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The Incipient Stage in Thrombin-Induced Fibrin Polymerization Detected by FCS at the Single Molecule Level

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We used fluorescence correlation spectroscopy (FCS) to study the activation of fibrinogen by thrombin and the subsequent aggregation of fibrin monomers into fibrin polymers at a very low and at physiological fibrinogen concentrations. In the labeling procedure used the fibrinogen was randomly labeled and the label was bound to the fibrinopeptide A and/or to the part of fibrinogen which after activation takes part in fibrin formation. We measured a diffusion coefficient for fibrinogen of $2.48 \times 10^{-7} \pm 0.10 \times 10^{-7}$ cm^2/s. After activation with thrombin both fibrinopeptide A and fibrin polymerization products could be demonstrated. From our findings we suggest a model for the formation of a three-dimensional network as two parallel processes, elongation and branching and that fibrin oligomers are not only intermediates in the polymerization process but also are substrates for branching.

The activation of fibrinogen by thrombin and the following polymerization of the fibrin monomers is the key event in humoral hemostasis (1, 2). Fibrinogen is a homodimeric protein with a molecular weight of 340 kDa, in which each half-molecule is composed of one Aα, one Bβ and one γ chain. The six chains are interconnected by 29 disulfide bonds. The two half-molecules are joined by three disulfide bonds close to their N termini. The tertiary structure of the whole fibrinogen molecule has not been determined yet, but electron micrographs (3), atomic force microscopy (4) and other methods suggest an elongated molecule with three domains. The three chains of each half-molecule reach out, in opposite directions, away from a central domain, fragment E, in a coiled coil fashion reaching two distal domains, fragment D. The tertiary structure of fragment D has been determined by X-ray crystallography (5, 6). The Bβ- and γ-chains end with their C-termini in fragment D whereas the α-chain makes a turn and folds back towards the center of the molecule. Thrombin activates fibrinogen by cleaving off two N-terminal peptides from the Aα and Bβ chains, fibrinopeptide A (FPA) and fibrinopeptide B (FPB). The conversion of fibrinogen into fibrin monomer can be described as $(A\alpha B\beta\gamma)_2 \rightarrow (\alpha\beta\gamma)_2 + 2$ FPA $+ 2$ FPB. Fibrin monomers polymerize and the polymers interact and form eventually a hydrated network, the fibrin gel. FPA is released faster ($k_{cat}/k_M = 11.6\ \mu M^{-1}\ s^{-1}$ (7)) than, and before, FPB ($k_{cat}/k_M = 0.2\ \mu M^{-1}\ s^{-1}$ (7)). The release of FPA is sufficient for the formation of a fibrin gel. Half-activated fibrinogen molecules have been isolated (8), i.e. fibrinogen from which only one FPA has been removed, but they do not seem to aggregate. The release of the second FPA occurs at a rate several times faster than the first FPA (8). The removal of FPA and FPB exposes polymerization sites called "A" and "B," respectively, which can interact with two complementary sites called "a" and "b." Site a is known to be located in the γ chain domain of fragment D and site b is most likely also located in fragment D (1). The A:a interaction causes longitudinal polymerization of the fibrin monomers in a half-staggered fashion, forming a protofibril. However, the half-staggered model has been disputed (9). The B:b interactions are thought to enhance lateral growth and/or branching of the protofibrils. The early stage of fibrin polymerization was studied by molecular sieving techniques (10, 11, 12, 13), chemical modifications (14, 15, 16), electron microscopy (17, 18, 19), dynamic light scattering (20) and small angle neutron scattering (21), where the fibrin polymerization was analyzed in bulk measurements, except for electron microscopy. Today, fluorescence correlation spectroscopy (FCS) is one of the most sensitive methods for single molecule detection. We are able to observe by FCS different species involved in the incipient polymerization process of fibrin.

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MATERIALS AND METHODS

Fibrinogen. The method of purifying human fibrinogen followed the method of Blombäck (22) with the exception that the starting material was Cohn's fraction I paste obtained from the Finnish Red Cross. The lyophilized fibrinogen was dissolved in bidistilled water and then filtered through a 0.8 μm filter to remove any large particles. After reconstitution of the lyophilized substance the solution contained fibrinogen at 20 mg/ml in 0.3 M NaCl. It was then dispensed in small fractions and stored at −80°C until used.

Thrombin. Bovine thrombin was a kind gift from Dr. B. Blombäck, Karolinska Institute, Stockholm, Sweden. The thrombin was stored lyophilized in sealed ampoules and was reconstituted just prior to use. The activity of the stock solution was 128 NIH units per ml.

Fluorescent dye. Cy5 (monofunctional) was purchased from Amersham Life Science, UK. The dye was dissolved in water free dimethyl sulfoxide (DMSO) obtained from Sigma, USA, at a concentration of 1 μg/μl. This solution was stored at −80°C.

Other reagents. All salts used were purchased from Merck, Germany (HPLC grade).

Protein determinations. Protein was determined according to Lowry (23) using bovine serum albumin (BSA) as standard.

Cy5-labeling of functionally active fibrinogen for single molecule detection. The labeling mixture consisted of 950 μl of 0.5 M $NaHCO_3/Na_2CO_3$, pH 9.3, (labeling buffer), 1 mg of fibrinogen and of dye at a molar ratio (dye/fibrinogen) of 0.43. The mixture was incubated for 15 minutes at room temperature. To remove the bulk of the free dye the labeling mixture was submitted to size exclusion chromatography on a PA 6 column, BioRad, USA, equilibrated with three column volumes of 25 mM Tris, 25 mM Imidazol and 100 mM NaCl, pH 7.4, (elution buffer). After application of the sample, the column was eluted with the elution buffer and fractions of 0.5 to 0.7 ml were collected manually. From each fraction was taken a sample of 120 μl which was then diluted five times in the elution buffer and the absorbance was measured spectrophotometrically at 280 nm, in a Pharmacia Biochrom 4060 spectrophotometer, Pharmacia, Sweden. An absorbance spectrum from 250 nm to 700 nm of the diluted sample of the main fraction was performed as a simple check for aggregation. In the presence of aggregates the baseline increased due to light scattering at a wavelength above 320 nm. To remove the very last of the dye a second size exclusion chromatography was performed, 200 μl of the peak fraction from the first size exclusion chromatography was applied to a Superose 6 column, Pharmacia, connected to a Pharmacia FPLC system. Before application the column was equilibrated with two volumes of elution buffer. The flow rate was 0.25 ml/min and 0.75 ml fractions were collected. The chromatogram showed one sharp peak at an elution volume of 11.2 ml, before this peak a less well defined peak emerged which had a height of less than four percent of the main peak. The fraction containing the main peak was used for the further studies. The overall yield was 9–11.5% with regard to protein.

Coating of the measurement chambers for FCS. We coated the measurement chamber to prevent any decrease in number of particles due to adsorption to the chamber walls during measurement. The chambers, Chambered Coverglass, Nunc, were incubated with BSA, 0.1 mg/ml in bidistilled water, for five hours at 37°C. After incubation the BSA solution was discarded and the chambers were dried for at least 30 minutes at 37°C.

Thrombin activation of fibrinogen. The labeled fibrinogen prepared as described above was diluted ten times in the elution buffer prior to measurements. 200 μl of the diluted sample was placed in a measurement chamber and five consecutive FCS measurements were performed. After these initial measurements thrombin was added to a final concentration of 2 NIH units/ml. Fifteen seconds after addition of thrombin measurements were made every minute for five minutes. One hour and twenty minutes after thrombin addition, another series of measurements was made.

FCS and data evaluation. The FCS setup used for autocorrelation measurements at the excitation wavelength of 633 nm in this study was based on the instrument ConfoCor, Zeiss/Evotec, Germany, as described previously (24, 25). The emission was recorded at 670 nm. When making FCS studies on large molecules, i.e. molecules with high diffusion times such as fibrinogen, care has to be taken of the laser power for excitation. This is because too high excitation intensity will cause photodestruction of the dye bound to slowly diffusing molecules (26). In this study we tried several different average excitation intensities and used the lowest intensity which still gave a good signal to noise ratio. The measurement time was 60 or 55 seconds. The theoretical expression of the intensity autocorrelation function G(t) describing the diffusion in a 3D-Gaussian volume element for three components (molecular species) is

$$G(\tau) = 1 + \frac{1}{N}\left(\frac{1-y-z}{\left(1+\frac{\tau}{\tau_{D1}}\right)\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\times\frac{\tau}{\tau_{D1}}}} + \frac{y}{\left(1+\frac{\tau}{\tau_{D2}}\right)\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\times\frac{\tau}{\tau_{D2}}}} + \frac{z}{\left(1+\frac{\tau}{\tau_{D3}}\right)\sqrt{1+\left(\frac{\omega_0}{z_0}\right)^2\times\frac{\tau}{\tau_{D3}}}}\right) \quad [1]$$

where N is the mean number of fluorescent molecules in the volume element, τ_{D1}–τ_{D3} are the characteristic diffusion times of the labeled molecules, y and z are the fractions of component 2 and 3, respectively, and (ω_0/z_0) is the ratio of the transverse and longitudinal half-axes of the volume element. Eqn. [1] was fitted to the data of the FCS experiments. The parameter (ω_0/z_0) was determined from measurements using only free Cy5 and fixed to the obtained value when fitting the experimental data. The data evaluation was performed using the program FCS Access, Evotec, Germany, and home made software at our laboratory.

The diffusion coefficient, D, was calculated from the measured diffusion time using the equation

$$\tau = \frac{\omega_0^2}{4D} \quad [2]$$

The diffusion coefficient of Cy5 was assumed to be 2.8×10^{-6} cm^2/s, which is the value known for Rhodamine 6G, another fluorescent dye of comparable size (27).

The distribution of translational diffusion times $P(\tau_D)$ was analyzed by the CONTIN algorithm (28, 29)

$$G(\tau) = 1 + \frac{1}{N}\int_{\tau_D}\frac{P(\tau_D)}{1+\frac{\tau}{\tau_D}}\left(\frac{1}{1+\left(\frac{\omega_0}{z_0}\right)^2\frac{\tau}{\tau_D}}\right)^{1/2} d\tau_D \quad [3]$$

and regularized for the distribution function in order to avoid solutions of false components in addition to the dominating real components.

RESULTS

Detection of Single Fibrinogen Molecules

Due to the risk of formation of artificial aggregates and/or precipitation of the fibrinogen during the label-

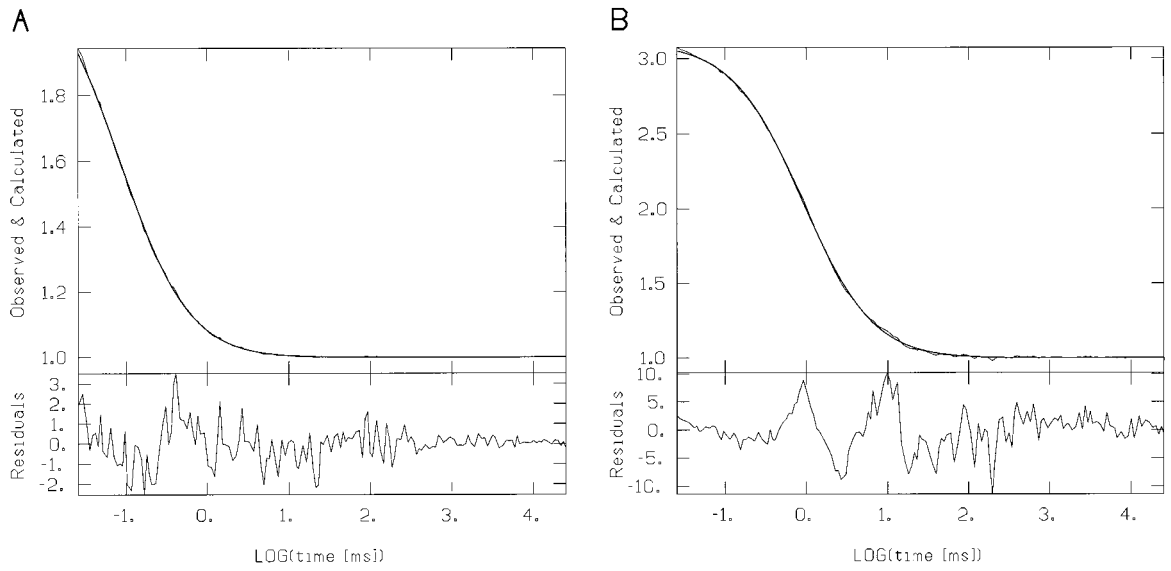

FIG. 1. (A) Free Cy5, the diffusion time is 0.085 ms. (B) Cy5-labeled fibrinogen. The diffusion time is 0.919 ms and the number of particles is 0.474. The free dye from the labeling reaction was completely removed by the preparative conditions described in Materials and Methods. The upper panels show the measured and the calculated autocorrelation curves, the lower panels show the difference between observed and calculated autocorrelation functions as weighted residuals.

ing and the separation of fibrinogen from free, unreacted Cy5 several different conditions were studied. The conditions tested were high (0.45 M) salt concentration in the elution buffer, exchange of NaCl for KBr, different molar ratios Cy5/fibrinogen in the labeling reaction, and addition of DMSO to the labeling buffer and the elution buffer. Once the aggregates were formed they could not be dissolved by using high salt concentration, chaotropic salt, or DMSO. Furthermore, formation of artificial aggregates was not a consequence of a too high labeling density. Instead, the conditions that influenced formation of artificial aggregates were the time and temperature of the labeling reaction. Using the conditions given in *Materials and Methods*, formation of artificial aggregates was completely suppressed (Fig. 1). Both FCS and spectrophotometry demonstrated the presence or absence of artificial aggregates. However, because of its high sensitivity, FCS could detect aggregates that could not be detected by spectrophotometry. Moreover, consecutive measurements of the same sample over extended periods of time did not show any decrease in number of particles when BSA coated chambers were used.

During the labeling reaction the dye bound covalently to amino groups on the fibrinogen molecule. The possible positions of the dye on the fibrinogen could only be on either the N-terminus of the Aα chain, which belongs to FPA, or on ϵ-amino groups of lysine on all chains or the N-terminus of the γ chain. It does not bind to the N-terminus of the Bβ chain as this has no free amino group, being a pyrrolidonecarboxylic acid residue (30). Given these conditions three cases were distinguished: (i) All dye bind to the N-termini of the Aα chains. (ii) All dye bind to $(\alpha\beta\gamma)_2$. (iii) Some dye bind to the N-termini of the Aα chains and some dye bind to $(\alpha\beta\gamma)_2$. In case of (i) the consequence of complete activation would be the same number of particles and no polymerization products being detectable, this was not supported by our experimental findings (see below) and was therefore excluded. In case of (ii) the consequence would be a decrease in number of particles and polymerization products being detectable but no faster diffusing species (i.e. FPA); the latter was not supported by our observations (see below). In case of (iii) the consequence would be the possibility to detect both polymerization products and a faster diffusing species. This was consistent with our experimental findings (see Fig. 2). When comparing the number of FPA molecules at 80 min after thrombin addition, 0.073 (in the volume element), with the number of fibrinogen molecules before thrombin addition, 0.474 (in the volume element), it can be concluded that about 15% of the dye molecules bound to FPA. The absence of a molecular species with the diffusion time of fibrinogen (or fibrin monomer) at 80 minutes after thrombin addition show that polymerization had occurred and that side chains essential for this process were not changed by the labeling procedure. The fibrinogen concentration in our experiments was 0.012 mg/ml (35 nM) as determined by protein determination and the average number of labeled fibrinogen molecules was 0.510/1.3 fL (0.82 nM), thus only one in approximately 42 fibrinogen molecules was carrying a label.

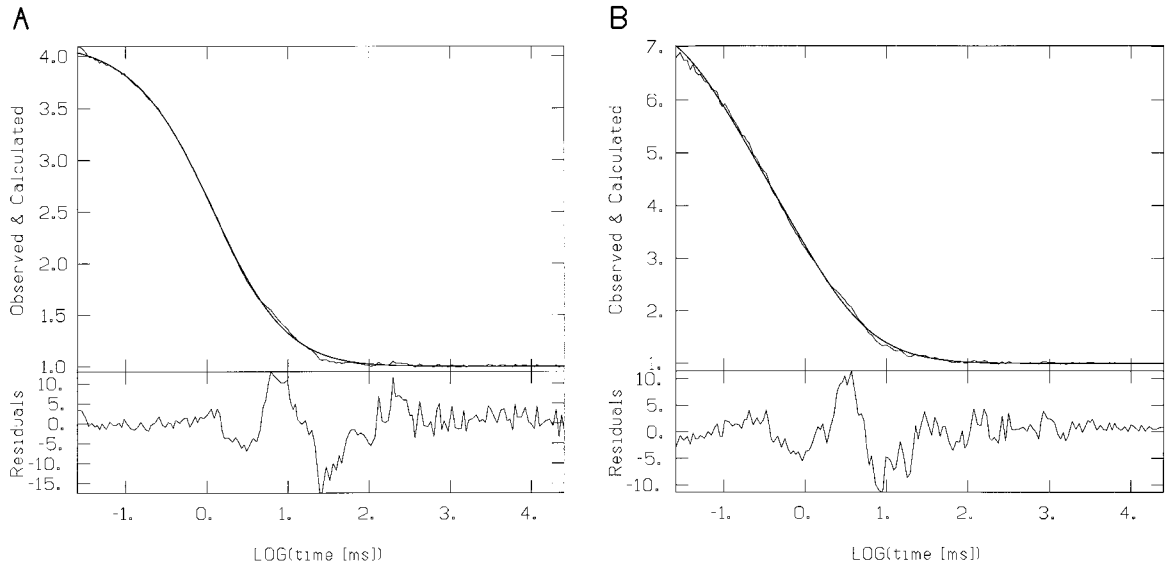

FIG. 2. (A) FCS measurement 4 minutes 15 seconds after thrombin addition. Here a three component model was used for the evaluation. τ_1 = 0.108 ms (0.018), τ_2 = 0.972 ms (0.209), and τ_3 = 2.371 ms (0.091); values within parentheses denotes the number of particles of the molecular species. (B) FCS measurement 80 minutes after thrombin addition. Here a two component model was used for the evaluation. τ_1 = 0.108 ms (0.073), τ_2 = 1.377 ms (0.075). The upper panel shows the observed autocorrelation curve together with the calculated, the lower panel shows the difference between observed and calculated as weighted residuals.

Diffusion Coefficient for Fibrinogen

The average diffusion time of fibrinogen from five consecutive measurements was 0.972 ms with a standard deviation of 0.039 ms (4.0%). This diffusion time corresponded to a diffusion coefficient of 2.48×10^{-7} $cm^2/s \pm 0.10 \times 10^{-7}$ cm^2/s (n = 5); this value is calculated from equation 2 using the average diffusion time of Cy5 (0.086 ms). One of the measurements is shown in Fig. 1. The diffusion coefficient for fibrinogen we report in this paper is slightly higher than that reported in refs. (20, 31), although the difference is not statistically significant. The difference could possibly be related to the use of a reference compound, i.e. free Cy5 dye, when calculating the diffusion coefficient.

Activation of Fibrinogen by Thrombin

After activation by thrombin the system was more complex, unlike the situation with fibrinogen alone, where only one molecular species was present. In our experiments three different molecular species could be observed. They are: (i) FPA, FPB can not be detected because it is not labeled. (ii) fibrin oligomers, long fibrin strands formed after thrombin addition could not frequently be seen because they precipitate, however, if they moved through the illuminated volume element during a measurement they gave rise to a significant increase in fluorescence intensity during several seconds, seen in the intensity profile (not shown). (iii) fibrinogen or fibrin monomers. Fibrinogen can not be distinguished from fibrin monomers because of the small relative difference in size (≈2%). In the study of complex systems with FCS, the different molecular species in the system are distinguished by their diffusion times, which are functions of their size and shape.

At 255 seconds after addition of thrombin, three molecular species could be observed. The diffusion time of 0.108 ms corresponded to the theoretical diffusion time of a molecule with the same molecular weight as FPA, when a globular shell model was used for calculation of the theoretical value. One species, 0.972 ms, corresponding to that of fibrinogen or fibrin monomer, and a third diffusion time, 2.371 ms, corresponding to fibrin oligomers. Furthermore a decrease in number of particles was seen, from 0.510 (in the starting situation) to 0.319 (Fig. 2A). At 80 minutes after addition of thrombin, two different molecular species could be distinguished, with diffusion times of 0.108 ms and 1.377 ms respectively. The number of particles was reduced to 0.155, Fig. 2(B). The diffusion time of 1.377 ms corresponds well to the theoretical value of a fibrin dimer according to the values reported by Larsson et al. (20) when taking into account the differences between their and our values for the fibrinogen monomer. The distribution of the diffusion times is depicted in Fig. 3, at 80 min after activation. The diffusion time component of FPA and a longer diffusion time component corresponding to oligomeric fibrin were obtained. In the measurements at 80 minutes after activation precipitated fibrin could sometimes be seen moving through the volume element as a several fold increase in fluorescence intensity during a few seconds (data not shown).

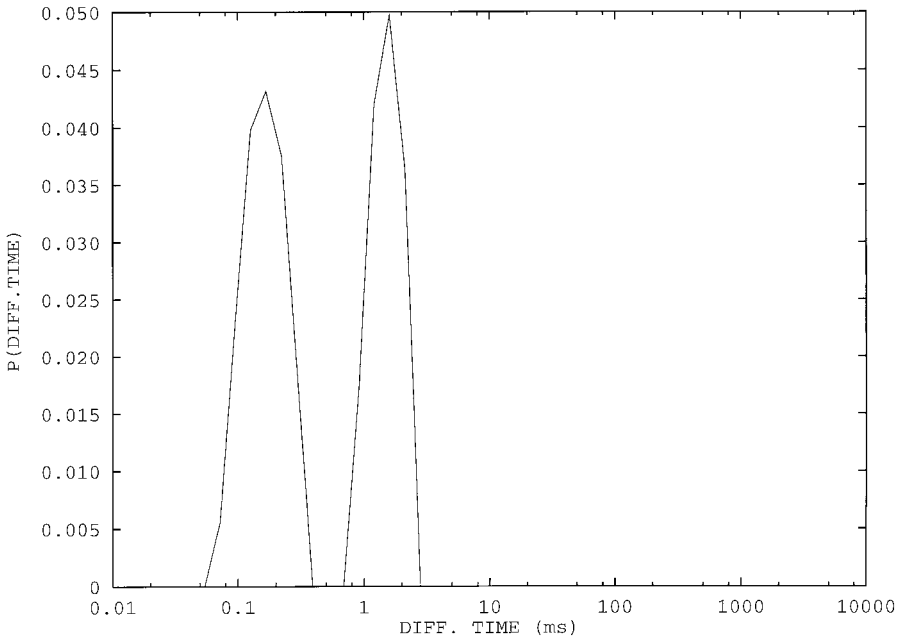

FIG. 3. Distribution of the translational diffusion times at 80 minutes after thrombin addition.

As the original fibrinogen concentration used in the above mentioned experiments was much lower than physiological, a series of experiments was also performed with the labeled fibrinogen diluted in the same buffer but with the addition of unlabeled fibrinogen to the buffer at 2 mg/ml. The thrombin concentration was the same as in the experiments without addition of unlabeled fibrinogen. These experiments yielded comparable results with a few exceptions. The most evident one was that a fibrin gel was formed in the reaction chamber. No slowly moving fibrin strands could be observed, this is because all formed polymeric fibrin is fixed in the network. The ratio fibrin oligomers/FPA was higher, approximately 9:1, when unlabeled fibrinogen was added instead of approximately 1:1, without addition of unlabeled fibrinogen. It should be noted that even when no unlabeled fibrinogen was added most of the fibrinogen molecules were not carrying a label. The number of oligomeric fibrin molecules present at 80 min after thrombin addition increased when unlabeled fibrinogen was added. The amount of oligomeric fibrin at 80 min after thrombin activation could thus be increased ca. 5-fold by the addition of ca. 160-fold excess of unlabeled fibrinogen.

DISCUSSION

Labeling of fibrinogen with Cy5 need special precautions to avoid formation of artificial aggregates, i.e. aggregates of fibrinogen molecules which are not activated by thrombin. The reaction time of labeling and the temperature at which it was performed were the crucial factors in avoiding formation of artificial aggregates. None of the other mentioned modifications, i.e. molar ratio of dye and protein, presence of DMSO, or use of chaotropic salts during size exclusion chromatography could suppress the formation of artificial aggregates. The labeling procedure used did not produce artificial aggregates, as proven by the following results: (i) The diffusion coefficient of our labeled fibrinogen is indistinguishable from that determined with dynamic light scattering (20) within the experimental errors. (ii) No sign of aggregates was seen in the intensity vs. time graph during the measurements. (iii) No turbidity was seen above 320 nm when a UV-vis wavescan was performed on the labeled fibrinogen. The disappearance, after activation with thrombin, of the molecular species with diffusion time corresponding to fibrinogen also shows that our labeled fibrinogen retains the ability to polymerize after thrombin activation.

The idea of half-staggered overlap (32) together with a trinodular starting unit (33) led to the model of interactions between the middle nodule of one unit and the distal nodule of another as shown in Fig. 4A (14, 34, 35, 36, 37). Kinetic models of fibrin polymerization (38, 39) suggest that the fibers grow in length when fibrin monomers are added one by one to the growing protofibril. Our findings of a relatively high number of diffusing fibrin oligomers at 80 minutes after activation does not fit in such a simple model, where, in the end stage, only large polymers would exist. From our finding of diffusing oligomers at the end stage of the polymerization process we suggest that the formation of

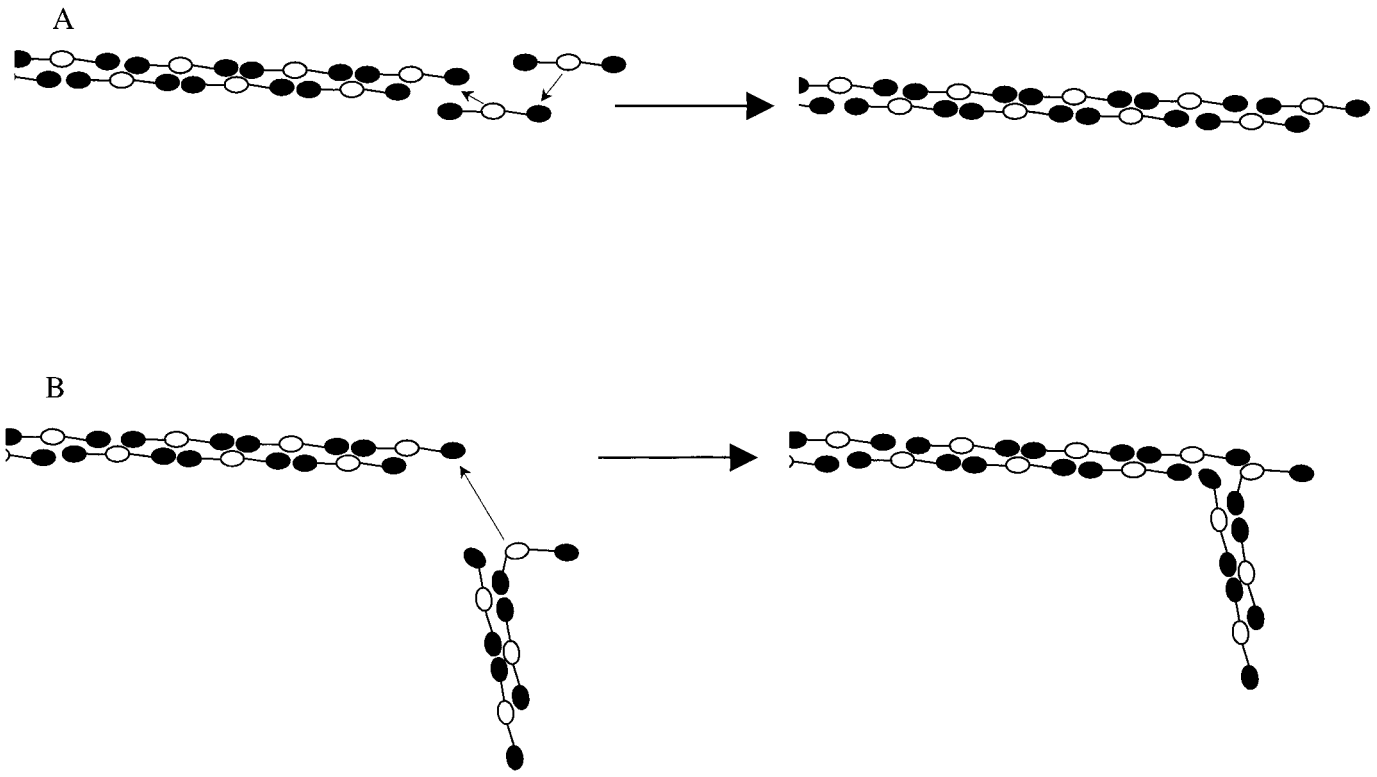

FIG. 4. Schematic view in the beginning of the two parallel processes. Panel (A) depicts the linear elongation of the fibrin strand and panel (B) our view of the formation of a branching point (node) the free ends of which can be further elongated as in panel (A). We are not able to give here kinetic data for the two parallel processes.

the three dimensional network is the result of two parallel processes using somewhat different substrates. The first process being elongation of a fibrin polymer by addition of single fibrin monomers and the second process being branching, requiring a fibrin polymer joining with a small fibrin oligomer (but not monomer). We therefore think that small fibrin oligomers have a more profound role than being merely intermediates between the activated monomer and full length protofibril. We assume that the so called three molecular branching points (2) are the most important for branching, this is supported by the finding that at most of the branching points (nodes) the number of connected polymer chains is three (40). We put forward the following role of fibrin oligomers in the polymerization process (see Fig. 4B): In the formation of the nascent network (nonlinear polymer chains) not only monomeric fibrin is involved as starting units. Small fibrin oligomers participate as the substrate for branching of the polymer chains. We suggest that their half-staggered ends are required for the formation of the three molecular branching points. The incorporation of small fibrin oligomers could occur at any time but if it should be in agreement with (41) it is in the beginning of the network formation. The amount of fibrin oligomers at a certain time in the beginning of the process would then dictate the degree of branching of the fibrin network. If we assume that formation of dimers requires a higher fibrin monomer concentration than elongation of an existing protofibril then the finding that shorter clotting times, i.e. higher activation rates, yields a more branched network (41), would be in line with our model. However, it could be argued that the diffusing oligomers we see at 80 minutes after thrombin addition is an experimental artifact caused by a subpopulation of our labeled molecules. We do not think this is the case because of the following. First, if a subpopulation of the labeled fibrinogen had impaired polymerization properties because of the attached dye molecule, the addition of cold fibrinogen would not change the ratio fibrin oligomers/FPA. On the contrary, there was a nine-fold increase in this ratio. Second, even though we do not know how many of the more than 200 lysine side chains in the fibrinogen molecule are available for coupling to the dye, we consider specific labeling of lysines close to the polymerization sites to be a low probability event.

Although most of our findings concerning fibrin polymerization are well in line with results by others, we suggest a special role of the fibrin oligomer in the polymerization process. The use of physiologically functional and fluorescently labeled fibrinogen together with FCS also aids other fields of study, e.g.

search for fibrinogen binding proteins and study of fibrinogen receptors.

CONCLUSION

Models describing linear polymerization can not explain the presence of oligomers as end products, as we find in this study. Models for the polymerization of fibrinogen, although not presented as being complete, do not explain the branching phenomenon. We propose that the oligomers formed during the initial phase of fibrin polymerization are necessary substrates for the branching process, and that their numbers determine the degree of branching.

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