Substitution of Tyrosine for Phenylalanine in Fibrinopeptide A Results in Preferential Thrombin Cleavage of Fibrinopeptide B from Fibrinogen†

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ABSTRACT: Phenylalanine at residue 8 in the Aα chain of fibrinogen is a highly conserved amino acid that is believed to be critical for binding and catalysis by the serine protease thrombin. We have examined the requirement for Phe at this position by constructing a variant recombinant fibrinogen with a conservative substitution of tyrosine for phenylalanine, Aα F8Y fibrinogen. We found that the variant fibrinopeptide A (F8Y 1−16) was cleaved by thrombin, in contrast to the lack of cleavage of an Aα 1−23 peptide and an Aα 1−50 fusion protein with the same substitution. This result indicates that fibrinogen residues other than Aα 1−50 participate in thrombin binding and fibrinogen proteolysis. We found, for the first time, that thrombin-catalyzed lysis of the fibrinogen Bβ chain preceded lysis of the Aα chain, such that fibrinopeptide B (FpB) was released prior to F8Y 1−16. Kinetic analysis demonstrated that F8Y 1−16 was a very poor substrate for thrombin, with a specificity constant 280-fold lower than normal fibrinopeptide A. FpB was also a poor substrate, but the specificity constant for FpB was only 4-fold lower than normal. Consequently, FpB was preferentially released from Aα F8Y fibrinogen. This "role reversal" had a dramatic effect on polymerization, such that the rate of Aα F8Y fibrinogen polymerization was 13% of the rate of normal recombinant fibrinogen. These results confirm the importance of phenylalanine at Aα chain residue 8 for efficient thrombin-catalyzed proteolysis of fibrinogen, and further demonstrate that sequential fibrinopeptide release has an important role in normal polymerization.

Thrombin is a serine protease with both procoagulant and anticoagulant roles. Fibrinogen is one of the procoagulant substrates for thrombin. It is made up of six polypeptide chains, two each of Aα, Bβ, and γ chains, with a combined molecular mass of 340 kDa. Fibrinogen is composed of three domains: two peripheral D domains, containing the carboxyl termini of the Bβ and γ chains, and a central E domain, containing the amino termini of all six polypeptide chains. The products of thrombin-catalyzed cleavage of fibrinogen are two fibrinopeptides A corresponding to residues Aα 1−16 (FpA),[1] two fibrinopeptides B corresponding to residues Bβ 1−14 (FpB), and the remaining fibrinogen molecule, called fibrin monomer. Fibrin monomers polymerize in a half-staggered, ordered fashion to form protofibrils that then laterally aggregate to form fibrin fibers. When thrombin cleaves fibrinogen, FpA is released first, at a rate that is described as a simple first-order reaction (*1, 2*). Initially, FpB release is slow, but subsequently the rate of FpB release is increased, such that FpB release is described as a simple first-order reaction that occurs after FpA release (*1, 2*). The delayed release of FpB has been attributed to a requirement for protofibril formation for efficient thrombin cleavage of FpB (*1, 3, 4*).

The residues required for thrombin binding and cleavage of the fibrinopeptides are found in the central domain of fibrinogen (see *5* for review). High-resolution structures of thrombin bound to fibrinogen fragments and thrombin inhibitors suggest that the Aα chain interacts with three sites on thrombin: an apolar pocket that interacts with Aα residues 7−15, the active site that interacts with Aα residues 16 and 17, and an exosite I that interacts with Aα residues 33−42 (*6*−*9*). Biochemical experiments with isolated dysfibrinogens, synthetic peptides, and recombinant Aα 1−50 fusion proteins support a model where these three fibrinogen sites have a critical role in the thrombin-catalyzed conversion of fibrinogen to fibrin (*5*).

One residue within FpA, Phe-8, has long been considered to be extremely important based on its conservation in many mammalian species (*10*, *11*). Peptide mapping studies demonstrated that peptides containing Phe-8 were considerably better substrates than those in which it was absent (*12*−*15*). When Phe-8 is replaced by Tyr in a recombinant Aα 1−50 fusion protein, thrombin does not cleave FpA, in contrast to the normal release of FpA from the Phe-8 substrate (*16*). Similarly, a 23 residue synthetic peptide containing this substitution is not cleaved by thrombin (*17*).

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[1] Abbreviations: CHO, Chinese hamster ovary; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

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A crystal structure of thrombin bound to this variant peptide, Aα 1−23 with Tyr at position 8, has been determined (*17*). A comparison of the normal and variant peptide data demonstrated that the carbonyl oxygen of Tyr was moved 1.8 Å relative to the carbonyl oxygen of Phe at residue 8 (*17*). This large shift was induced by the formation of a novel hydrogen bond between the tyrosine side-chain hydroxyl and the amide nitrogen of glycine at residue 14. As a result, the geometry of the Tyr-8 peptide was distorted at the catalytic site, such that the scissile bond was not effectively oriented for nucleophilic attack by the catalytic serine and the variant peptide was not cleaved (*17*). These experiments confirm that Phe-8 has a critical role in thrombin-catalyzed FpA cleavage.

Previously, we have demonstrated the utility of engineered recombinant fibrinogen variants to examine the role of specific fibrinogen residues in thrombin binding and catalysis (*18*). To verify the importance of Phe-8 in FpA, we constructed and examined a variant fibrinogen with the conservative substitution of Tyr for Phe. This variant, called Aα F8Y fibrinogen, permitted examination of the impact of this substitution on the release of FpA in the context of the entire fibrinogen molecule. In addition, Aα F8Y fibrinogen allowed studies on the effect of this substitution on the thrombin-catalyzed release of FpB. Surprisingly, our data showed that thrombin catalyzed the release of FpA from Aα F8Y fibrinogen, albeit at a very slow rate compared to normal recombinant fibrinogen. In addition, our data demonstrated that substitution of Tyr for Phe resulted in preferential cleavage of FpB, in contrast to the preferential cleavage of FpA from normal fibrinogen. Finally, we found that the reversal of the order of fibrinopeptide release, FpB before FpA, resulted in extremely delayed fibrin polymerization.

EXPERIMENTAL PROCEDURES

Materials. Plasma fibrinogen was purchased from Calbiochem (La Jolla, CA). Human α-thrombin was a generous gift of Dr. Frank Church, University of North Carolina (Chapel Hill, NC). Plasmids, tissue culture medium, bacteria, and Chinese hamster ovary (CHO) cells have been described (*19*). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). IF-1 monoclonal antibody, which specifically binds fibrinogen in the presence of calcium, was purchased from Iatron Laboratories (Tokyo, Japan) (*20*).

Synthetic Peptides. Peptides were synthesized using an automated synthesizer (Protein Chemistry Laboratory of UNC−CH). The following three peptides, corresponding to regions of the Aα or Bβ chains of fibrinogen, were synthesized: residues 1−16 of the Aα chain (FpA), residues 1−14 of the Bβ chain (FpB), and fibrinopeptide F8Y 1−16 corresponding to residues 1−16 of the Aα chain with Tyr substituted for Phe at residue 8 (F8Y 1−16). Stock solutions of all three peptides were prepared at 2 μM in buffer A (25 mM NaH_2PO_4/Na_2HPO_4, pH 6.0). These solutions were passed through a 0.2 μm filter, dispensed into 250 μL aliquots, and stored at −20 °C.

Construction of Plasmids. Plasmids containing the cDNAs of all three fibrinogen chains, pMLP-Aα, pMLP-Bβ, and pMLP-γ, have been described (*19*). A plasmid encoding the replacement of Phe at residue eight of the Aα chain with Tyr (called pMLP-Aα F8Y) was constructed using the polymerase chain reaction (PCR) with Vent polymerase. Two sets of PCR primers were prepared (University of North Carolina Oligonucleotide Facility):

1. TACCTGACGTCTAAGGCCTCTTCCACTG (forward primer)

   TTCAGATCTGGCCATACACT (reverse primer)

2. GCAGATTCCGGAGAAGGTGACTA*TCT-AGCTGA (forward primer)

   TGTGAAGATGCCAGATTC (reverse primer)

The underlined sequence introduced a unique *Bsp*EI restriction site without altering the encoded sequence. The asterisk indicates the point mutation that altered the codon for residue eight to encode for Tyr, rather than Phe. A 1.6 kb fragment from primer set 1 was digested with *Bsp*EI and *Mlu*I, and the resulting 1.3 kb fragment was isolated by electroelution. Similarly, a 0.2 kb fragment from primer set 2 was digested with *Sal*I and *Bsp*EI, and the resulting 69 base pair fragment was isolated by electroelution. These two fragments were ligated to the Aα chain vector, pMLP-Aα, previously digested with *Sal*I and *Mlu*I. The ligation mixture was used to transform DH5αF′, resistant colonies were selected and grown in overnight cultures, and plasmids were isolated using the Qiagen mini-prep kit (Chatsworth, CA). A positive plasmid was identified by digestion with *Bsp*EI and sequenced using an Applied Biosystems automated sequencer (Foster City, CA). The new plasmid, named pMLP-Aα F8Y, was used for synthesis of Aα F8Y fibrinogen in CHO cells following cotransfection with pMSVhis into cells previously transfected with plasmids pMLP-Bβ, pMLP-γ, and pRSV-neo, as described (*19*). Clones were selected based on neomycin and histidinol resistance and screened for fibrinogen secretion using an enzyme-linked immunosorbant assay. The clone secreting the highest fibrinogen concentration was grown in roller bottles in serum-free medium and the medium harvested as described (*19*). The medium was pooled and stored at −70 °C. The same procedure was used to grow CHO cells expressing normal recombinant fibrinogen.

Fibrinogen Purification. Normal recombinant fibrinogen and Aα F8Y fibrinogen were purified as described (*21*). Briefly, fibrinogen was precipitated from the media by adding $(NH_4)_2SO_4$ to 40% final concentration. The precipitate was collected by centrifugation at 16000*g* at 4 °C for 30 min, and was resuspended in 20 mM Tris, pH 7.4, with 300 mM NaCl, 10 mM $CaCl_2$, and a cocktail of protease inhibitors (5 mM ϵ-aminocaproic acid, 5 μM leupeptin, 5 μM pepstatin, 100 μM phenylmethanesulfonyl fluoride, 5 mM benzamidine, and 10 units/mL soybean trypsin inhibitor). The sample was centrifuged at 27000*g* at 20 °C for 20 min and the supernatant applied to a Sepharose 4B column coupled with an IF-1 monoclonal antibody and equilibrated with loading buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$) (20). The column was washed with loading buffer followed by washing buffer I (20 mM Tris, pH 7.4, 1 M NaCl, 1 mM $CaCl_2$) and washing buffer II (50 mM sodium acetate, pH 6.0, 300 mM NaCl, 1 mM $CaCl_2$). Fibrinogen was eluted with 20 mM Tris, pH 7.4, 300 mM NaCl, 5 mM EDTA and dialyzed against one change of 20 mM Tris, pH 7.4, 150

mM NaCl, 1 mM $CaCl_2$, followed by extensive dialysis with Tris-buffered saline (TBS, 20 mM Tris, pH 7.4, 150 mM NaCl). The concentration of the dialyzed protein was measured by the OD_{280}, using a molar extinction coefficient of 1.51, and the protein was aliquoted and stored at −70 °C. Purified fibrinogen was analyzed by SDS−PAGE, according to the method of Laemmli (*22*).

Thrombin Digests. Digestion of fibrinogen by thrombin was performed as described (*18*). Fibrinogen solutions were diluted to give an OD_{280} of 0.1, corresponding to a fibrinogen concentration of 0.2 μM. Thrombin (4.3 units/mL) was added to the fibrinogen solutions to give a final concentration of 0.043 unit/mL (plasma and normal recombinant fibrinogens) or 0.43 unit/mL (Aα F8Y fibrinogen). The tubes were mixed by inversion and the digests aliquoted into 260 μL portions. All of the manipulations following thrombin addition were completed within 1 min. The aliquots were incubated at ambient temperature for the indicated times, and the reaction was halted by immersion in boiling water. The samples were centrifuged for 15 min at 4 °C in a Fisher Model 235C microcentrifuge (Pittsburgh, PA), and the supernatant was removed and stored at −70 °C until it was analyzed by reversed-phase high-performance liquid chromatography (HPLC). Zero time point controls were prepared by adding TBS to the fibrinogen solution and by immediately heating. The infinity time controls were prepared by adding 1 μL of 4300 units/mL thrombin to a 260 μL aliquot of 0.2 μM fibrinogen (final thrombin concentration of 16 units/mL) and incubating for 25 min prior to heating the samples. These controls were then used to calculate the peak area for FpA, F8Y 1−16, and FpB corresponding to 0% and 100% release, respectively.

Analysis of Fibrinopeptide Release. Fibrinopeptide release was monitored by reversed-phase HPLC as described (*18*). Briefly, thrombin digests were loaded onto an Isco system HPLC (Lincoln, NE) with a Vydac C_{18} column (Hesperia, CA) equilibrated with buffer A. Fibrinopeptides were eluted with a linear gradient from 100% buffer A to 40% buffer B (25 mM NaH_2PO_4/Na_2HPO_4, pH 6.0, with 50% acetonitrile) and monitored by the absorbance at 205 nm. The differences in molar absorptivities for FpA, F8Y 1−16, and FpB were not part of our analysis, even though the molar absorptivities of FpA and FpB are slightly different at OD_{205} (*23*). Fibrinopeptide peak area was determined using the accompanying ISCO software. Progress curves were prepared by plotting the percent release versus time, as described (*18*).

The FpA data for normal fibrinogen and the F8Y 1−16 and FpB data for Aα F8Y fibrinogen were fitted with a simple first-order rate equation. The FpB data for normal fibrinogen were fitted to a standard equation describing two consecutive first-order processes, as described (*23*). The curves described by these equations were plotted using Delta Graph (DeltaPoint, Inc, Monterey, CA). Specificity constants, k_{cat}/K_M, were determined by dividing first-order rate constants by the thrombin concentration, as described (*18*).

Polymerization Analysis. Polymerization of normal recombinant fibrinogen and Aα F8Y fibrinogen was monitored in 100 μL microcuvettes (StarnaCells, Inc., Atascadero, CA) as the change in turbidity at 350 nm, as described (*21*). At time zero, thrombin (10 μL) was added to 0.2 μM fibrinogen (90 μL in 20 mM Tris, pH 7.4, 150 mM NaCl) and mixed systematically. The final thrombin concentration was 0.043 unit/mL for normal recombinant fibrinogen and 0.43 unit/mL for Aα F8Y fibrinogen. All polymerization experiments were performed at ambient temperatures.

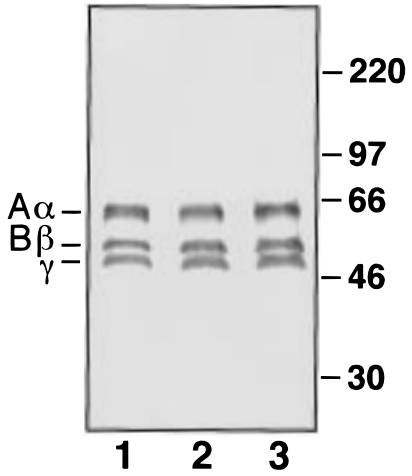

FIGURE 1: SDS−PAGE analysis of purified fibrinogens. Fibrinogen samples were prepared according to the method of Laemmli (*22*) and resolved on an 8% gel run under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250. The lane assignments are (1) plasma fibrinogen, as purchased from Calbiochem, (2) normal recombinant fibrinogen, and (3) recombinant Aα F8Y fibrinogen.

RESULTS

Synthesis and Purification of Fibrinogen. Normal recombinant fibrinogen and Aα F8Y fibrinogen were synthesized and secreted from CHO cells and purified from the media by immunoaffinity chromatography, as described under Experimental Procedures. The purified recombinant fibrinogens were analyzed by SDS−PAGE on an 8% gel run under reducing conditions. Both recombinant fibrinogens contained only three polypeptide chains that migrated to the same extent as the Aα, Bβ, and γ chains of plasma fibrinogen (Figure 1).

Time Course of Thrombin Digests. The release of fibrinopeptides was measured by reversed-phase HPLC as described (*18*). Synthetic peptides corresponding to FpA, FpB, and F8Y 1−16 were used to identify the corresponding peaks from thrombin digests. Representative chromatograms shown in Figure 2A,C demonstrate that all three synthetic peptides eluted as single sharp peaks. Synthetic F8Y 1−16 peptide had a shorter retention time than FpA, as expected from the increased hydrophilicity of Tyr relative to Phe. Thrombin digests of normal recombinant fibrinogen contained two peptides with the same elution profile as a mixture of synthetic FpA and FpB (Figure 2A versus Figure 2B). Thrombin digests of Aα F8Y fibrinogen contained two peptides, corresponding to F8Y 1−16 and FpB (Figure 2A,C versus Figure 2D). The identification of the HPLC peaks was confirmed by spiked controls; the addition of synthetic peptides to thrombin digests resulted in larger peak areas than for the digest alone and did not introduce any further peaks (data not shown).

The data were plotted as percent fibrinopeptide released, and representative progress curves are shown in Figure 3. FpA release preceded FpB release when normal recombinant fibrinogen was incubated with thrombin; this is similar to the release of fibrinopeptides observed following incubation

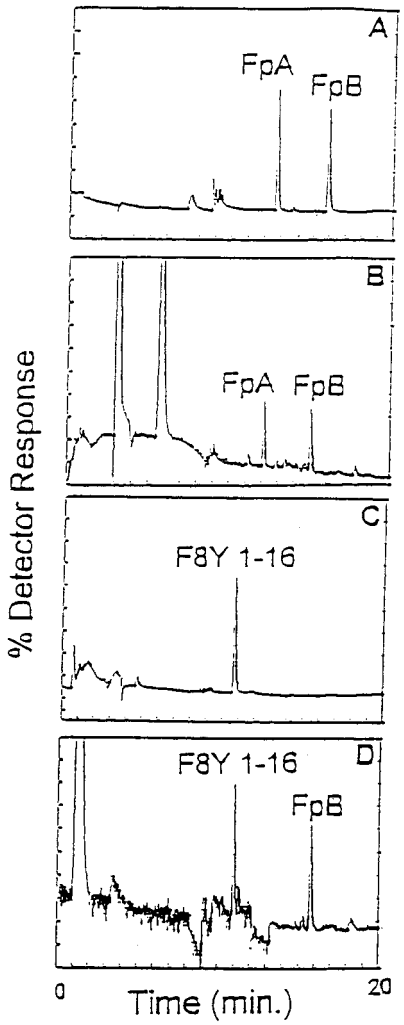

FIGURE 2: HPLC elution profiles. Fibrinopeptide samples were loaded onto a C_{18} column with solvent A (sodium phosphate buffer, pH 6.0) and eluted with a linear gradient of solvent B (sodium phosphate buffer, pH 6.0, in 50% acetonitrile). The elution was monitored at 205 nm. Samples were (A) an equimolar mixture of synthetic FpA and FpB, (B) supernatant from a 16 min thrombin (0.043 unit/mL) digest of normal recombinant fibrinogen, (C) synthetic F8Y 1−16 peptide, and (D) supernatant from a 30 min thrombin (0.43 unit/mL) digest of Aα F8Y recombinant fibrinogen. The data are presented as percent detector response, in arbitrary units.

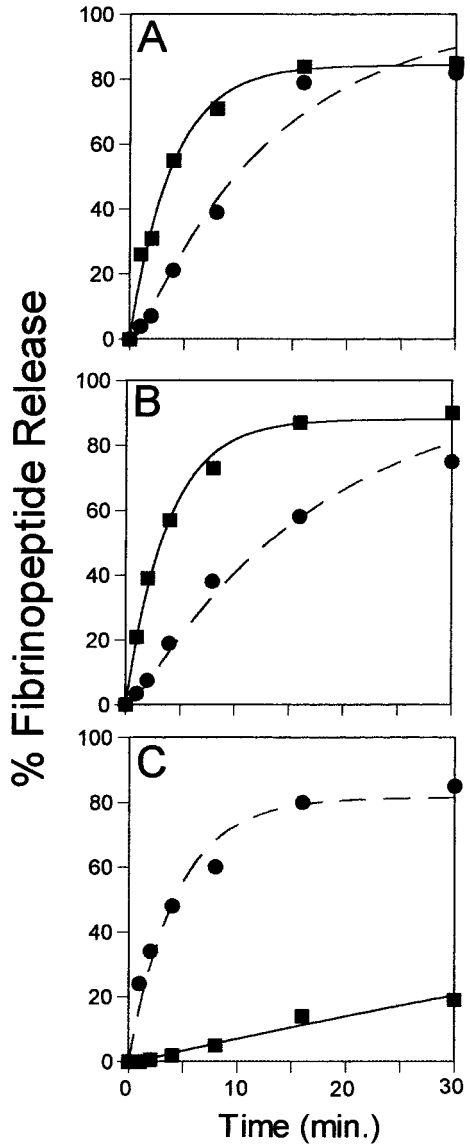

FIGURE 3: FpA and FpB progress curves. Peak areas for the FpA, F8Y 1−16, and FpB peptides released following thrombin digestion were converted to percent release using an infinity time point as described under Experimental Procedures. The data were plotted and fitted to first-order equations using Delta graph, as described under Results. Representative progress curves are shown for each of the following: (A) plasma fibrinogen; (B) normal recombinant fibrinogen; and (C) Aα F8Y recombinant fibrinogen. FpA and F8Y-16 release are represented as solid lines. FpB release is represented by dashed lines. The final thrombin concentrations were (A and B) 0.043 unit/mL and (C) 0.43 unit/mL.

of plasma fibrinogen with thrombin (Figure 3A,B). In addition, the amount of fibrinopeptides A and B released from normal recombinant fibrinogen at the final observation point, 30 min, was similar to that seen using plasma fibrinogen. These results demonstrated the similarities between recombinant fibrinogen and plasma fibrinogen and, along with previous data (*21*), supported the use of normal recombinant fibrinogen as the control for studies with Aα F8Y fibrinogen.

Fibrinopeptide release from Aα F8Y fibrinogen was significantly impaired, such that a 10-fold increase in thrombin was required to generate the progress curve shown in Figure 3C. In contrast to normal recombinant fibrinogen, FpB was released preferentially from Aα F8Y fibrinogen (Figure 3B versus 3C). In addition, unlike previous experiments with fusion proteins and synthetic peptides, F8Y 1−16 was cleaved from Aα F8Y fibrinogen. However, the percentage of F8Y 1−16 released was dramatically reduced compared to normal recombinant fibrinogen, 20% versus 85% FpA, respectively, at the longest observation point (30 min). The percentage of FpB released at the final time point was similar for both Aα F8Y fibrinogen and normal recombinant fibrinogen, with approximately 80% FpB released from both proteins.

The first-order rate constants for fibrinopeptide release were obtained from the progress curves as described (*18*, *23*); the results are presented in Table 1. For normal fibrinogen, k_1 values were determined from the FpA data and k_2 from the FpB data, with the assumption that FpB release occurred only after FpA release. Because this assumption was not true for Aα F8Y fibrinogen, we fit the variant FpB data to the equation used for FpA release from

Table 1: First-Order Rate Constants and Specificity Constants for Fibrinopeptide Release[a]

fibrinogen	First-Order Rate Constants k_1	k_2
plasma	4.3 ± 0.4	1.7 ± 0.2
normal recombinant	4.4 ± 0.3	1.4 ± 0.2
Aα F8Y	0.16 ± 0.01	3.8 ± 0.6
fibrinogen	Specificity Constants k_{catFpA}/K_{MFpA}	k_{catFpB}/K_{MFpB}
plasma	9.2 ± 0.9	3.6 ± 0.5
normal recombinant	9.3 ± 0.6	2.9 ± 0.5
Aα F8Y	0.033 ± 0.003	$0.80 + 0.13$

[a] First-order rate constants are the average values from seven experiments and are expressed as 10^{-3} s^{-1}. The specificity constants are the average from seven experiments and are expressed as 10^6 M^{-1} s^{-1}.

normal fibrinogen; this constant is k_2 in Table 1. The rate constant for FpA release from Aα F8Y fibrinogen was determined by fitting the data to a simple first-order equation, the same as for normal fibrinogen.

To compare the relative efficiency of the three fibrinogens as thrombin substrates, we calculated the specificity constants from the first-order rate constants, dividing by the thrombin concentration (Table 1). Comparison of the specificity constants for both FpA and FpB from normal recombinant and plasma fibrinogens demonstrated that plasma fibrinogen and normal recombinant fibrinogen had similar properties as substrates for thrombin. Compared to the normal recombinant fibrinogen control, Aα F8Y fibrinogen was a very poor substrate, as indicated by the reduced specificity constants. For F8Y 1−16 release, the specificity constant was 280 times lower than that for FpA release from normal recombinant fibrinogen. For FpB release, the specificity constant was 4 times lower than that for FpB release from normal recombinant fibrinogen, bearing in mind that the two equations used to determine the rate constants, and, hence, the specificity constants, were different (see above).

Thrombin-Catalyzed Polymerization. Thrombin-catalyzed polymerization was monitored by the change in turbidity at 350 nm (*21*). Because previous experiments demonstrated that polymerization of recombinant fibrinogen was the same as polymerization of plasma fibrinogen, we used recombinant fibrinogen as the normal control for these experiments (*21*). The polymerization of Aα F8Y fibrinogen was significantly slower than the polymerization of normal fibrinogen, even when a 10-fold higher concentration of thrombin was used with Aα F8Y fibrinogen. This higher thrombin concentration was chosen to approximate the same rate of FpB release from both Aα F8Y fibrinogen and normal fibrinogen. As shown in Figure 4, polymerization of normal fibrinogen neared completion within 40 min, whereas polymerization of Aα F8Y fibrinogen approached completion after 200 min. The turbidity curves were used to generate two quantitative values of polymerization, lag time and maximum rate (*24*); the data are presented in Table 2. The lag time reflects the period of protofibril formation required for the initiation of lateral aggregation (*3*, *4*, *24*). The average lag time for polymerization of Aα F8Y fibrinogen was 8.5 times longer than the lag time for normal recombinant fibrinogen. The maximum rate (V_{max}) of polymerization corresponds to the maximum rate of lateral aggregation. The average V_{max} for polymerization of Aα F8Y fibrinogen was 8-fold lower than the average V_{max} for normal fibrinogen polymerization.

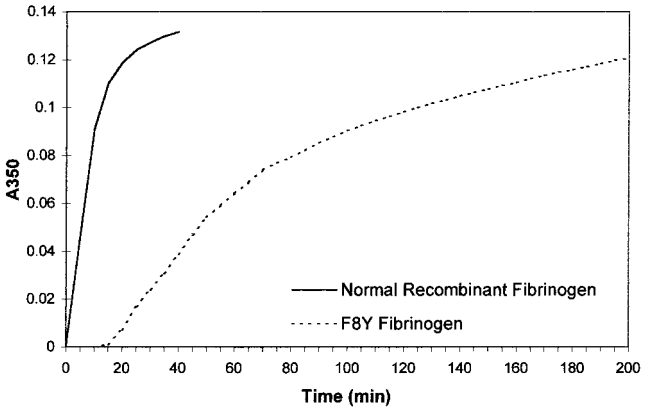

FIGURE 4: Polymerization of normal recombinant and Aα F8Y fibrinogens. Polymerization was initiated by addition of thrombin at time zero to normal recombinant fibrinogen (0.2 μM; 0.043 unit/mL thrombin) or Aα F8Y fibrinogen (0.2 μM; 0.43 unit/mL thrombin). Polymer formation was measured as the change in OD_{350} versus time (representative curves).

Table 2: Polymerization Parameters[a]

fibrinogen	lag time (s)	V_{max} ($\times 10^{-5}$ s^{-1})
normal recombinant	120 ± 7	24 ± 6.8
Aα F8Y	1013 ± 85	3.0 ± 0.4

[a] Lag time and maximum rate (V_{max}) are the average values from three experiments. Values were determined from a line tangent to the steepest section of the turbidimetric curve; the lag time was the point where the tangent line crossed the *x*-axis, and V_{max} was the slope of the tangent line (*24*).

DISCUSSION

We describe, for the first time, a fibrinogen variant where thrombin-catalyzed FpB release preceded FpA release. The altered order and decreased rate of fibrinopeptide release resulted in an extreme delay in the polymerization of this variant. These data confirm the critical role of Phe at residue 8.

Previous high-resolution NMR and X-ray crystallography studies identified several interactions between FpA and thrombin, and showed that Phe-8 participates in multiple interactions that are likely relevant to thrombin catalysis (*7*, *8*, *25*, *26*). Further, the crystal structure of a variant peptide with Tyr at position 8 showed an altered peptide geometry at the thrombin catalytic site, consistent with the data demonstrating that this peptide is not a substrate. In view of the structural data, we were surprised that F8Y 1−16 was cleaved from Aα F8Y fibrinogen. These results imply that the substitution of Tyr for Phe-8 in the context of the fibrinogen molecule was less detrimental to the orientation of the substrate than this substitution in the context of the Aα 1−51 fusion protein or the Aα 1−23 peptide (*16*, *17*). As the Bβ chain was not present in the fusion protein or synthetic peptides, and a role for the Bβ chain in thrombin−fibrinogen interactions has previously been noted (*18*, *27*), we suggest that the differences in orientation arise from the presence of the Bβ chain in fibrinogen. Perhaps the prior

release of FpB from Aα F8Y fibrinogen alters the enzyme−substrate conformation to improve the orientation of F8Y 1−16 at the active site.

Because it is known that the rate and the order of fibrinopeptide release are important for polymerization, we examined the effect of preferential FpB release. We anticipated that the polymerization of Aα F8Y fibrinogen would resemble the polymerization of the congenital dysfibrinogen Metz I, an Aα Arg-16→Cys substitution (*28*) found in an individual homozygous for this substitution. Thrombin did cleave FpB, but not FpA, from fibrinogen Metz I, giving rise to desB-fibrin (*29*). DesB-fibrin did not form a clot at ambient or higher temperatures, but did form a clot at 14 °C, although polymerization was delayed relative to normal fibrinogen even at 14 °C (*30*). A direct comparison of our data to dysfibrinogen Metz I was not possible because the experiments were done with different conditions. Specifically, the fibrinogen concentration (2 mg/mL for dysfibrinogen Metz I versus 0.1 mg/mL for Aα F8Y fibrinogen), thrombin concentration (5 units/mL versus 0.43 unit/mL), temperature (14 °C versus 25 °C), and the presence of calcium in the clots formed from Metz I were different. Bearing that in mind, the following were noted. Overall, Aα F8Y fibrinogen resembles Metz I fibrinogen in that polymerization is delayed compared to normal fibrinogen controls. The magnitude of the delay, however, was greater for dysfibrinogen Metz I. The Aα F8Y fibrinogen turbidity curve reached a plateau within 200 min (Figure 4), whereas the fibrinogen Metz I turbidity curve was still rising at this time (*30*). The fibrinogen Metz I lag time approached 40 min, but the Aα F8Y fibrinogen lag time was closer to 17 min. As all the differences in experimental conditions, except temperature, would favor more effective polymerization of dysfibrinogen Metz I, we concluded that polymerization of Aα F8Y fibrinogen was much more normal than this dysfibrinogen.

The fibrinopeptide release data offer some insight into the differences between the Aα F8Y fibrinogen and Metz I fibrinogen. Only desB-fibrin was formed from the Metz I fibrinogen, whereas desAB-fibrin was formed from Aα F8Y fibrinogen (*30*). Furthermore, the lag time for Aα F8Y fibrinogen polymerization (17 min) corresponded to the time in the thrombin digest when appreciable F8Y 1−16 appeared (16 min; 14% release). Similarly, the rate of polymerization appears to depend on the rate of F8Y 1−16 release. We noted that the V_{max} for polymerization of Aα F8Y fibrinogen was 8-fold slower than the V_{max} for normal fibrinogen under conditions where the k_1 for the release of F8Y 1−16 was nearly 30-fold slower than the k_1 for the release of FpA. Although these rates are quantitatively different, it is reasonable to conclude that the rate of F8Y 1−16 cleavage controls the maximum rate of polymerization. Taken together, the increased lag time and slowed rate of polymerization are consistent with the conclusion that release of F8Y 1−16 from Aα F8Y fibrinogen is the rate-limiting step in the polymerization of this protein. Thus, polymerization of Aα F8Y fibrinogen was impaired because F8Y 1−16 is a poor thrombin substrate.

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