THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 33, Issue of August 18, pp. 25239–25246, 2000
Printed in U.S.A.

Recombinant Fibrinogen Studies Reveal That Thrombin Specificity Dictates Order of Fibrinopeptide Release*

Received for publication, May 15, 2000
Published, JBC Papers in Press, June 2, 2000, DOI 10.1074/jbc.M004142200

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During cleavage of fibrinogen by thrombin, fibrinopeptide A (FpA) release precedes fibrinopeptide B (FpB) release. To examine the basis for this ordered release, we synthesized A′β fibrinogen, replacing FpB with a fibrinopeptide A-like peptide, FpA′ (G14V). Analyses of fibrinopeptide release from A′β fibrinogen showed that FpA release and FpA′ release were similar; the release of either peptide followed simple first-order kinetics. Specificity constants for FpA and FpA′ were similar, demonstrating that these peptides are equally competitive substrates for thrombin. In the presence of Gly-Pro-Arg-Pro, an inhibitor of fibrin polymerization, the rate of FpB release from normal fibrinogen was reduced 3-fold, consistent with previous data; in contrast, the rate of FpA′ release from A′β fibrinogen was unaffected. Thus, with A′β fibrinogen, fibrinopeptide release from the β chain is similar to fibrinopeptide release from the α chain. We conclude that the ordered release of fibrinopeptides is dictated by the specificity of thrombin for its substrates. We analyzed polymerization, following changes in turbidity, and found that polymerization of A′β fibrinogen was similar to that of normal fibrinogen. We analyzed clot structure by scanning electron microscopy and found that clots from A′β fibrinogen were similar to clots from normal fibrinogen. We conclude that premature release of the fibrinopeptide from the N terminus of the β chain does not affect polymerization of fibrinogen.

Fibrinogen is a 340-kDa plasma protein that is involved in the final phase of the coagulation cascade. Fibrinogen consists of two pairs of three polypeptide chains (Aα, Bβ, and γ) that fold to produce a trinodular protein with two distal (D) nodules connected to a central nodule (E) by coiled-coil regions. The central nodule of the molecule consists of the N termini of all six polypeptide chains, and the D nodules consist predominantly of the C termini of the β and γ chains, each folded into a globular domain. To initiate polymerization, the serine protease thrombin cleaves four specific Arg–Gly bonds at the N termini of both the Aα and Bβ chains, releasing fibrinopeptides A (FpA)[1] and B (FpB), respectively. The release of FpA, a 16-residue peptide, exposes the "A" site, which noncovalently interacts with the "a" site in the γ chain of the D nodule of another molecule. This A:a interaction results in the linear arrangement of half-staggered, double-stranded protofibrils (1). The release of FpB, a 14-residue peptide, exposes the "B" site (2–4), which presumably interacts with a "b" site in the β chain of the D nodule of another molecule (5). This B:b interaction is thought to be responsible for lateral aggregation of protofibrils to form fibers (2) and to be analogous to the A:a interaction; however, the mechanism of this interaction is not yet well understood. The final product of this polymerization is a complex, branching network of fibers.

The interactions of thrombin with fibrinogen have been extensively studied (Refs. 6 and 7; for a review, see Ref. 8). Thrombin contains three domains that interact with fibrinogen: the active site, an apolar specificity pocket, and a fibrinogen-binding exosite. The exosite, also called the fibrinogen recognition site, confers the specificity with which thrombin binds to fibrinogen. Upon binding, thrombin cleaves FpA and initiates polymerization. FpA release from fibrinogen follows first-order kinetics, described by the kinetic constant k_1. In contrast, FpB is released from fibrinogen at a slow initial rate (3, 9, 10), which is then increased upon polymerization (2, 9–11) and the depletion of FpA as a substrate for thrombin. This efficient release of FpB, subsequent to FpA release, follows first-order kinetics and is described by the kinetic constant k_2, assuming that the release of fibrinopeptides A and B from thrombin occurs through two successive first-order processes.

Crystallographic data have depicted the contacts between FpA and thrombin (12); but, to date, similar data are not available for thrombin and FpB. The sequence of FpB is different from that of FpA (13) such that FpB should require different contacts with thrombin (12). Because we do not have structural information, our understanding of FpB release is indirect and based on kinetic studies that examined the timing of FpB release. These studies have shown that the majority of FpB is released from fibrin after FpA has been removed (3, 14) and that the rate of FpB release is enhanced upon polymerization of des-A polymers (2–4, 15). To date, however, the mechanism responsible for the delay in FpB release remains undetermined.

To extend our understanding of the mechanism of thrombin on fibrinopeptide B and its resulting effect on polymerization, we have designed a variant recombinant fibrinogen (A′β) that contains a fibrinopeptide A-like substrate on the N termini of the β chains. This fibrinogen was designed to probe the mechanism of fibrinopeptide release and more specifically to deter-

* This work was supported by National Institutes of Health Grant R01-HL31048 (to S. T. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FpA, fibrinopeptide A; FpB, fibrinopeptide B; FpA′, fibrinopeptide A′ (FpA with a G14V mutation); A′α, FpA′ substituted on the N terminus of the α chain; A′β, FpA′ substituted on the N terminus of the β chain; bp, base pair(s); HPLC, high performance liquid chromatography; GPRP, Gly-Pro-Arg-Pro acetate salt peptide.

mine whether the delayed release of FpB is a consequence of its affinity for thrombin or of its location on the N terminus of the β chain. Our studies have revealed that it is the specificity of thrombin for FpB that is responsible for the order of fibrinopeptide release during fibrin polymerization.

EXPERIMENTAL PROCEDURES

Materials—HEPES, ε-aminocaproic acid, sodium phosphate, and all other reagents were obtained from Sigma unless otherwise noted. Human α-thrombin was purchased from Enzyme Research Labs (South Bend, IN); two different lot numbers were used for this work: HT1330, 1.85 mg/ml equivalent to 5655 units/ml; and HT1540PA, 1.96 mg/ml equivalent to 6392 units/ml. Human α-thrombin (4300 units/ml, 1.71 mg/ml) was a generous gift from Dr. Frank Church (University of North Carolina, Chapel Hill). Recombinant hirudin was purchased from Calbiochem-Novabiochem. All restriction enzymes were obtained from New England Biolabs Inc. (Beverly, MA). Glutaraldehyde, osmium tetroxide, and sodium cacodylate were all electron microscopy-grade materials obtained from Electron Microscopy Sciences (Fort Washington, PA). Eight-well strip polymerase chain reaction tubes were obtained from NalgeNunc (Naperville, IL). UV-transparent 96-well microtiter plates (catalog no. 3635) were purchased from Corning Costar. All plasmid vectors, Chinese hamster ovary cells, bacteria, and culture medium have been previously described (16). Monoclonal antibody IF-1 (17) was obtained from Iatron Lab, Inc. (Tokyo).

Expression Vector Construction—Cloning procedures and vectors were as described previously (16). The expression plasmid for the variant A′α chain (pMLP-A′α) was assembled from two encoding fragments and the expression vector p284. A Gly-to-Val substitution at position 14 was introduced by oligonucleotide-directed mutagenesis of the human Aα chain cDNA cloned in a single-stranded phage using the T7-GEN *in vitro* mutagenesis kit (U. S. Biochemical Corp.) and the oligonucleotide 5′-AAC GCG TGG GCC ACG CAC GAC TC. The resulting clone encoded Val at position 14 of the Aα chain and contained a new *Mlu*I site (underlined). The altered segment was isolated from this clone as an 870-bp *Pvu*II/*Bgl*II fragment (40 bp of 5′-untranslated sequence, the signal sequence, and codons for A′α residues 1–174) and ligated to a 1360-bp *Bgl*II/*Ssp*I fragment (codons for A′α residues 174–625 plus 10 bp of 3′-untranslated sequence) isolated from a plasmid containing the Aα cDNA; the product fragment (2230 bp) was ligated into expression vector p284 cleaved with *Sma*I, and the products were transformed into competent DH5α cells. Plasmid DNAs from individual colonies were screened by restriction enzyme analysis, including the newly introduced *Mlu*I site, and candidate clones were sequenced as described (16). The complete A′α coding segment was sequenced to demonstrate that the desired codon change was present, and no unanticipated changes were introduced.

The expression plasmid for the variant A′β chain (pMLP-A′β) was prepared by ligation of a polymerase chain reaction fragment encoding the α chain signal sequence and FpA′ and a fragment encoding Bβ residues 15–461 into expression vector p284. The polymerase chain reaction fragment was prepared by amplification of a segment from pMLP-A′α with oligonucleotides 5′-TCCACAACCCTTGGGCCACG-GACG (which introduced a *Dsa*I site, underlined) and 5′-TTCAGATCT-GGCCATACACTT; the product was cleaved with *Sal*I and *Dsa*I, and the 156-bp fragment was isolated by electroelution following agarose gel electrophoresis. The fragment encoding Bβ residues 15–461, along with the stop codon and 10 bp of 3′-untranslated sequence, was isolated from pMLP-Bβ as a 1350-bp fragment following cleavage with *Dsa*I and *Sna*BI. The p284 vector was cleaved with *Sma*I and *Sal*I. Following ligation into the vector, individual colonies were screened by restriction enzyme analysis, and candidate clones were sequenced. The sequence demonstrated that the junction between FpA′ and Bβ residue 14 was as intended and that no unanticipated changes were introduced.

The new plasmids, named pMLP-A′α and pMLP-A′β, were used for synthesis of A′α and A′β fibrinogens, respectively, in Chinese hamster ovary cells. pMLP-A′α was cotransfected with pMSVhis into cells previously transfected with plasmids pMLP-Bβ, pMLP-γ, and pRSV-neo as described (16). Similarly, pMLP-A′β was cotransfected with pMSVhis into cells previously transfected with plasmids pMLP-Aα, pMLP-γ, and pRSV-neo. Clones were selected based on neomycin and histidinol resistance and screened for fibrinogen secretion by enzyme-linked immunosorbent assay.

Recombinant Fibrinogen Synthesis and Purification—The normal, A′α, and A′β clones secreting the highest fibrinogen concentration were selected, and fibrinogens were synthesized as described previously (18). Briefly, Chinese hamster ovary cell lines expressing fibrinogen were grown in roller bottles in serum-free medium, and the medium was harvested and stored at −70 °C with protease inhibitors (16). Of note, the expression of A′α fibrinogen was approximately one-tenth of the expression of normal or A′β fibrinogen. These proteins were purified as described (19). In brief, the medium was thawed at 37 °C, and fibrinogen was precipitated with a 40% ammonium sulfate cut. The precipitate was redissolved in buffer containing 10 mM $CaCl_2$ and applied to a Sepharose 4B column coupled with monoclonal antibody IF-1, specific for fibrinogen. Fibrinogen was eluted with buffer containing 5 mM EDTA; dialyzed against 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$ for one change and then extensively against 20 mM HEPES (pH 7.4) and 150 mM NaCl; and stored at −70 °C. The fibrinogen concentration was determined at A_{280} using the extinction coefficient $\epsilon_{280} = 1.506$ for a 1 mg/ml fibrinogen solution (20). Purity of the proteins was analyzed by 9% reducing SDS-polyacrylamide gel electrophoresis following the method of Laemmli (21).

Thrombin-catalyzed Fibrinopeptide Release—The thrombin-catalyzed release of fibrinopeptides A, B, and A′ from normal, A′α, and A′β fibrinogens was performed essentially as described (18). Fibrinogen solutions were diluted to 0.3 μM in 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM ε-aminocaproic acid, and 1 mM $CaCl_2$. ε-Aminocaproic acid was included to inhibit any possible plasmin contaminant activity. Thrombin was added to the fibrinogen solutions to a final concentration of 0.005 units/ml (0.054 nM). The tubes were mixed by inversion, and the digests were aliquoted into 440-μl portions for the 1-, 2-, and 5-min time points and into 220-μl aliquots for the 10-, 40-, 80-, 120-, and 180-min time points. All of the manipulations following thrombin addition were completed within 1 min. The aliquots were incubated at ambient temperature, and the reaction was halted by immersion in boiling water for 15 min. The samples were kept on ice for the remainder of the time course. At the end of the reaction, all samples were centrifuged for 15 min at 4 °C, and the supernatants were removed and stored at −70 °C prior to analysis by reversed-phase HPLC. Zero-time point controls were prepared by boiling 430 μl of the fibrinogen solution in the absence of thrombin.

To increase the sensitivity at early time points in the reaction, the assays were also performed in larger volumes, with less thrombin. This macroscale assay on normal and A′β fibrinogens was performed as described above, with the following modifications. The thrombin concentration was 0.002 units/ml (0.018 nM), and the fibrinogen solution was diluted in 20 mM HEPES (pH 7.4) and 150 mM NaCl. For this assay, the aliquots at the 0-, 1-, 2-, and 5-min time points each contained 2.2 ml; those at the 10-, 15-, and 30-min time points each contained 1.1 ml; and those at the 45-, 60-, and 180-min time points each contained 220 μl. These reactions were stopped by the addition of 1 μl of 2000 units/ml hirudin. The samples were boiled and centrifuged, and the supernatants were stored for later HPLC analysis as described above.

Similar assays were performed in the presence of Gly-Pro-Arg-Pro to examine the role of polymerization in the kinetics of fibrinopeptide release from normal and A′β fibrinogens. Fibrinogen was diluted to a final concentration of 0.3 μM in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM Gly-Pro-Arg-Pro acetate salt peptide (GPRP); this concentration of GPRP was sufficient to abolish an increase in turbidity at 350 nm, due to polymerization, for 3 h. Thrombin was added to a final concentration of 0.002 units/ml (0.017 nM), and the samples were mixed by inversion and aliquoted into 440-μl aliquots for 2- and 5-min time points and into 220-μl aliquots for 10-, 15-, 30-, 45-, 60-, and 180-min time points. The samples were set in boiling water for 15 min, centrifuged, and stored for later HPLC analysis as described above.

Fibrinopeptide release was monitored by reversed-phase HPLC as described (18). Briefly, the samples were loaded onto an Isco HPLC system with a Vydac C_{18} column equilibrated with buffer A (25 mM NaH_2PO_4/Na_2HPO_4 (pH 6.0)). Samples containing 200 μl, 400 μl, 1 ml, or 2 ml were loaded onto this column, depending on the experiment and the time point. 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 absorbance at 206 nm. The retention times for FpA′ differed from those for FpA and FpB (22). The differences in molar absorption for FpA, FpA′, and FpB were not part of our analysis, even though the molar absorption of FpA and FpB is slightly different at A_{206} (23). Fibrinopeptide peak area was determined using the accompanying Isco software (Chemresearch Version 2.4). Fibrinopeptide release curves were prepared by plotting the percent release *versus* time as described (18).

All FpA and FpA′ data were fitted with a simple first-order equation. The FpB data from normal and A′α fibrinogens were fitted to a stand-

ard 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 of Recombinant Fibrinogens—Polymerization was monitored at 350 nm in a SpectraMax-340PC 96-well microtiter plate reader (Molecular Devices, Sunnyvale, CA) at ambient temperature. Three 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 A′β fibrinogen. To each reaction well was added 90 μl of normal recombinant or A′β fibrinogen in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$. To initiate the polymerization reaction, 10 μl of thrombin (4 or 1 units/ml) was added to all reaction wells with a multichannel pipette such that all reactions began simultaneously. Immediately after the addition of enzyme, the samples were automixed by the instrument for 5 s. Turbidity was monitored every 10 s for 1 h. 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 thrombin (0.4 units/ml) and 0.3 μM fibrinogen (0.1 mg/ml) with 0.8 nM thrombin (0.1 unit/ml).

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 of the steepest part of the polymerization curve.

Scanning Electron Microscopy—Clots were formed under the same conditions as described for polymerization. For each condition, scanning electron microscopy was performed on two clots each with at least two separate microscopy preparations. The scanning electron microscopy preparation was performed as described (24) with minor modifications. In short, clots were polymerized in caps of eight-well strip polymerase chain reaction 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 the samples were mixed with the pipette tip. Polymerization proceeded in a moist environment at ambient temperature for 4 h. The Parafilm was gently removed, and the caps were rinsed in 0.05 M sodium cacodylate buffer (pH 7.3) for 15 min with three changes. The clots were then fixed in 2% glutaraldehyde overnight, rinsed again in sodium cacodylate with three changes, and stained with 2% osmium tetroxide for 30 min. The clots were rinsed with distilled water and dehydrated with a series of increasing concentrations of ethanol for 10 min each, up to 100% ethanol. The samples were critical point-dried in a Balzers CPD020 for ~1 h, mounted, sputter-coated with ~20 nm of gold-palladium, and viewed on a Cambridge StereoScan S200 (LEO Electron Microscopy, Thornwood, NY). All images were taken at ×16,200 with a 17.0-mm working distance and 20.0-kV accelerating voltage. Fiber diameters were calculated using ScionImage (Scion Corp., Frederick, MD). Statistical analysis comparing fiber diameters was carried out by unpaired t test using StatView. A difference is significant when $p < 0.05$.

RESULTS

Characterization of Recombinant Fibrinogens—We synthesized two variant fibrinogens with altered fibrinopeptides A′ where glycine at position 14 was changed to valine. We chose the G14V substitution based on the previous result that the specificity of thrombin for the Aα-(1–51) fusion substrate with this substitution was not significantly altered compared with normal FpA (22). In A′α fibrinogen, FpA′ replaced FpA on the two α chains, and in A′β fibrinogen, FpA′ replaced FpB on the two β chains. SDS-polyacrylamide gel electrophoresis analysis run under reducing conditions showed that each variant had the expected bands corresponding to the Aα, Bβ, and γ chains of fibrinogen (Fig. 1) and that no contaminating proteins were detected. The expected fibrinopeptides were released following digestion with thrombin, confirming the presence of the intended sequence substitutions. The HPLC chromatograms shown in Fig. 2 demonstrated that the G14V substitution in FpA altered the HPLC retention time relative to that for FpA or FpB as described previously (22). As expected, the fibrinopeptides from A′α fibrinogen had retention times characteristic of FpA′ and FpB (Fig. 2*B*), and the fibrinopeptides from A′β fibrinogen had retention times characteristic of FpA and FpA′ (Fig. 2*C*).

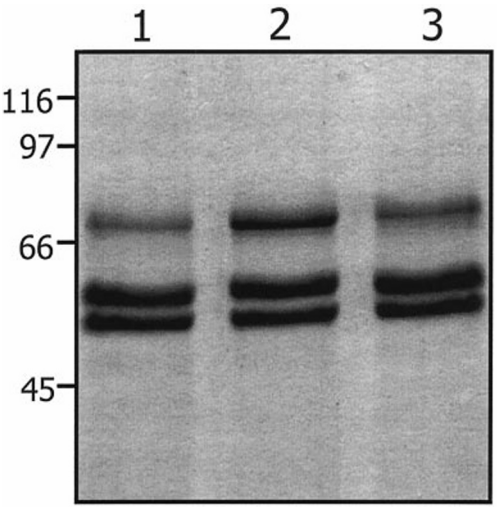

FIG. 1. **SDS-polyacrylamide gel of recombinant proteins.** A 9% reducing gel was used following the method of Laemmli (21) and stained with Coomassie Blue. *Lane 1*, normal fibrinogen; *lane 2*, A′α fibrinogen; *lane 3*, A′β fibrinogen. Molecular masses (in kilodaltons) are indicated to the left.

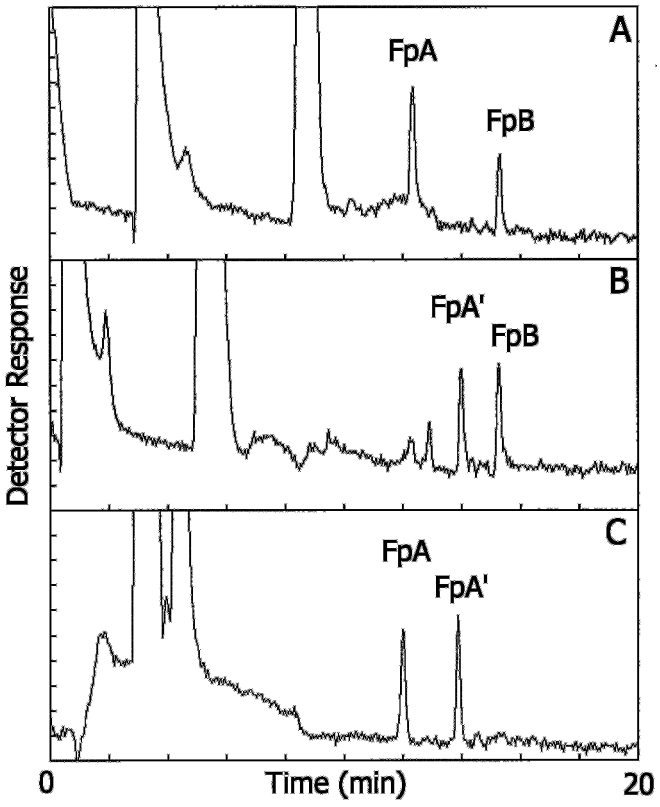

FIG. 2. **HPLC chromatograms depict retention times of fibrinopeptides released from normal (*A*), A′α (*B*), and A′β fibrinogens (*C*).**

Thrombin-catalyzed Fibrinopeptide Release—We examined the rate of thrombin-catalyzed fibrinopeptide release by measuring the peak areas of FpA, FpA′, and FpB as detected by HPLC and plotting the data as the percent fibrinopeptide release with time. Representative curves are shown in Figs. 3–5; specificity constants from multiple averaged experiments are presented in Table I.

A′α fibrinogen was synthesized to test the effect of introduc-

TABLE I

Specificity constants of normal, A′α, and A′β fibrinogens under microscale conditions (part A), macroscale conditions (part B)*, and in the presence of 1 mM GPRP* (part C)

All units are 10^6 M^{-1} s^{-1} · k_{cat}/K_m values for FpA and FpA′ were calculated as k_1/[thrombin]. k_{cat}/K_m for FpB was calculated as k_2/[thrombin]. Each set of experiments was performed with a different thrombin stock and different thrombin dilutions, which may contribute to the differences in specificity. These values represent the mean ± S.D.

Fibrinogen	$k_{cat}/K_{m(FpA)}$ ± S.D.	$k_{cat}/K_{m(FpB)}$ ± S.D.	$k_{cat}/K_{m(FpA')}$ ± S.D.
A. Microscale fibrinopeptide release by thrombin (n = 4)			
Normal	14.7 ± 4.5	4.6 ± 1.4	
A′α		8.7 ± 1.1	9.7 ± 1.3
A′β	8.1 ± 2.4		6.9 ± 2.1
B. Macroscale fibrinopeptide release by thrombin (n = 2)			
Normal	20.2 ± 2.9	9.3 ± 0.2	
A′β	16.5 ± 6.7		8.6 ± 0.1
C. Microscale fibrinopeptide release by thrombin in the presence of GPRP (n = 2)			
Normal	13.5 ± 1.4	1.9 ± 0.4	
A′β	7.2 ± 3.5		4.7 ± 1.3

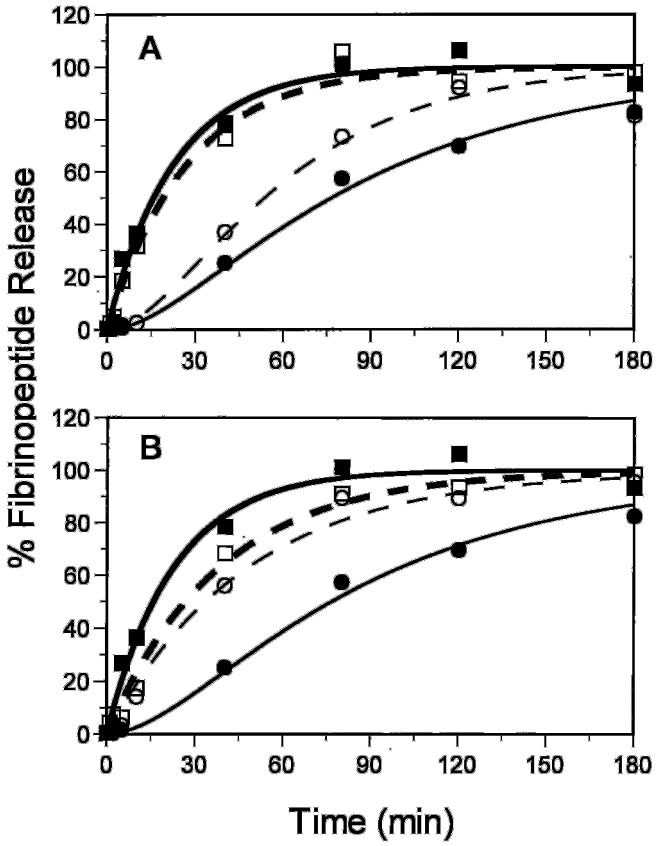

FIG. 3. **Thrombin-catalyzed fibrinopeptide release from normal, A′α, and A′β fibrinogens.** Shown are representative curves of fibrinopeptide release with 0.005 units/ml thrombin and 0.3 μM fibrinogen in 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM ε-aminocaproic acid, and 1 mM $CaCl_2$. All FpA and FpA′ release curves were fit to a first-order rate equation, whereas FpB release curves were fit to an equation describing two consecutive first-order equations. *A*, fibrinopeptide release from normal (*solid lines*) and A′α (*dashed lines*) fibrinogens. ■ and ●, FpA and FpB release, respectively, from normal fibrinogen; □ and ○, FpA′ and FpB release, respectively, from A′α fibrinogen. *B*, fibrinopeptide release from normal (*solid lines*) and A′β (*dashed lines*) fibrinogens. ■ and ●, FpA and FpB release, respectively, from normal fibrinogen; □ and ○, FpA and FpA′ release, respectively, from A′β fibrinogen.

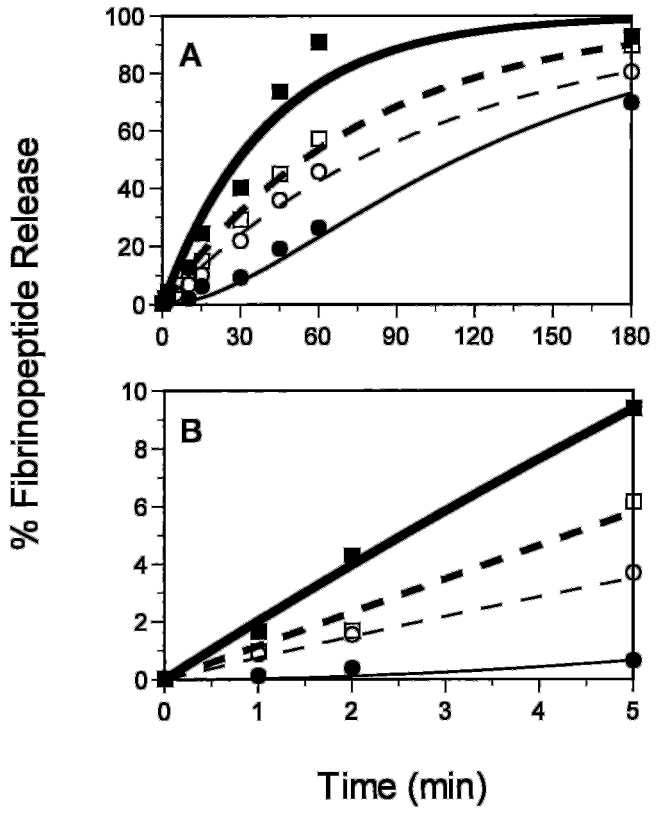

FIG. 4. **Thrombin-catalyzed fibrinopeptide release from normal and A′β fibrinogens.** Shown are representative curves of macroscale fibrinopeptide release with 0.002 units/ml thrombin and 0.3 μM fibrinogen in 20 mM HEPES (pH 7.4) and 150 mM NaCl. All FpA and FpA′ release curves were fit to a first-order rate equation, whereas FpB release curves were fit to an equation describing two consecutive first-order equations. ■ and ●, FpA and FpB release, respectively, from normal fibrinogen; □ and ○, FpA and FpA′ release, respectively, from A′β fibrinogen. *A*, entire time course of reaction of normal (*solid lines*) and A′β (*dashed lines*) fibrinogens; *B*, magnification of the early time points of this reaction. The volume applied to the HPLC column for these points was 5-fold higher than in the early time points for Fig. 3.

ing the G14V substitution on FpA release. When comparing A′α fibrinogen with normal fibrinogen, FpA and FpA′ release data were fit to a first-order rate equation, whereas FpB release data were fit to a standard equation describing two consecutive first-order processes (23). Specificity constants (k_{cat}/K_m) were calculated as the rate constant (k_1 for FpA and FpA′ and k_2 for FpB) divided by the thrombin concentration as described previously (23). Representative data, presented in Fig. 3*A*, showed that the release of FpA′ from A′α fibrinogen was modestly slower than FpA release from normal fibrinogen. Nevertheless, the specificity constants, presented in Table I (part A), were similar (k_{cat}/K_m for normal FpA = (14.7 ± 4.5) × 10^6 M^{-1} s^{-1} and for FpA′ = (9.7 ± 1.3) × 10^6 M^{-1} s^{-1}). These results demonstrated that FpA′ was comparable to normal FpA as a substrate for thrombin. We found that the rate of FpB release from A′α fibrinogen was faster than FpB release from normal fibrinogen (Fig. 3*A*). The specificity constant for FpB

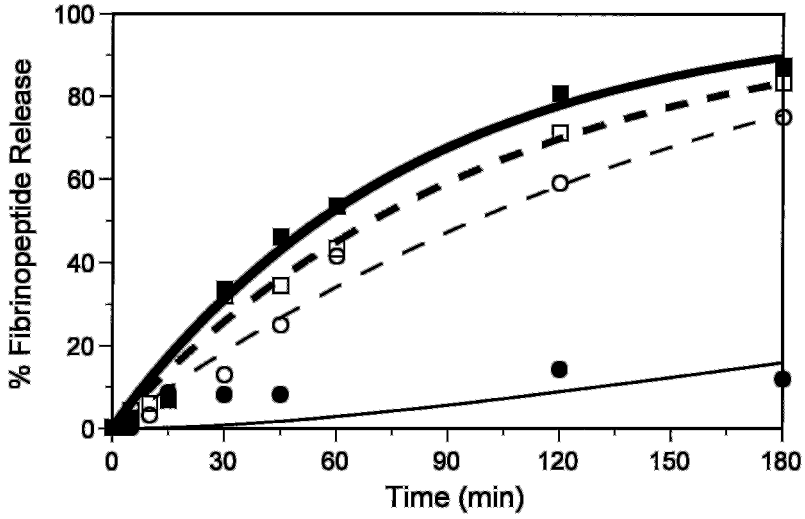

FIG. 5. **Thrombin-catalyzed fibrinopeptide release from normal and A′β fibrinogens in the presence of 1 mM Gly-Pro-Arg-Pro.** Shown are representative curves of normal (*solid lines*) and A′β (*dashed lines*) fibrinopeptide release initiated with 0.002 units/ml thrombin and 0.3 μM fibrinogen in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM GPRP. All FpA and FpA′ release curves were fit to a first-order rate equation, whereas FpB release curves were fit to an equation describing two consecutive first-order equations. ■ and ●, FpA and FpB release, respectively, from normal fibrinogen; □ and ○, FpA and FpA′ release, respectively, from A′β fibrinogen.

release from A′α fibrinogen ($(8.7 \pm 1.1) \times 10^6$ M^{-1} s^{-1}) was twice that from normal fibrinogen ($(4.6 \pm 1.4) \times 10^6$ M^{-1} s^{-1}). The significance of this finding remains unresolved.

A′β fibrinogen was synthesized to determine the effect of substituting FpA′ for FpB on the kinetics of fibrinopeptide release. The fibrinopeptide release data showed that the shape of the curve describing FpA′ release from A′β fibrinogen more closely resembled an FpA release curve than a FpB release curve (Fig. 3*B*). Residual analyses of curves fit (25) to either a first-order rate equation or two consecutive first-order equations revealed that the release of FpA′ from A′β fibrinogen was best described by a first-order rate equation. Thus, all specificity constants were calculated with first-order equations for FpA and FpA′ and with two consecutive first-order equations for FpB. Comparing the specificity constants (Table I, part A) for the release of FpA ($(8.1 \pm 2.4) \times 10^6$ M^{-1} s^{-1}) and FpA′ ($(6.9 \pm 2.1) \times 10^6$ M^{-1} s^{-1}) from A′β fibrinogen showed that the release of these fibrinopeptides was similar. Representative curves describing FpA and FpA′ release were similar (Fig. 3*B*); however, the amount of FpA′ release was *always* less than the amount of FpA released. In comparison with normal fibrinogen, the specificity constant for FpA cleavage from A′β fibrinogen ($(8.1 \pm 2.4) \times 10^6$ M^{-1} s^{-1}) was about half that of FpA release from normal fibrinogen ($(14.7 \pm 4.5) \times 10^6$ M^{-1} s^{-1}) (Table I, part A). FpA′ cleavage from A′β fibrinogen ($(6.9 \pm 2.1) \times 10^6$ M^{-1} s^{-1}) was similar to FpB cleavage from normal fibrinogen ($(4.7 \pm 1.4) \times 10^6$ M^{-1} s^{-1}). Representative curves describing the release of FpA′ from A′β fibrinogen and FpB from normal fibrinogen are qualitatively different from one another, as depicted in Fig. 3*B*, despite their similar specificity constants.

To better examine the early time points, we increased the sensitivity of the assay by 1) slowing down the reaction with lower thrombin concentrations and 2) performing the assay on a macroscale, by increasing the reaction volume and thus increasing the amount of fibrinopeptides measured. The results of this assay (Fig. 4) revealed that fibrinopeptides from both fibrinogens were released from the beginning of the reaction, although the amount of FpB released was much lower than the amount of each of the other fibrinopeptides (Fig. 4*B*). Specifically, we noted that there was not a delayed enhancement of the rate of FpA′ release from A′β fibrinogen, as seen in FpB release from normal fibrinogen. Comparing the specificity constants (Table I, part B), the rate of FpA release from A′β fibrinogen ($(16.5 \pm 6.7) \times 10^6$ M^{-1} s^{-1}) was less than the rate of FpA release from normal fibrinogen ($(20.2 \pm 2.9) \times 10^6$ M^{-1} s^{-1}), but greater than the rate of FpA′ release from A′β fibrinogen ($(8.6 \pm 0.1) \times 10^6$ M^{-1} s^{-1}). The rate of FpA′ release from A′β fibrinogen was similar to the rate of FpB release from normal fibrinogen ($(9.3 \pm 0.2) \times 10^6$ M^{-1} s^{-1}), although the curve describing FpA′ release from A′β fibrinogen was qualitatively different from the curve describing FpB release from normal fibrinogen.

To examine whether polymerization influences the release of fibrinopeptides from A′β fibrinogen, as it does from normal fibrinogen (3, 14), we monitored fibrinopeptide release in the presence of GPRP, a known inhibitor of fibrin polymerization (26). In agreement with previous reports (3, 14), we found that the release of FpA from normal fibrinogen was unchanged, but the release of FpB was markedly slower in the presence of GPRP (Fig. 5). In contrast, with A′β fibrinogen, neither FpA release nor FpA′ release was changed in the presence of GPRP. Comparing the specificity constants from the averaged data (Table I, part C), we saw that with normal fibrinogen, the release of FpB was 3-fold slower (compare $(1.9 \pm 0.4) \times 10^6$ M^{-1} s^{-1} with $(4.6 \pm 1.4) \times 10^6$ M^{-1} s^{-1}) in the presence of GPRP, whereas FpA was unchanged (compare $(13.5 \pm 1.4) \times 10^6$ M^{-1} s^{-1} with $(14.7 \pm 4.5) \times 10^6$ M^{-1} s^{-1}). In contrast, with A′β fibrinogen, the release of FpA′ was similar in the presence of GPRP (compare $(4.7 \pm 1.3) \times 10^6$ M^{-1} s^{-1} with $(6.9 \pm 2.1) \times 10^6$ M^{-1} s^{-1}), as was the release of FpA (compare $(7.2 \pm 3.5) \times 10^6$ M^{-1} s^{-1} with $(8.1 \pm 2.4) \times 10^6$ M^{-1} s^{-1}). We conclude that unlike the release of FpB, the release of FpA′ from the N terminus of the β chain was not influenced by polymerization.

Thrombin-catalyzed Fibrin Polymerization—To determine whether the altered kinetics of fibrinopeptide release were associated with altered polymerization, we monitored the polymerization of normal and A′β fibrinogens. The polymerization of A′β fibrinogen as measured by turbidity at 350 nm was similar to the polymerization of normal fibrinogen, as shown in Fig. 6. We measured the polymerization parameters lag time, V_{max}, and final turbidity for both proteins and found no significant differences between these parameters for A′β and normal fibrinogens (Table II).

Scanning Electron Microscopy Results—We examined the final fibrin clot structures for both normal and A′β fibrinogens by scanning electron microscopy. The two clots were similar in appearance (Fig. 7), and measurement of the fiber diameters showed that there was not a significant difference between the diameters of A′β fibers (136 ± 26 nm) and normal fibrin fibers (148 ± 27 nm; $p = 0.16$). This finding was consistent with the polymerization curves for normal and A′β fibrinogens, which had similar final A_{350} values, indicative of similar fiber diameters (27).

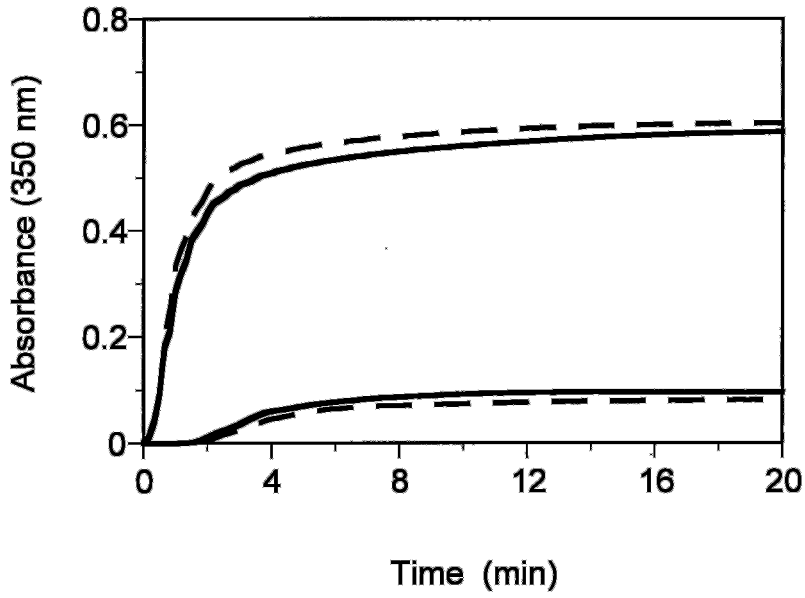

FIG. 6. **Thrombin-catalyzed polymerization of normal and A′β fibrinogens.** Shown are average curves of normal (*solid lines*) and A′β (*dashed lines*) polymerization with 1.2 μM fibrinogen and 0.4 units/ml thrombin (*upper curves*; $n = 12$) and with 0.3 μM fibrinogen and 0.1 unit/ml thrombin (*lower curves*; $n = 8$) in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$.

TABLE II
Polymerization parameters of normal and A′β fibrinogens

Lag time was measured as the time elapsed until an increase in turbidity was seen, and V_{max} was calculated as the slope of the steepest part of the polymerization curve. Final absorbance at 350 nm was determined after polymerization for 1 h. These values represent the mean ± S.D.

	Lag time	V_{max}	Final A_{350}
	s	$\times 10^{-3}$ *s*$^{-1}$	*s*
A. 0.4 mg/ml fibrinogen ($n = 12$)			
Normal	25 ± 13	8.1 ± 0.2	0.61 ± 0.11
A′β	21 ± 5	8.5 ± 0.2	0.60 ± 0.10
B. 0.1 mg/ml fibrinogen ($n = 8$)			
Normal	123 ± 24	0.7 ± 0.2	0.10 ± 0.01
A′β	135 ± 30	0.6 ± 0.1	0.09 ± 0.02

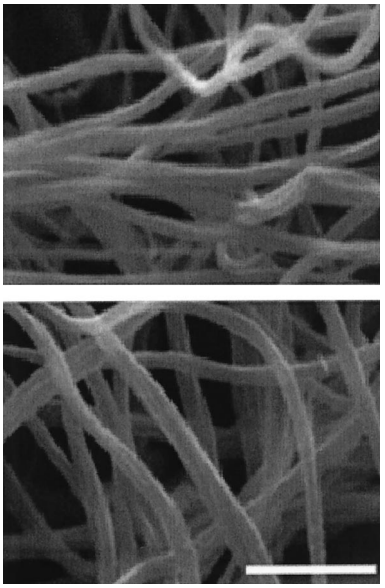

FIG. 7. **Scanning electron micrographs of normal and A′β fibrin clots.** Normal (*upper*) and A′β (*lower*) fibrin clots were formed with 1.2 μM fibrinogen and 0.4 units/ml thrombin in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM $CaCl_2$. The clots were fixed, stained, dehydrated, critical point-dried, and sputter-coated as described previously (23). Images were viewed on a Cambridge StereoScan S200 at a 17.0-mm working distance, 20-kV accelerating voltage, and magnification × 16,200. The *magnification bar* represents 1 μm.

DISCUSSION

We have synthesized a recombinant fibrinogen (A′β) to determine what effect the substitution of FpA′ on the N terminus of the β chain has on the kinetics of fibrinopeptide release. The release of FpA and FpA′ from A′β fibrinogen occurred from the beginning of the reaction and followed first-order kinetics, indicating that both FpA and FpA′ are equally competitive substrates for thrombin and have cleavage sites that are accessible to thrombin at the beginning of the reaction. Thus, the assumption used to characterize FpB release, that FpB release depends on prior FpA release, does not accurately describe the release of FpA′ from A′β fibrinogen. In addition, the similar rate of release of either FpA′ or FpB from the β chain indicates that despite the fibrinopeptide placed on the N terminus of the β chain, the rate of cleavage of the Arg–Gly bond by thrombin remains constant. Taken together, we conclude that it is thrombin, in its interaction with FpB, that is responsible for the delay in efficient FpB release during fibrin polymerization, *i.e.* the specificity of thrombin for FpB, and not its location on the N terminus of the β chain, accounts for the kinetics of fibrinopeptide release from fibrinogen.

Additional experiments support the conclusion that FpA′ release from A′β fibrinogen is dictated by thrombin specificity and not fibrin polymerization. We found that FpA′ release is qualitatively different from FpB release from normal fibrinogen, indicating that the release of FpA′ from the β chain is not dependent on prior polymerization of des-A monomers. This conclusion is supported by our studies in the presence or absence of GPRP, an inhibitor of fibrin polymerization. Our results showed that FpB release was impaired in the presence of GPRP, whereas FpA′ release was not affected. Together, these results indicate that FpA′ release from A′β fibrinogen is independent of fibrin polymerization. Thus, we have created a fibrinogen in which the delay in the rate enhancement of fibrinopeptide release from the β chain has been eliminated.

We present the following models of the interaction of thrombin with fibrinogen, which accommodate our findings with A′β and normal fibrinogens. Initially, thrombin is oriented such that all three fibrinogen interaction sites (the apolar specificity pocket, fibrinogen-binding exosite, and active site) are aligned to accommodate FpA as the preferred substrate. Upon binding to A′β fibrinogen, thrombin is best suited for FpA or FpA′

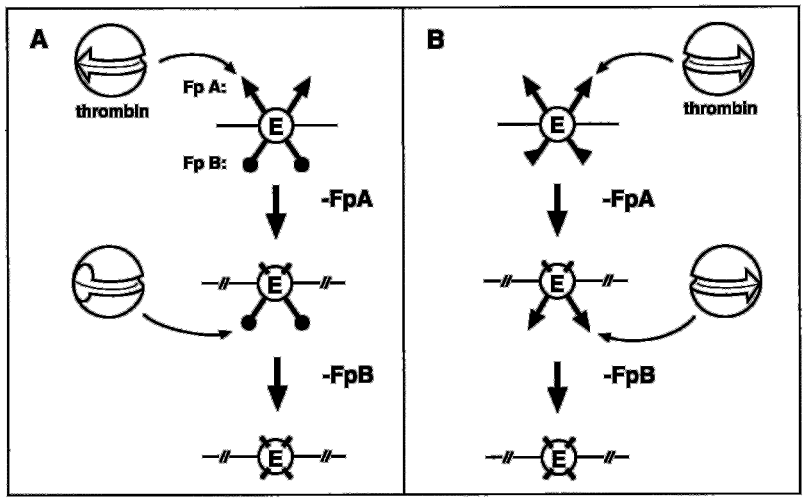

FIG. 8. **Schematic representation of the possible mechanisms for the enhanced cleavage of FpB during fibrin polymerization.** *A*, initially, the conformation of thrombin preferentially accommodates FpA (*arrow*) and initiates cleavage. FpB maintains a conformation that is different from FpA and does not fit into the thrombin active site. Upon cleavage of FpA, the conformation of thrombin changes (represented by a conversion from an *arrow* to a *circle*) during polymerization such that it then accommodates FpB (*circle*) and initiates efficient cleavage. *B*, initially, the conformation of thrombin preferentially accommodates FpA (*arrow*). FpB (*skewed arrow*) is unavailable for cleavage by thrombin because its conformation is not compatible with the thrombin active site. Upon cleavage of FpA, the conformation of FpB changes (*arrow*) and is capable of being cleaved by thrombin.

cleavage, regardless of the placement of the peptide on the α or β chain; thus, all four fibrinopeptides are cleaved simultaneously and with similar affinity. In normal fibrinogen, the thrombin active site is specific for FpA, cleaving it efficiently to initiate polymerization, whereas thrombin cleavage of FpB is much less efficient. During polymerization, however, efficient cleavage of FpB becomes favorable, *i.e.* FpB release becomes more efficient upon the appearance of des-A polymers (2–4, 15). We propose two possible mechanisms, as depicted in Fig. 8, for this shift to efficient cleavage of FpB. Either 1) upon the release of FpA, thrombin changes its conformation such that FpB can be accommodated and cleaved efficiently, or 2) the local conformation of FpB is altered over the course of polymerization, which correctly orients the Arg^{14}–Gly^{15} bond for efficient cleavage within the active site of thrombin.

Although our results do not give us reason to favor one possibility over the other, previous studies lend credence to both. Studies on thrombin binding to exosite-binding fragments of hirudin, heparin cofactor II, and the thrombin receptor have shown that exosite interaction can allosterically modify the active site of thrombin (28–32). Because thrombin binds to fibrinogen in the exosite, it is possible that conformational changes that occur in fibrinogen during polymerization can indirectly affect the thrombin active site. Alternatively, conformational changes in the fibrinogen molecule have been proposed (2, 3, 14, 23, 33–35) and measured (36) during fibrin polymerization, and these changes could reposition the E domain such that the scissile bond in FpB is properly oriented for efficient cleavage by thrombin. Whether it is conformational changes in fibrinopeptide B, thrombin, or both, these studies suggest that either model is possible and reemphasize that we can only conclude from our studies that it is the specificity of thrombin for the fibrinopeptides that dictates the rate and timing of their release.

Production of A′β fibrinogen also allowed us to evaluate the effect of early exposure of the B site on fibrin polymerization. If early exposure of the B site did affect polymerization, we would expect a polymerization curve similar to that for fibrin monomer polymerization, when the A and B sites are exposed from the start of the reaction. Our results did not follow this pattern, thus suggesting that exposure of the B site alone does not directly influence polymerization. Rather, the participation of the B site in polymerization likely depends on certain polymerization events. Previous studies (33, 37) suggest that this event is the polymerization of des-A monomers to form protofibrils of a critical length.

In summary, by making this fibrinogen with essentially four fibrinopeptides *A*, we have synthesized a model substrate that eliminated the delay in fibrinopeptide release from the β chain. Thus, the normal delay in fibrinopeptide B release likely arises from a specific interaction between thrombin and FpB. We therefore conclude that the kinetics of fibrinopeptide release are dictated by the affinity of thrombin for its substrates. In addition, our work suggests that early exposure of the B site does not affect the polymerization process.

Acknowledgments—We gratefully acknowledge Li Fang Ping and Kasim McLain for excellent technical assistance in protein purification and production. We also gratefully acknowledge John Weisel, Chandrasekaran Nagaswami, and Yuri Veklich for teaching us the electron microscopic techniques and Victoria Madden and Bob Bagnell for technical assistance in this endeavor. We thank Frank Church for the generous donation of human α-thrombin.

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