Transient Intermediates in the Thrombin Activation of Fibrinogen

EVIDENCE FOR ONLY THE desAA SPECIES*

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The structure of a fibrin gel depends on the nature of the fibrinogen activation products produced by thrombin and the physical condition under which assembly occurs. Two different structures of the intermediate fibrin protofibril have been proposed, the production of which requires different extents of fibrinopeptide A (FpA) cleavage from fibrinogen. The fibrin activation intermediates must be stable since time is required for the intermediates to diffuse to growing protofibrils. The classic Hall-Slayter model requires cleavage of both FpAs to form a desAA intermediate. The Hunziker model requires cleavage of only one FpA to form an AdesA intermediate. Electrophoretic quasi elastic light scattering has been used to show the time-dependent production of the relevant fibrinogen activation intermediates that includes desAA but not AdesA.

Since the first description of fibrin structure by Ferry and Morrison (1), controversy has existed over the exact mechanism of fibrin assembly. The issues include the order of release of the fibrinogen activation peptides, fibrinopeptide A (FpA)[1] and fibrinopeptide B (FpB), the importance of the extent of removal of fibrinopeptide A, the manner in which the fibrin monomers are organized into protofibrils, and the manner of bundling of the protofibrils into fibrin fibers. The complexity of the three-step assembly process including fibrinogen activation, protofibril formation, and fiber bundling lends itself to the diversity of fibrin structure originally noted by Ferry (2). The final fibrin structure depends on many factors such as the rate of monomer production, fibrin monomer concentration, the number of polymerization sites present on the fibrin monomer, pH, ionic strength, solution viscosity, the presence of other charged molecules, and volume exclusion effects (1, 3–8). FpA is released before FpB, and the release of FpA is sufficient to initiate fibrin assembly (9–11). Different models for fibrin assembly have been proposed. Support for the classical Hall-Slayter model (12) for fibrin assembly in which fully activated fibrin monomers with both FpAs cleaved (desAA) are added in an overlapping half-staggered manner to growing protofibrils has been derived from assembly kinetics, light scattering studies, and electron micrographs (13–15). A recent challenge to the Hall-Slayter model by Hunziker *et al.* (16) refutes the overlapping monomer sequence in the protofibril. Protofibril assembly in the Hunziker model requires the existence of a fibrin monomer with only one FpA removed (AdesA) in sufficient concentrations and with a sufficient lifetime to assemble a non-overlapping protofibril. The fundamental difference in these two models arises from differences in the protofibril structure, which depends on the predominance and stability of AdesA compared with desAA fibrin monomers. Report for the existence of the AdesA monomer is based on gel exclusion chromatography and electron microscopy (16–19). Other investigators using similar methods and peptide sequencing experiments do not find the AdesA fibrin monomer in either sufficient quantity or lifetime to be a significant factor in fibrin assembly (20–22).

It is the purpose of this report to apply a methodology that permits direct observation and evaluation in real time of the transient intermediates that form during the activation of fibrinogen. Electrophoretic quasi elastic light scattering (ELS) can resolve structural differences between fibrin monomers because of differences in the columbic charge on the different activation intermediates. Quasi elastic light scattering (QLS) without electrophoresis reports on changes in the diffusion coefficients resulting from differences in the mass of the molecule. Thus, ELS should be better suited to study activation products of fibrinogen activation than QLS, since the fibrinopeptide cleavage changes the charge substantially but does not reduce the mass appreciably.

MATERIALS AND METHODS

Fibrinogen Activation—Highly purified human band I fibrinogen (less than 10% band II) was used in these experiments (23). A working solution of 0.5 mg/ml stock fibrinogen was prepared from a freshly thawed fibrinogen stock solution at 0.3 M NaCl by diluting with 10 mM NaCl, 5 mM Hepes at pH 7.4. Contaminating dust and large fibrinogen aggregates in the working solution were reduced by filtration through a 0.22-mm filter followed by centrifugation at $48,000 \times g$ for 90 min. The final fibrinogen concentration was determined from the solution optical density at 280 nm in a Cary 3E UV/VIS spectrophotometer using an extinction coefficient of 1.6 ml/mg. Further dilution of the working solution to obtain a desired fibrinogen concentration was made with 10 mM NaCl, 5 mM Hepes at pH 7.4. Fibrinogen was activated with 0.005 NIH units/ml human α-thrombin (Sigma). A low concentration of thrombin was used to slow fibrinopeptide cleavage to a rate that could be observed in our experiment. The fibrinogen-thrombin mixture was sampled at various times (2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 60, and 90 min), and thrombin was inhibited by the addition of phenylalanyl prolyl arginine chloromethyl ketone (final concentration of 2.5 μM) at each stage. Fibrinogen quenched at various stages of activation was then examined in the ELS spectrometer for the presence of intermediate activation species.

Experiments to examine the release of only fibrinopeptide A were carried out using Atroxin, an enzyme derived from Bothrops atrox (Sigma). In these experiments, Atroxin was added at a final concentration of 1.25 ng/ml, in place of thrombin, which gave equivalent fibrin gelation times. Fibrinopeptide B removal was examined in a similar

[1] The abbreviations used are: FpA, fibrinopeptide A; FpB, fibrinopeptide B; ELS, electrophoretic quasi elastic light scattering; QLS, quasi elastic light scattering; ACV, *A. controtrix* venom; desAA, fibrin assembly in which fully activated fibrin monomers with both FpAs are cleaved; AdesA, a fibrin monomer with only one FpA removed.

experiment by adding an enzyme purified from Agkistrodon controtrix venom (Sigma) at 2 μg/ml. Although this enzyme removes predominately fibrinopeptide B, 30% fibrinopeptide A is also cleaved (9, 16).

Electrophoretic Quasi Elastic Light Scattering (ELS)—ELS measurements were made on a multi-angle quasi elastic light scattering spectrometer (DELSA 440, Coulter Electronics, Inc., Hialeah, FL) mounted on a Newport vibration isolation table. Simultaneous measurements were made at four different scattering angles. The electrophoretic effect was obtained by superimposing a uniform electric field (usually 150–500 volts/cm) across the sample. The field was pulsed and its polarity alternated to avoid mass accumulation. The scattered intensity (I_s) from a moving particle at a fixed scattering angle (θ_s) is observed as an oscillating intensity described in the heterodyne experiment as a second order field autocorrelation function (24–26),

$$G^2_{Lhet}(\tau) = I^2_L + 2I_L <I_s> cos(K \cdot v_d \tau)e^{-DK^2\tau} \qquad \text{(Eq. 1)}$$

where τ is the time increment, I_L is the intensity of the reference beam (local oscillator), and $<I_s>$ is the average intensity of the scattered light. K is the scattering vector defined by Equation 2 where θ_s is the scattering angle, n is the refractive index, λ is the wavelength of the incident light, v_d is the velocity of the scattering particle, and D is the diffusion coefficient. The important quantity in this expression is $K \cdot v_d$, the Doppler shift of the signal resulting from the particle motion.

$$K = \frac{4\pi n \sin(\theta_s/2)}{\lambda} \qquad \text{(Eq. 2)}$$

The magnitude of the Doppler shift is determined from the power spectrum, which is calculated from the Fourier transform of the autocorrelation spectrum. The Doppler shift can then be related to the electrophoretic mobility by Equation 3,

$$\delta v = [2v_o v_d \sin(\theta_s/2)]/C \qquad \text{(Eq. 3)}$$

where δv is the Doppler shift, v_o is the frequency of the incident light, and C is velocity of light in the medium. The electrophoretic mobility is related to the velocity of the scattering particle by the simple equation, $v_d = \mu E$, where μ is the electrophoretic mobility and E is the applied electric field (25). Temperature, ionic strength, pH, and conductivity affect the electrophoretic mobility of the scattering particle and were therefore carefully controlled by monitoring the conductivity of each sample. Joule heating was governed by regulation of the pulse duration and the pulse frequency of the electric field. Thermal lensing was avoided by control of the incident laser power. Snell's law corrections were made for all scattering angles.

RESULTS

Fig. 1 shows the changes in the electrophoretic mobility spectrum for fibrinogen at a concentration of 0.05 mg/ml (*peak A*) at intermediate times during a 90-min activation by thrombin. At 10 min, a new peak (*peak B*) with a mobility of −0.6 (μ-cm)/(V-s) is observed. At 25 min, the electrophoretic spectrum shows the appearance of a third peak (*peak C*) with a mobility of −1.0 (μ-cm)/(V-s). Both peaks B and C continue to increase in intensity with time as shown by the spectra at 40 and 60 min. Other contributions to the mobility spectra are seen at higher mobilities. These experiments show activation intermediates at times of 10, 25, 40, and 60 min. Under conditions of very dilute thrombin concentrations, peak B emerges well ahead of peak C.

In Fig. 2, the effect of thrombin, which removes both FpA and FpB, is compared with Atroxin, which removes only FpA. Thus, by using Atroxin, a homogeneous desAA can be produced. The experimental conditions are identical to those used in Fig. 1. The 25-min mobility spectrum using thrombin from Fig. 1 has been superimposed and is shown as a *dashed line* so that assignment of peak B can be identified as removal of fibrinopeptide A, yielding desAA. The same fibrinogen mobility (*peak A*) is observed in both the thrombin and Atroxin experiments. The difference occurs in the absence of peak C. Since Atroxin, the *solid curve* in Fig. 2, only releases fibrinopeptide A and since the morphology of the single new peak is symmetric, the new mobility shown as peak B must represent the desAA fibrin
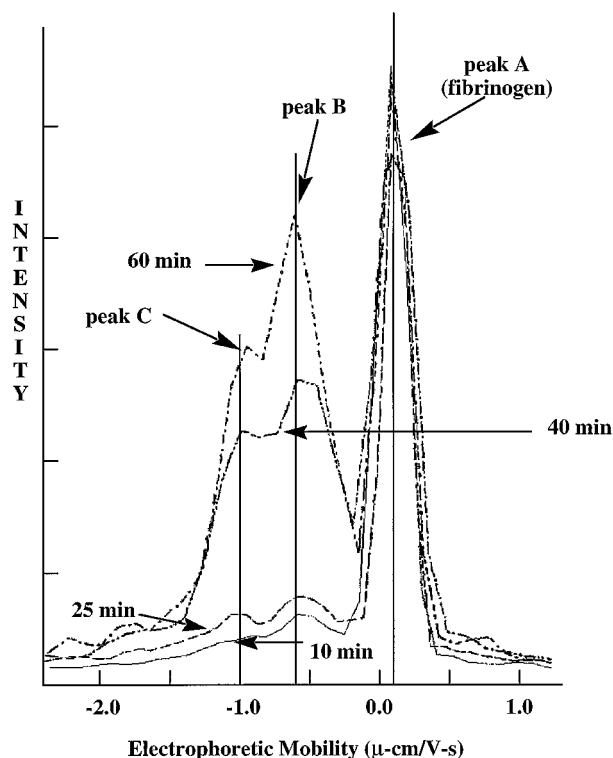

FIG. 1. **Detection of fibrinogen intermediates.** ELS spectra of fibrinogen and fibrinogen activation intermediates generated by the addition of 0.005 NIH units/ml of human α-thrombin and sampled at 10, 25, 40, and 60 min. Thrombin removes both FpA and FpB from fibrinogen. *Peak A* is fibrinogen. *Peaks B* and *C* represent activation intermediates of fibrinogen. Note that the appearance of peak B precedes that of peak C, which provides further support that FpA is the first fibrinopeptide released and that desAA fibrin monomer is the stable intermediate.

intermediate. Some investigators have postulated the existence of an AdesA fibrin intermediate. The inset shows a plot of the line width at half height, Γ, for peak B *versus* the scattering vector, *K*, and confirms that the line broadening is due to diffusion ($\Gamma/2 = K^2_D$) and not sample heterogeneity ($\Gamma/2 = K_D$) (24). Thus, contribution to peak B from AdesA is highly unlikely. Furthermore, since the mobility difference between peak A for native fibrinogen and peak B for desAA is so large, if AdesA was present, it should appear as an easily identifiable peak between peak A and peak B. Since no peaks are observed in this region of the mobility spectra, and since we can show the appearance of peak B ahead of peak C (see the spectrum for the 10-min sample time in Fig. 1), we take this as evidence that AdesA does not exist as a significant intermediate. If it exists at all, it must be extremely short lived or at a very low concentration. These observations are consistent with the observation of Janmey *et al.* (14, 15, 27) that the second FpA is removed 16 times faster than the first FpA, suggesting that the possibility of a stable AdesA intermediate is low.

To identify the species responsible for the mobility represented by peak C produced by the thrombin activation of fibrinogen shown in Fig. 1, the following experiments were performed. The experiments shown in Fig. 3 are identical to those shown in Figs. 1 and 2 except that fibrinogen is activated by an enzyme from the venom of the Southern copperhead, *Agkistrodon controtrix*, which cleaves FpB at a much faster rate than FpA. Again, the 25-min spectrum from Fig. 1, shown as a *dashed line*, has been superimposed so that the identification of FpB removal can be established. As expected, no desAA peak (*peak B* in Fig. 1) is observed because the FpB is removed first. A new peak, peak C′, with a slightly slower mobility than the peak C from Fig. 1, is
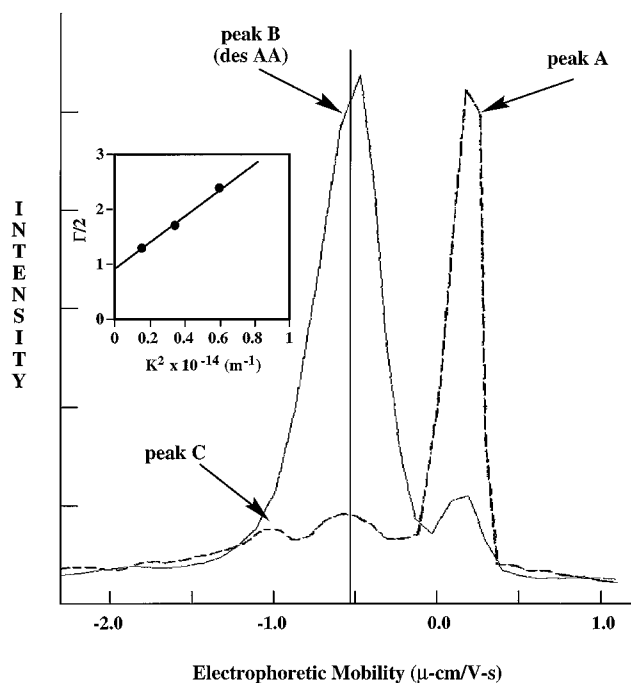
FIG. 2. **Identification of peak B as the desAA intermediate.** ELS spectra of fibrinogen (0.05 mg/ml) incubated with 1.25 ng/ml Atroxin (*solid line*), which removes FpA. Note that only one new peak is observed. Thus, Peak B is identified as desAA by the superimposition of the 25-min spectra from Fig. 1 (*dashed line*). The *inset* shows a plot of the line width at half height, Γ, *versus* the scattering vector, K, and confirms that the line broadening is due to diffusion ($\Gamma/2 = K^2_D$) and not sample heterogeneity ($\Gamma/2 = K_D$) (24). If AdesA was present, it would be seen as a specific mobility between peaks A and B. No mobility is observed in the region between peaks A and B.

produced. The activation product from *A. controtrix* should produce both desBB and desAAdesBB. Additional help to resolve the assignment of peak C was obtained from the production of a homogeneous desAAdesBB generated by first treating fibrinogen with Atroxin followed by *A. controtrix* venom, which had identical mobility with peak C from Fig. 1 (data not shown). Linewidth analysis on peak C generated by both venoms, *i.e.* desAAdesBB, and from thrombin (Fig. 1) both have a K^2 dependence, confirming a single species identified as desAAdesBB. Linewidth analysis of the slower mobility peak C′ from Fig. 3 shows a K dependence, indicating a mixture of species (see Fig. 3, *inset*). The conclusion from these experiments is that peak C represents desAAdesBB and is supported by the fact that we do not expect to find desBB in thrombin activation. These results also suggest that desAAdesBB has a faster mobility than desBB. Finally, peak C in Fig. 1 is definitely not AdesA, since it would be highly unlikely that removal of one FpA would produce a faster mobility than removal of both FpAs and since no peak C is seen in Atroxin activation. When both desBB and desAAdesBB are present, a slower peak C′ is seen that results from contamination by the slower moving desBB.

The experiments shown in Fig. 4 are identical to those shown in Fig. 1, except that the concentration of fibrinogen is higher, 2 mg/ml, since it represents the normal human plasma concentration of fibrinogen and is also identical to the fibrinogen concentration used by Smith (17) and Hunziker *et al.* (16). Under these experimental conditions, the activation rate is thrombin limited. As shown in Fig. 4, an activation profile is seen similar to that in Fig. 1 with no evidence for AdesA.

DISCUSSION

Fibrin assembly is initiated by thrombin cleavage of the N-terminal Aα-chain, fibrinopeptide A, which exposes one of
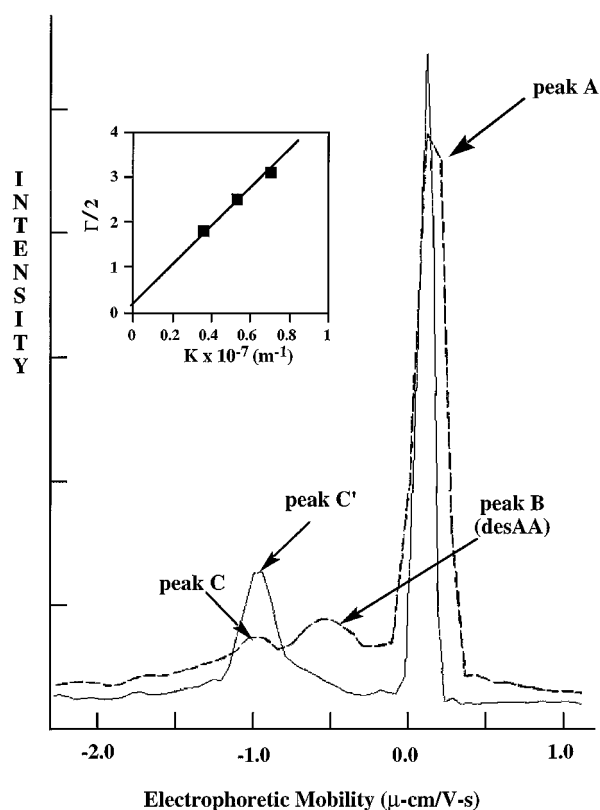

FIG. 3. **Effect of fibrinopeptide B removal on peak C.** ELS spectra of fibrinogen (0.05 mg/ml) incubated with 2.0 μg/ml *A. controtrix* venom (ACV) (*solid line*). Although ACV removes both FpA and FpB, FpB is removed at a much greater rate than FpA. Linewidth analysis of peak C from Fig. 1 shows a K^2 dependence and indicates a single species, desAAdesBB. When superimposed on Fig. 1, peak C generated by both Atroxin and ACV, also desAAdesBB, has an identical mobility to peak C in Fig. 1 (data not shown). In contrast, peak C′ generated with ACV alone (shown in this figure) has a slightly slower mobility. The *inset* shows the linewidth of peak C′ is dependent on K, which indicates more than one species is present in peak C′. ACV is known to produce desBB and desAAdesBB. Thus, the slightly slower moving peak C′ generated with ACV alone results from the presence of desBB in peak C′ and indicates that desBB has a slower mobility than desAAdesBB.

the two polymerization site "A's" on the E-domain (28–30). Structural information on the chemical nature of "A" polymerization site is limited, but His[16] on the Bβ-chain and contributions from α-chain are necessary for polymerization to occur (31–34). The polymerization "A" site on the E-domain interacts with the constitutively present "a" site on the D-domain of an adjacent monomer. In contrast to the "A" site, the critical amino acid sequence in the "a" site is better defined and located on the C-terminal γ-chain between amino acid residues 356 and 411 (30, 35–37).

Once the fibrin monomer is generated following FpA release, fibrin assembly ensues. The classical mechanism for fibrin assembly as described by the Hall-Slayter model suggests that fibrin monomers polymerize in a half-staggered manner so that the D-domain of one monomer interacts with the centrally located E-domain of the adjacent monomer to form protofibrils 2 monomers thick (12). In this model, polymerization symmetry permits monomer addition to either end of the growing protofibril (bipolar growth), but only if an "A" site faces the "a" site, which implies rotational symmetry about the minor hemi-axis (equivalent ends) but *not* about the major hemi-axis with respect to polymerization sites (38). An additional critical factor is the structural contribution of the dihedral angle present in fibrinogen that introduces helical structure to the protofibril. However, the exact details of the interaction and packing re-

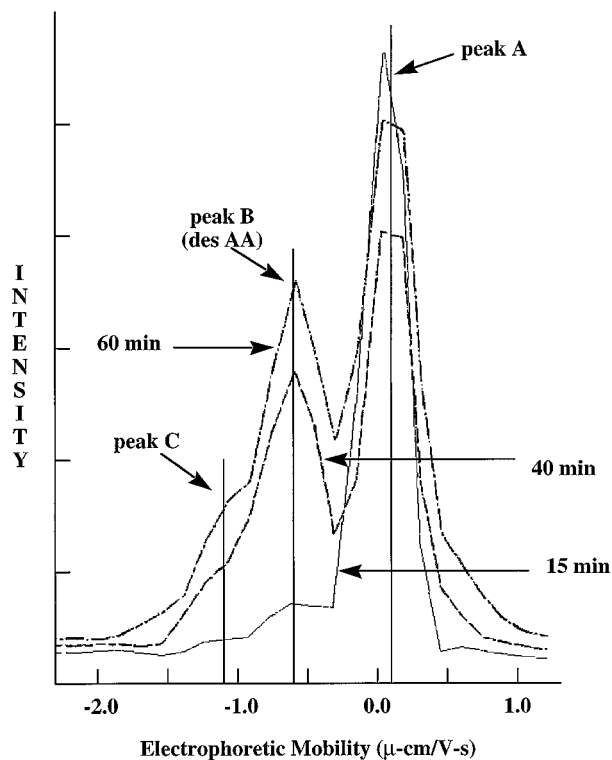*Detection of Fibrinogen Activation Intermediates*

FIG. 4. **Fibrinogen activation at physiologic concentrations of fibrinogen.** ELS spectra of fibrinogen at the normal physiologic concentration (2 mg/ml) and fibrinogen activation intermediates generated by addition of 0.005 NIH units/ml human α-thrombin sampled at 15, 40, and 60 min are shown. The designations of the peaks are the same as for Fig. 1.

main unknown (38).

Recently, the Hall-Slayter model for fibrin assembly has been challenged (16). A fundamental feature of the alternative model described by Hunziker is that only one D-domain from each fibrin monomer is involved in the initial polymerization process. The second D-domain is then left free to form branches. For this model to be possible, the second "A" polymerization site on the E-domain must not be activated, *i.e.* the AdesA species must predominate and be stable long enough to diffuse to the surface of the assembling fibrin protofibril. If the second FpA is cleaved, protofibril assembly will proceed in an overlapping bipolar manner as postulated in the Hall-Slayter model. Assembly requires close proximity of monomers and growing fibrin oligomers and sufficient time for both rotational and lateral diffusion to occur so that the correct spatial orientation occurs. Thus, the monomer must be stable long enough for diffusive processes to bring monomers and oligomers together. If the AdesA species is the important fundamental monomeric species, it must not encounter a second thrombin molecule that would result in the cleavage of its second FpA before its assembly into the growing fibrin protofibril. These critical interactions between monomer and growing oligomer are highly important in the determination of fiber assembly kinetics and structure (39).

Thus, a fundamental issue in the differentiation between the Hall-Slayter and Hunziker models is proof of the existence of the AdesA *versus* the desAA intermediate as the predominant species during fibrin assembly. The existence of a transient AdesA fibrin monomer is controversial and is dependent on the nature of interaction between thrombin and fibrinogen. For example, the AdesA could be produced through one thrombin bound for each FpA so that removal of each FpA is a temporally independent event. The desAA species could be produced by

either two thrombin molecules bound simultaneously to fibrinogen or by the sequential removal of FpAs by one bound thrombin molecule. In favor of the latter model, a 16-fold increased rate of removal for the second fibrinopeptide A has been observed (14, 15).

The existence of an AdesA intermediate in the early stage of fibrin formation was first proposed by Smith (17), and his analysis, based on N-terminal amino acid analysis of gel chromatography isolated fibrinogen activation intermediates, reported that the interior of fibrin oligomers was composed of desAA monomers but that the oligomer was capped by AdesA monomers. It is not clear from this model how desAA monomers can be added to the growing oligomer if each end is capped by an AdesA. Based on his analysis, Smith postulated that AdesA was the early predominant species. A similar finding also using chromatography to isolate fibrin intermediates was reported by Alkjaersig and Fletcher (18). Dietler *et al.* (19) used light scattering to arrive at a similar conclusion. It should be emphasized that quasi elastic light scattering gives highly accurate diffusion coefficients but only for monodispersed solutions (40). Sample heterogeneity caused by fibrinogen or fibrin monomer aggregates will produce uncertainty in the result as evidenced by a large second moment in the cumulant analysis (40). More recently, Hunziker *et al.* (16) have used electron microcopy to examine fibrin oligomers that appear to contain AdesA intermediates. Electron microscopy studies offer the possibility of analyzing individual monomers and oligomers, but it is not clear if the drying process alters the monomers so that their original solution appearance is altered. Monomer aggregation artifacts may also occur during the drying process. Important species present in solution may not be represented in the observed species.

Other investigators do not find evidence for the AdesA fibrin monomer. Wilf and Minton (21) used gel permeation chromatography and only found desAA fibrin monomers. Wilf and Minton (21) also examined Smith's original proposed assembly mechanism and found that Smith's predictions did not agree with either their analysis or with Shainoff's sedimentation analysis (41). In addition, only desAA was observed by Janmey *et al.* (14, 27) in their light scattering studies. Henschen (22) was not able to detect AdesA intermediates using a more direct analysis of the FpA and amino acid sequencing of the central, dimeric fragments derived from the N-terminal region of all Aα-chains and α-chains present in the thrombin digest.

In this report, we have used light scattering to examine fibrinogen activation intermediates. However, we have avoided the problem of sample heterogeneity by adding electrophoresis to quasi elastic light scattering as described by Ware and Flygare (42). Differences between the molecular weight and hence the diffusion coefficient of fibrinogen and fibrin monomer using standard quasi elastic light scattering may not resolve subtle differences present in fibrinogen activation intermediates. On the other hand, substantial differences may be present in the molecular charge for each activation species, which would be highly sensitive to detection by electrophoresis. We view the results from QLS and ELS as complementary. We have shown that ELS is well suited for the study of fibrinogen activation and protofibril formation. ELS reports the surface charge of a particle and is observed as the electrophoretic mobility. The ELS method can measure the mobilities of a mixture of multiple particles with different structures and charges. In the present case, differences in the mobility of fibrin intermediates depend on small changes in the surface charge in activation intermediates. When the fibrinopeptides are removed and fibrin monomers are produced, only a small change in the molecular weight occurs, but a large difference in the

electrophoretic mobility occurs. In fact, the change is so large that the existence of the AdesA species would be easily observed between peaks A and B, which is not seen (Fig. 1). We have also used limiting concentrations of thrombin to enhance detection of the AdesA intermediate, if it is present. In separate experiments, physiologic concentrations of fibrinogen were examined (Fig. 4). Our data strongly support the hypothesis that desAA fibrin monomer is the significant intermediate in fibrin assembly. If AdesA does exist, its lifetime and concentration are insufficient to exert a significant role in fibrin assembly.

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