed for the decrease in the rate of polymerization after gelation in citrate-treated plasma containing re-added Ca^{2+} to which reptilase has been added.

In all conditions studied, except for pure fibrinogen solutions (i.e. no Ca^{2+} or albumin added), the gels appeared to be nearly fully aligned in the polarizing microscope. Also, the final birefringence is similar to that obtained with thrombin in comparable conditions (Torbet, 1986), so that the thickness of the fibres formed cannot be very different (Hantgan *et al.*, 1980).

Fibrin structure

The low-angle neutron-diffraction pattern obtained from magnetically oriented human fibrin formed by reptilase catalysis is shown in Fig. 4. The first and third orders of a 22.5 nm axial repeat are visible, there is a weak equatorial peak at about 9.0 nm and there are off-equatorial reflexions on the first-order layer-line. A second equatorial peak appears at 18.0 nm when the resolution in the low-angle region is improved. This diffraction pattern is virtually identical with that given by human fibrin, which indicates that the axial repeat (Hantgan *et al.*, 1980) and also the lateral packing within fibres are constant. Thus at low resolution the fibre

structure does not depend on whether fibrinopeptide A only or both fibrinopeptides A and B are released. The position of the maxima are within experimental accuracy the same as was obtained from bovine fibrin (Torbet *et al.*, 1981; Freyssinet *et al.*, 1983), although the quality of Fig. 4 is inferior, owing to poorer orientation and weaker neutron flux. However, the first and third orders of the axial repeat from bovine fibrin have a similar peak intensity, whereas from human fibrin the third order is much weaker. Thus the packing arrangements in both fibrins are alike, but there is a difference in the distribution of scattering density along the fibre axis. This correlates with the different banding patterns seen in electron micrographs of negatively stained human and bovine fibrins (Williams, 1983).

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