Decreased Lateral Aggregation of a Variant Recombinant Fibrinogen Provides Insight into the Polymerization Mechanism†

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ABSTRACT: We analyzed the polymerization of BβA68T fibrinogen, the recombinant counterpart of fibrinogen Naples, a variant known to have decreased thrombin binding. When polymerized with equal thrombin concentrations, BβA68T fibrinogen had a longer lag time and lower rate of lateral aggregation, V_{max}, than normal recombinant fibrinogen, but a similar final turbidity. At thrombin concentrations that equalized the rates of fibrinopeptide A release, BβA68T fibrinogen polymerized with a lag time and V_{max} similar to normal, but reached a significantly lower final turbidity. Similar results were produced when BβA68T was polymerized with Ancrod, which cleaves fibrinopeptide A at the same rate from either fibrinogen, and when BβA68T desA monomers were polymerized. The polymerization of desAB fibrin monomers, which circumvents fibrinopeptide release, was the same for both fibrinogens. We confirmed that turbidity was indicative of fiber thickness by scanning electron microscopy of fibrin clots. Here, we present the first experimental evidence of fibrin polymerization with a normal period of protofibril formation and rate of lateral aggregation, but with a significantly decreased extent of lateral aggregation. We conclude that the decreased lateral aggregation seen in BβA68T fibrinogen is due to an altered step in the enzymatic phase of its polymerization process. We propose that during normal polymerization a subtle conformational change in the E domain occurs, between the release of FpA and FpB, and that this change modulates the mechanism of lateral aggregation. Without this change, the lateral aggregation of BβA68T fibrinogen is impaired such that variant clots have thinner fibers than normal clots.

Fibrinogen is a 340 kDa plasma protein that consists of two sets of three polypeptide chains, Aα, Bβ, and γ. Fibrinogen is a trinodular molecule with two distal nodules, called D, containing the C-termini of the β and γ chains, and one central nodule, called E, containing the N-termini of all six chains. To initiate polymerization, the serine protease, thrombin, cleaves the N-terminal 16 residue peptide, FpA,[1] from the Aα chain. Cleavage of FpA exposes the "A" polymerization site, which noncovalently interacts with an already exposed "a" polymerization site in the D nodule of another molecule. This A:a interaction results in spontaneous polymerization, which leads to the formation of half-staggered, double-stranded protofibrils (*1*). As these protofibrils grow in length, thrombin cleaves the Bβ chain, releasing the N-terminal 14 residue peptide, named FpB, to expose the "B" polymerization site (*2*−*4*). The "B" polymerization site presumably interacts with the "b" site, located within the D nodule of another molecule (*5*), in a fashion similar to the A:a interaction. This B:b interaction was first proposed to be responsible for the lateral aggregation of protofibrils by Blomback et al. (*2*), although the mechanism for this interaction is still not well understood. The end result of this two-step polymerization process is the formation of a complex, branching network of fibers.

Previous studies have shown that the polymerization of fibrin can occur with the removal of only FpA, by Ancrod, to form desA fibrin, or only FpB, by Agkistrodon Contortrix Thrombin-like Enzyme, to form desB fibrin. DesA fibrin consists of thinner fibers than desAB fibrin (*6*), and polymerization of desB fibrin only occurs at low temperatures (14 °C) (*7, 8*). While conditions for the formation of these clots differ from normal, the fact that clots are formed emphasizes that the release of both FpA and FpB is not *required* for polymerization, but rather that the release of both fibrinopeptides is necessary for optimal polymerization. Thus, the formation and lateral aggregation of protofibrils are not specific to the release of either fibrinopeptide, but rather all factors necessary for polymerization are present in fibrin(ogen) even when only one fibrinopeptide is cleaved.

As the initiator of polymerization, thrombin plays an important role in the final fibrin clot structure. Thrombin has three important domains with respect to fibrinogen: an active site, an apolar specificity pocket, and a fibrinogen recognition site (*9*). Fibrinogen Naples, a naturally occurring

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[1] Abbreviations: BβA68T, Thr substituted for Ala at position 68 in the Bβ chain; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; SEM, scanning electron microscopy.

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variant fibrinogen with a Thr substituted for Ala at position 68 in the Bβ chain (BβA68T), was previously found to have a significant reduction in the binding affinity for thrombin compared to normal fibrinogen (*10*). The present studies with recombinant BβA68T fibrinogen are focused on the effects that this mutation imposes on thrombin and Ancrod-catalyzed polymerization. In this paper, we present polymerization of the variant BβA68T fibrinogen which has a normal period of protofibril formation and rate of lateral aggregation but which has a significantly reduced extent of lateral aggregation and thus forms thinner fibers. Our results indicate that the region of the fibrinogen molecule that contains this mutation is important for normal polymerization and that its importance is imposed during the enzymatic phase of the reaction. To accommodate our data, we propose a model by which BβA68T fibrinogen forms thin fibers and incorporate this model into the existing knowledge of the polymerization process.

EXPERIMENTAL PROCEDURES

Materials. Ancrod, HEPES, and all other reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted. Human α-thrombin was obtained from Enzyme Research Labs (South Bend, IN). Glutaraldehyde, osmium tetroxide, and sodium cacodylate were all EM-grade materials obtained from Electron Microscopy Sciences (Fort Washington, PA). PCR 8-well cap strips were obtained from NalgeNunc (Naperville, IL). UV-transparent 96-well microtiter plates (catalog #3635) were purchased from Corning-Costar. All plasmid vectors, CHO cells, bacteria, and culture medium have been previously described (*11*). IF-1 monoclonal antibody (*12*) was obtained from Iatron Lab, Inc. (Tokyo, Japan).

Recombinant Fibrinogen Expression and Purification. Normal recombinant and BβA68T proteins were expressed as previously described (*13*). Briefly, CHO cell lines expressing normal or BβA68T fibrinogen were grown in roller bottles in serum-free medium, and the medium was harvested, mixed with protease inhibitors, and stored at −70 °C (*11*). These proteins were purified as described (*14*). In brief, the medium was thawed at 37 °C, and fibrinogen was precipitated with 40% ammonium sulfate. The precipitate was redissolved in a buffer containing 10 mM $CaCl_2$ and was applied to a Sepharose 4B column coupled with an IF-1 monoclonal antibody specific for fibrinogen. Fibrinogen was eluted with a buffer containing 5 mM EDTA and was dialyzed against 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ for one change and then extensively against 20 mM HEPES, pH 7.4, 150 mM NaCl and stored at −70 °C. The fibrinogen concentration was determined at A_{280}, using the extinction coefficient of $\epsilon = 1.506$ for a 1 mg/mL fibrinogen solution (*15*). Purity of the proteins was analyzed by SDS−PAGE gel, by the method of Laemmli (*16*).

Polymerization of Recombinant Fibrinogens. Polymerization of normal recombinant and BβA68T fibrinogen was monitored at 350 nm in a SpectraMax-340PC 96-well microtiter plate reader at ambient temperatures (Molecular Devices, Sunnyvale, CA). Two separate experiments were performed in quadruplicate for each polymerization condition. For each row used in the plate, four wells contained normal fibrinogen and four wells contained BβA68T fibrinogen. To each reaction well was added 90 μL of normal recombinant or BβA68T fibrinogen in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$. To initiate the polymerization reaction, 10 μL of enzyme (thrombin or Ancrod) was added to all reaction wells with a multichannel pipettor such that all reactions began simultaneously. In most cases, no more than two rows were used in one reaction to minimize the time between addition of enzyme to the first row and the last. Immediately after addition of enzyme, the samples were automixed by the instrument for 5 s. Turbidity was monitored every 9 s for either 1 or 4 h. To avoid the effects of evaporation during the longer runs, a glass slide was firmly sealed with silica gel on top of the rows in use, immediately after the addition of thrombin. All turbidity readings were normalized to a 1 cm path length by the PathCheck sensor within the instrument. Final concentrations for each set of reactions were 1.2 μM fibrinogen (0.4 mg/mL), with 3.6 nM (0.4 unit/mL) or 97 nM (11 units/mL) thrombin or 0.4 unit/mL Ancrod. The Ancrod and thrombin units of activity were not normalized to one another.

Analysis of Polymerization Results. Lag time and V_{max} were determined for each polymerization reaction. Lag time was measured as the time elapsed until an increase in turbidity was seen, and V_{max} was calculated as the slope at the steepest part of the polymerization curve (*17*). Statistical values comparing normal recombinant and BβA68T fibrinogen were determined using unpaired *t*-tests. A difference is significant when the p value is <0.05.

Fibrin Monomer Preparation. Fibrin monomers were prepared as described (*14, 18*). In brief, fibrinogen was clotted by thrombin (desAB fibrin monomer) or Ancrod (desA fibrin monomer). Twenty-seven times more thrombin was used for preparation of BβA68T fibrin monomer than normal. To prepare desA fibrin monomer, the same concentration of Ancrod was used for normal and BβA68T fibrinogens. Reverse phase HPLC analysis of clot supernatant was used to ensure that fibrinopeptide cleavage was complete from the two fibrinogens. Both desA fibrins had up to 8% of molecules with both fibrinopeptides cleaved. The clots were wrapped around a glass rod, washed in 0.15 M NaCl, and dissolved in 0.125% acetic acid on ice. The dissolved fibrin monomers were repolymerized by dilution (10-fold) in 40 mM HEPES, pH 7.4, 0.2 M NaCl buffer. The resultant clots were wrapped around a glass rod, washed in 0.15 M NaCl, and dissolved in 0.125% acetic acid on ice; repolymerization was repeated twice. The resulting fibrin monomers were kept in 0.125% acetic acid at 4 °C and used within 1 month of preparation. An SDS−PAGE gel was run on the fibrin monomers to ensure that degradation had not occurred.

Fibrin Monomer Polymerization. Fibrin monomer polymerization was performed in microtiter plates essentially as described above. In each reaction, 10 μL of fibrin monomer (desA or desAB) was added to each well containing 90 μL of buffer with a final concentration of 40 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and polymerization monitored for 1 h. The final concentration of fibrin monomer was 0.6 μM (0.2 mg/mL).

Scanning Electron Microscopy (SEM). Clots were formed at the same conditions as described for the turbidity experiments. For each polymerization condition, SEM was performed on two clots each with at least two separate microscopy preparations. The SEM preparation was per-

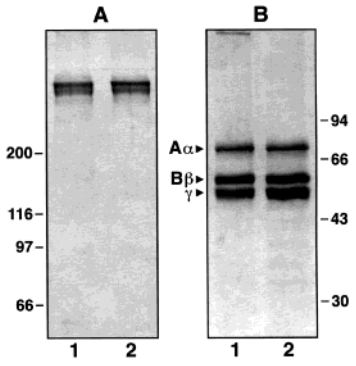

FIGURE 1: SDS−PAGE of normal and BβA68T fibrinogen. (A) Coomassie-stained 6% SDS−PAGE run under nonreducing conditions: lane 1, normal fibrinogen; lane 2, BβA68T fibrinogen. Size markers are indicated on the left. (B) Coomassie-stained 10% SDS−PAGE run under reducing conditions: lane 1, normal fibrinogen; lane 2, BβA68T fibrinogen. Fibrinogen chains are labeled on the left, and size markers are indicated on the right.

formed as described (*19*), with a few minor modifications. In short, clots were polymerized in caps of 8-well strip PCR tubes from which the bottoms had been cut. One side of each cap was sealed with Parafilm, and 45 μL of fibrinogen solution was added. A 5 μL sample of enzyme was added to each well and mixed by repeated pipetting. Polymerization proceeded in a moist environment at ambient temperature for 4 h. The Parafilm was then gently removed from each of the clots, and the caps were rinsed in 0.05 M sodium cacodylate buffer, pH 7.3, for 15 min with 3 changes. The clots were then fixed in 2% glutaraldehyde overnight, rinsed again in sodium cacodylate with 3 changes, and stained with 2% osmium tetroxide for 30 min. The clots were rinsed with distilled water, and dehydrated with a series of ethanol dehydrations of 10 min each, up to 100% ethanol. The samples were then critical-point-dried in a Balzers CPD020 for ~1 h, mounted, sputter-coated with approximately 20 nm gold−palladium, and viewed on a Cambridge StereoScan S200 (LEO Electron Microscopy, Thornwood, NY). All images were taken at 16200× with a 17.0 mm working distance and 20.0 kV accelerating voltage. Fiber diameters were calculated using NIH Image Version 1.62.

RESULTS

Characterization of Recombinant Fibrinogens. Normal and BβA68T fibrinogens were synthesized in CHO cells and purified as described under Experimental Procedures. Analysis of the purified proteins by SDS−PAGE showed the characteristic double bands of fibrinogen when run under nonreduced conditions (Figure 1A) and the three bands representing the Aα, Bβ, and γ chains when run under reduced conditions (Figure 1B). The data demonstrated that all three polypeptides were present and assembled into the fibrinogen molecule, and that there were no significant contaminating proteins present.

Thrombin-Catalyzed Polymerization. Polymerization of normal and BβA68T fibrinogens was examined at two thrombin concentrations, 3.6 and 97 nM. Because previous experiments demonstrated that polymerization of normal recombinant fibrinogen typifies polymerization of plasma fibrinogen (*14*), we used normal recombinant fibrinogen as the control in each of these experiments. Polymerization of both fibrinogens was monitored as the change in turbidity at 350 nm, and the lag time and V_{max} were determined as described under Experimental Procedures. When comparing polymerization at equal thrombin concentrations, we found that BβA68T had longer lag times, lower V_{max} values (Table 1A), and comparable final turbidities relative to normal fibrinogen (Figure 2A,C). As shown in Figure 2A, with 3.6 nM thrombin, BβA68T fibrinogen reached the same turbidity as normal fibrinogen only after an extended incubation: 120 min for BβA68T versus 20 min for normal.

Table 1: Polymerization Parameters of Normal and BβA68T Fibrinogens under Varying Thrombin and Ancrod Concentrations[a]

	lag time (s) ± SD	V_{max} ($\times 10^{-3}$ s^{-1}) ± SD
(A) Normal and BβA68T Fibrinogens Polymerized with 3.6 nM (0.4 unit/mL) Thrombin ($n = 3$)		
normal	25 ± 5	5.1 ± 0.8
BβA68T	643 ± 119	0.33 ± 0.04
p value	0.0008	0.0005
(B) Normal Fibrinogen Polymerized with 3.6 nM (0.4 unit/mL) and BβA68T Fibrinogen Polymerized with 97 nM (11 units/mL) Thrombin, To Normalize the Rate of Release of FpA ($n = 3$)		
normal	25 ± 5	5.1 ± 0.8
BβA68T	36 ± 14	5.3 ± 1.3
p value	0.26	0.88
(C) Normal and BβA68T Fibrinogens Polymerized with 0.4 unit/mL Ancrod ($n = 5$)		
normal	90 ± 23	3.3 ± 0.2
BβA68T	94 ± 5	2.7 ± 0.3
p value	0.68	0.0006

[a] All experiments were conducted with 1.18 μM fibrinogen in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$.

To compare the kinetics of polymerization relative to the rate of FpA release, we followed polymerization under conditions that equalized the rate of FpA release between normal and BβA68T fibrinogens. As previously determined, 97 nM thrombin was necessary to equalize the rate of FpA release of BβA68T fibrinogen to normal fibrinogen at 3.6 nM thrombin (*13*). As shown in Figure 2B, polymerization of BβA68T fibrinogen with 97 nM thrombin proceeded with a lag time and V_{max} similar to normal fibrinogen with 3.6 nM thrombin (Table 1B). These results demonstrated that the rate of fibrinopeptide A release was the predominant determinant of the length of the lag phase and the rate of V_{max}. In contrast, after a 15 min incubation, the turbidity with BβA68T fibrinogen was considerably lower than with normal fibrinogen (Figure 2B). After longer incubation, the turbidity with BβA68T fibrinogen did not approach that of normal fibrinogen (data not shown). Thus, similar rates of fibrinopeptide A release did not lead to comparable final turbidities. This observation, where two fibrinogens polymerize with similar lag times and V_{max} values, but different final turbidities, has not been previously reported experimentally.

The polymerization curves shown in Figure 2C present the results from all four conditions. This figure emphasizes that at equivalent thrombin concentrations, the initiation of polymerization was extremely delayed for BβA68T fibrinogen relative to normal fibrinogen. Nevertheless, under the two concentrations of thrombin tested here, the final turbidities were comparable when the thrombin concentrations were the same.

Ancrod-Catalyzed Polymerization. To remove the contribution of FpB release, we examined the polymerization of these fibrinogens with Ancrod, an enzyme which preferen-

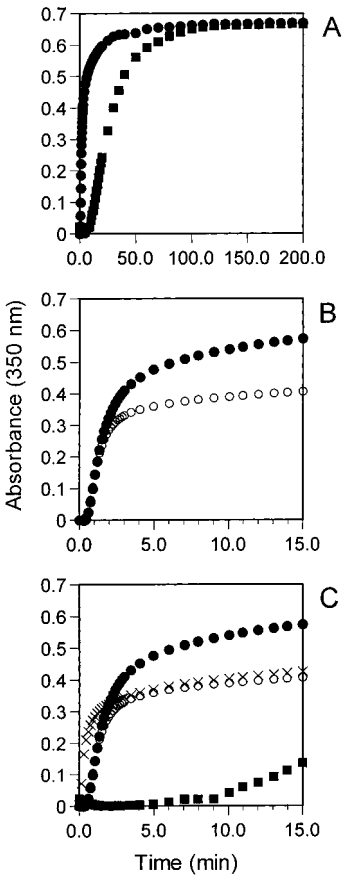

FIGURE 2: Thrombin-catalyzed polymerization of normal and BβA68T fibrinogen. Average polymerization curves ($n = 3$) of normal and BβA68T fibrinogen with (A) equal thrombin concentrations (3.6 nM), (B) thrombin concentrations that equalize the rate of FpA release (3.6 nM for normal fibrinogen and 97 nM for BβA68T fibrinogen), and (C) superposition of data in panels A and B; note the different time scales in panel A vs panel C. Normal fibrinogen with 3.6 nM thrombin (●) or with 97 nM thrombin (×), and BβA68T fibrinogen with 3.6 nM thrombin (■) or with 97 nM thrombin (○). All polymerizations were carried out with 1.2 μM fibrinogen in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, at ambient temperature.

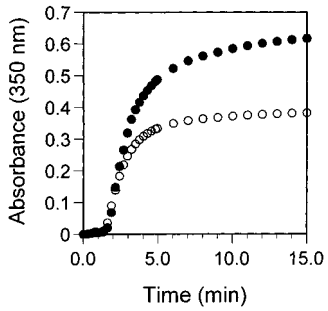

FIGURE 3: Ancrod-catalyzed polymerization of normal and BβA68T fibrinogen. Average polymerization curves ($n = 5$) of normal (●) and BβA68T (○) fibrinogens polymerized with 0.4 unit/mL Ancrod for 1 h. All polymerizations were carried out with 1.2 μM fibrinogen in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, at ambient temperature.

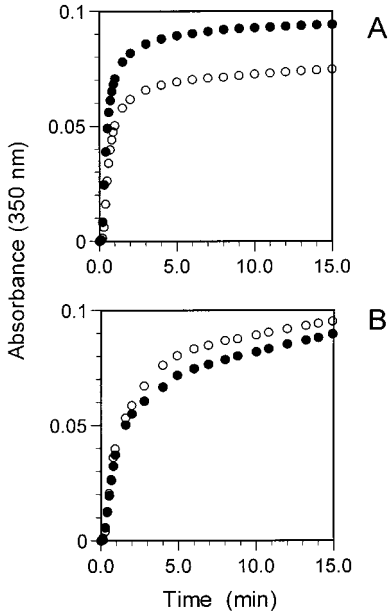

FIGURE 4: Polymerization of normal and BβA68T fibrin monomers. (A) Average polymerization curves ($n = 6$) of desA fibrin monomer of normal (●) and BβA68T (○). (B) Average polymerization curves ($n = 4$) of desAB fibrin monomers of normal (●) and BβA68T (○). All polymerizations were carried out at 0.6 μM fibrin monomer concentration in 40 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, at ambient temperature.

tially cleaves FpA (*20*). We found that the rate of Ancrod-catalyzed FpA release from BβA68T fibrinogen was comparable to the rate of Ancrod-catalyzed FpA release from normal fibrinogen, and that less than 8% of FpB was released from either fibrinogen (data not shown). As shown in Figure 3, polymerization of BβA68T fibrinogen proceeded with the same lag time as normal fibrinogen, but with a statistically lower V_{max} value (Table 1C) and final turbidity. Significantly, these polymerization curves closely resembled the thrombin-catalyzed curves where the rates of FpA release were normalized with different thrombin concentrations (Figure 2B; Table 1B). Thus, the data obtained with Ancrod were consistent with the conclusion that the rate of FpA release was a dominant determinant of the length of the lag phase and the rate of V_{max}.

Fibrin Monomer Polymerization. To completely separate polymerization from the rate of fibrinopeptide release, we examined polymerization of desA and desAB fibrin monomers of both BβA68T fibrinogen and normal fibrinogen. Fibrin monomers were prepared as described under Experimental Procedures; SDS−PAGE analysis of each fibrin monomer preparation showed that neither fibrinogen was degraded during the preparation process. The percent yield of each preparation was similar. As shown in Figure 4A, BβA68T desA fibrin monomers polymerized with a modestly longer lag time and lower V_{max} than normal fibrinogen (Table 2), but reached a considerably lower final turbidity. In contrast, polymerization curves of desAB fibrin monomers from BβA68T and normal fibrinogen were similar to one another (Figure 4B, Table 2). This finding is in agreement with past reports on plasma fibrinogen Naples (*10*, *21*).

Scanning Electron Microscopy (SEM) of Fibrin Clots. To confirm that the turbidities measured with BβA68T fibrinogen were consistent with fiber thickness, as previously shown for normal fibrinogen (*22*), we examined the fibrin clots by scanning electron microscopy. As shown in Figure 5, images of clots prepared with BβA68T fibrinogen showed that the overall fiber thickness was similar to that seen with clots prepared with normal fibrinogen, when comparing clots prepared with the same thrombin concentration (compare panel A to B, or panel C to D). In contrast, when clots were

Table 2: Polymerization Parameters of Normal and BβA68T Fibrin Monomers[a]

	lag time (s) ± SD	V_{max} ($\times 10^{-3}$ s^{-1}) ± SD
(A) Polymerization of Normal and BβA68T DesA Fibrin Monomers ($n = 6$)		
normal	9 ± 1	5.4 ± 1.0
BβA68T	14 ± 2	3.4 ± 1.5
p value	0.001	0.001
(B) Polymerization of Normal and BβA68T DesA Fibrin Monomers ($n = 4$)		
normal	13 ± 5	1.5 ± 1.1
BβA68T	19 ± 6	1.2 ± 0.1
p value	0.09	0.59

[a] All experiments were conducted with 0.6 μM fibrin monomer in 40 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$.

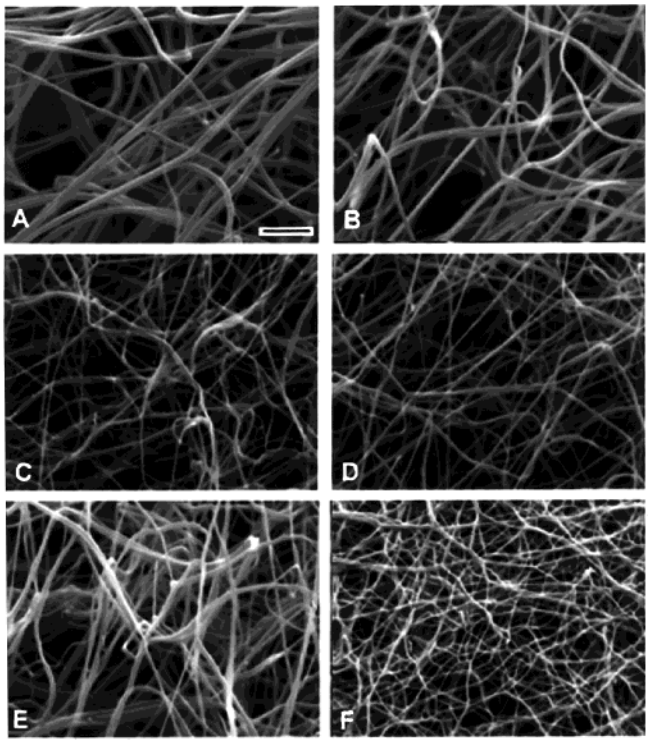

FIGURE 5: Scanning electron microscopy of fibrin clots formed with both thrombin and Ancrod. All clots were polymerized, fixed, stained, critical-point-dried, and mounted as specified under Experimental Procedures. (A, C, E) Normal fibrinogen polymerized with 3.6 nM (0.4 unit/mL) thrombin (A), 97 nM (11 units/mL) thrombin (C), or 0.4 unit/mL Ancrod. (B, D, F) BβA68T fibrinogen polymerized with 3.6 nM (0.4 unit/mL) thrombin (B), 97 nM (11 units/mL) thrombin (D), or 0.4 unit/mL Ancrod (F). All micrographs were taken at the same magnification; the bar represents 1 μm.

prepared under conditions with equalized FpA release (compare panel A to D), the overall thickness of fibers formed from BβA68T fibrinogen is clearly thinner than the thickness of fibers formed with normal fibrinogen. We measured fiber diameters using NIH image, as described under Experimental Procedures, and found that the BβA68T fibers are thinner than normal (Table 3) under these conditions. Thus, the SEM results confirmed the turbidity data, that lower turbidity with BβA68T fibrinogen was predictive of smaller fiber diameters. We also examined clots formed with Ancrod, and, as shown in panels E and F, clots formed with BβA68T fibrinogen had thinner fibers than clots formed with normal fibrinogen. NIH image analyses of these data are also presented in Table 3. The data with Ancrod, again,

Table 3: Diameter Measurements of Fibers Formed with Normal and BβA68T Fibrinogens under Varying Thrombin and Ancrod Concentrations[a]

	normal (nm)	BβA68T (nm)	*p* value
(A) equal thrombin concn	101 ± 19[b]	106 ± 23[b]	0.38
	74 ± 13[c]	71 ± 15[c]	0.28
(B) normalized FpA release	101 ± 19[b]	71 ± 15[c]	<0.0001
(C) Ancrod expt	100 ± 16[d]	59 ± 13[d]	<0.0001

[a] All measurements were made on clots formed with 1.2 μM fibrinogen in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and the indicated amounts of thrombin. [b] With 3.6 nM thrombin. [c] With 97 nM thrombin. [d] With 0.4 unit/mL Ancrod.

demonstrated that the turbidity data were predictive of fiber thickness. As expected, SEM images of the clots made from normal and BβA68T desAB fibrin monomers were visually the same (data not shown).

DISCUSSION

The correlation between the rate and sequence of thrombin-catalyzed fibrinopeptide release and the kinetics of fibrin polymerization has been extensively studied (*17, 23*−*27*). Based on these analyses, we anticipated that thrombin-catalyzed polymerization of BβA68T fibrinogen compared to normal fibrinogen at equal thrombin concentrations would mirror the polymerization curves of normal fibrinogen at low thrombin concentrations since the affinity of thrombin for the variant is decreased about 10-fold relative to normal (*10*). That is, at equal thrombin concentrations, we expected that the polymerization of BβA68T fibrinogen would proceed with a longer lag time, lower V_{max}, and higher final turbidity as would be seen with a 10-fold lower thrombin concentration and normal fibrinogen (*22*, *28*). Although polymerization of BβA68T fibrinogen did proceed with a longer lag time and lower V_{max}, we were surprised to find that the polymerization curve reached the same final turbidity as normal fibrinogen. This was the first indication that the final fiber thickness of polymers formed from BβA68T fibrinogen was lower than we expected, if assuming that the differences in polymerization were due solely to the decreased thrombin affinity.

To probe the basis for this unanticipated finding, we compared thrombin-catalyzed polymerization curves obtained when the rates of fibrinopeptide A release were equalized between the two fibrinogens. Under these conditions, the lag times and V_{max} values for the variant and normal fibrinogen were similar, as expected, indicating that the exposure and interactions of the A:a sites were comparable. In contrast, the final turbidity of the BβA68T clots was significantly lower than normal under these conditions. Thus, with this variant we have found that the kinetics that regulate the early stages of polymerization, characterized by the lag time and V_{max}, do not solely determine the final fiber thickness.

Two possible contributors to the decreased final turbidity reached by BβA68T fibrinogen are inherent in the reaction. First, increasing the enzyme concentration such that there is 1 thrombin molecule to every 12 fibrinogen molecules may have altered the polymerization process, as measured by turbidity. Previous experiments have shown that the addition of active-site-inhibited thrombin altered the rate of fibrin polymerization as measured in turbidity experiments (*34*). Thus, the increased concentration of thrombin per se, not

the increased rate of catalysis, may contribute to the increased rate of polymerization of BβA68T fibrinogen at high thrombin concentration (Figure 2B, Table 1B). Second, while the rates of FpA release were equalized at higher thrombin concentrations, the rate of FpB release from BβA68T fibrinogen was still 2 times slower than normal (*13*). As the cleavage of FpB has been associated with the rate of lateral aggregation (*2*, *6*, *7*, *29*, *30*), it is possible that the differences in final turbidity could be related to this delay in FpB release from BβA68T fibrinogen.

To eliminate the influence of both of these possibilities, the increased enzyme concentration and delayed FpB release of BβA68T fibrinogen, we polymerized both fibrinogens with Ancrod, an enzyme that preferentially cleaves FpA. Polymerization with this enzyme eliminated the need for high enzyme concentrations and removed the contribution of FpB release from the reaction. Despite eliminating these possible interfering variables, however, we still found that BβA68T fibrinogen polymerized with a similar lag time and V_{max} value, but reached a markedly lower final turbidity than normal. Similarly, BβA68T desA fibrin monomer polymerization produced clots with lower turbidity compared to normal, thus demonstrating that neither the difference in the rate of FpB release nor the high enzyme concentration accounted for the thinner fibers formed with BβA68T fibrinogen. Furthermore, when we compared desAB fibrin monomer polymerization, we did not see significant differences, demonstrating that the A:a and B:b interactions in BβA68T fibrin are occurring normally. Taken together, these data suggest that the variation in polymerization seen with BβA68T fibrinogen is due to a change during the enzymatic phase of polymerization, and that this change is irrespective of both the rate of FpA release, as it was equal between both fibrinogens, and the rate and occurrence of FpB release.

Although these differences in polymerization have never been reported experimentally, similar curves were produced in a model in which thrombin catalysis was incorporated into the kinetics of fibrin polymerization (*24*, *28*). In this model, a polymerization profile with similar lag time and V_{max} value, but lower final turbidity, was obtained when the rate at which two protofibrils aggregate to initiate a fiber was increased compared to normal. That is, the rate at which two protofibrils initiated a new fiber was increased relative to the rate at which one protofibril was added to a growing fiber. This model provides the possible molecular mechanism by which thinner fibers form upon polymerization of BβA68T fibrinogen, but it does not explain *why* such a mechanism should occur. Here, we propose a mechanistic situation that could cause the initiation of fibers to be preferred over lateral aggregation of existing fibers in the case of BβA68T fibrinogen. This mechanism accommodates all of our polymerization data and is consistent with the current process of polymerization.

Our model is based on our finding that the extent of lateral aggregation is reduced in BβA68T fibrinogen despite normal rates of FpA release and irrespective of the rate or occurrence of FpB release. These results localize the alteration in BβA68T fibrinogen polymerization to the period of time between FpA release and FpB release, or exposure of the "B" site. The only changes that have been proposed during this period to date are conformational in nature and have been attributed to both the kinetics of fibrinopeptide release (*31*, *32*) as well as the polymerization process (*2*, *3*, *17*, *24*, *33*). Direct evidence for such a conformational change between FpA and FpB release in normal fibrin polymerization has been reported in photooxidation studies (*35*). We propose that it is the lack or alteration of this conformational change that impairs the downstream processes of FpB release and lateral aggregation in BβA68T fibrinogen. Incorrect positioning within the molecule after normal A:a interactions, due to the nature of the BβA68T mutation, could explain (1) the delayed FpB release seen in thrombin-catalyzed polymerization, due to improper conformation, (2) the preferential initiation of fibers over lateral aggregation, if improper conformation discourages the proper lateral aggregation contacts, and (3) the normal fibrin monomer polymerization of BβA68T polymerization, as fibrin monomer polymerization circumvents the enzymatic phase of polymerization. While we do not have direct evidence for a conformational change, or lack thereof, we present this as the most logical interpretation of our data. This possibility is in accordance with previous reports of conformational changes occurring during the fibrin polymerization process. It is possible, of course, that other yet unidentified changes could occur during fibrin polymerization, resulting in the altered polymerization profile of BβA68T fibrinogen.

This paper presents the novel experimental finding of a variant recombinant fibrinogen that polymerizes via normal protofibril formation and rate of lateral aggregation but with a significantly decreased extent of lateral aggregation. This result is attributed to the substitution of Thr for Ala at position 68 of the Bβ chain. Because the A:a and B:b interactions involved in the polymerization of this variant are normal, and the decreased lateral aggregation is evidenced under equalized FpA release rates and irrespective of the rate or occurrence of FpB release, we conclude that the BβA68T substitution has altered the enzymatic phase of the polymerization process between the release of FpA and FpB.

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