10-25-1990

The Polymerization and Thrombin-Binding Properties of Des-(Bβ1-42)-Fibrin

Kevin R. Siebenlist
Marquette University, kevin.siebenlist@marquette.edu

James P. DiOrio
Sinai Samaritan Medical Center

A. Z. Budzynski
Sinai Samaritan Medical Center

Michael W. Mosesson
Blood Center of Wisconsin

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 265, No. 30, Issue of October 25, pp. 18650-18655, 1990
Printed in U.S.A.

The Polymerization and Thrombin-binding Properties of Des-(Bβ1–42)-fibrin*

(Received for publication, April 13, 1990)

Kevin R. Siebenlist‡§, James P. DiOrio‡, Andrei Z. Budzynski¶, and Michael W. Mosesson‡

From the ‡Sinai Samaritan Medical Center, University of Wisconsin Medical School, Milwaukee Clinical Campus, Milwaukee, Wisconsin 53233 and the ¶Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Multiple factors affect the thrombin-catalyzed conversion of fibrinogen to fibrin, including: fibrinopeptide (FPA and FPB) release leading to exposure of two types of polymerization domains ("A" and "B," respectively) in the central portion of the molecule, and exposure of a noncatalytic "secondary" thrombin-binding site in fibrin. Fibrinogen containing the FPA sequence but lacking the Bβ1–42 sequence ("des-(Bβ1–42)-fibrinogen"), was compared to native fibrinogen (containing both FPA and FPB) to investigate the role played by Bβ1–42 in the polymerization of α-fibrin (*i.e.* fibrin lacking FPA), to compare reptilase and thrombin cleavage of FPA from fibrinogen, and to explore the location and function of the secondary thrombin-binding site. Electron microscopy of evolving polymer structures (μ, 0.14; pH 7.4) plus turbidity measurements, showed that early thin fibril formation as well as subsequent lateral fibril associations were impaired in des-(Bβ1–42)-α-fibrin, thus indicating that the Bβ1–42 sequence contributes to the A polymerization site. Reptilase-activated des-(Bβ1–42)-α-fibrin polymerized even more slowly than thrombin-activated des-(Bβ1–42)-α-fibrin, differences that disappeared when repolymerization of preformed fibrin monomers was carried out. Since existing data indicate that thrombin releases FPA in a concerted manner, resulting in relatively rapid evolution of fully functional divalent α-fibrin monomers, it can be inferred that delayed fibrin assembly of reptilase fibrin is due to slower formation of divalent α-fibrin monomers. Thrombin-activated des-(Bβ1–42)-α-fibrin polymerized more rapidly at low ionic strength (μ, 0.04) than did native α,β-fibrin, a reversal of their behavior at physiological ionic strength (μ, 0.14). Concomitant measurement of FPA release revealed modest slowing of release at low ionic strength from des-(Bβ1–42)-fibrinogen ($t_{½}$, 36.5 *versus* 21.5 min) and marked slowing from native fibrinogen ($t_{½}$, 138 *versus* 22.2 min). This behavior correlated with increased thrombin binding to native α,β-fibrin at low ionic strength, coupled with weak thrombin binding to des-(Bβ1–42)-α-fibrin, and indicates that secondary thrombin binding plays an important role in regulating thrombin diffusion and catalytic activity. Des-(Bβ1–42)-fibrinogen lacks or has a markedly defective secondary thrombin-binding site, from which we conclude that the Bβ15–42 sequence in fibrin plays a major role in forming or providing this site.

Under physiological conditions, α,β-fibrin assembly commences with thrombin-catalyzed release of FPA[1] from the amino termini of fibrinogen Aα chains accompanied by slower release of FPB from Bβ chains (2, 3). Selective release of FPA by reptilase, which is capable of releasing only this fibrinopeptide (4), or release of FPB by copperhead venom procoagulant enzyme (5), is sufficient to initiate fibrin polymerization, forming α- or β-fibrin, respectively. Mature α- and α,β-fibrin clots evidently assemble from a network of thin fibrils which undergo lateral aggregation resulting in increased fiber thickness and branching (6–11). The β-fibrin clot matrix shares many structural features with α- and α,β-fibrin, including formation of twisting fibrils, trimolecular branch points (11), and ultimately, thick striated fibers (10). However, the β-fibrin matrix structure is weaker than α- or α,β-fibrin and is readily dissociable at 37 °C (5).

Release of FPA and FPB exposes two types of polymerization sites in the amino-terminal regions of fibrinogen molecules (12) which appear to function cooperatively in fibrin self-assembly (5). FPA release exposes an "A" polymerization site in its central region (E domain) which subsequently aligns with a complementary "a" site in the outer region (D domain) of another molecule (3, 13) to form staggered overlapping two-stranded fibrils. FPB release uncovers an independent "B" polymerization site (5). The complementary "b" site is apparently formed through cooperative interactions resulting from participation and/or proper alignment of two D domains in the α,β-fibrin polymer (5, 13).

Existing evidence suggests that the A polymerization site is comprised of more than a single peptide sequence, one portion of which is located at the amino-terminal region of the fibrin α-chain, beginning at Aα 17, the site of thrombin cleavage. Peptide sequences that are homologous with Aα-17–20 (*e.g.* Gly-Pro-Arg-Val) inhibit fibrin polymerization (14, 15). Another portion of the A site is probably in the amino-terminal region of the fibrinogen Bβ chain. Photo-oxidation of Bβ-His-16 impaired binding of the E domain of fibrin to fibrinogen (A to a polymerization) but did not impair its

* This investigation was supported by National Heart, Lung, and Blood Institute Grants HL-28444 and HL-36221. An abstract of this work was presented at the XII Congress of the International Society on Thrombosis and Haemostasis (1), August, 1989 and at the Ninth International Workshop on Fibrinogen, Kyoto, Japan, August, 1989. 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.

§ To whom reprint requests should be addressed: Sinai Samaritan Medical Center, 836 North 12th St., Milwaukee, WI 53233.

[1] The abbreviations used are: FPA, fibrinopeptide A; FPB, fibrinopeptide B; α-fibrin, fibrinogen lacking FPA; β-fibrin, fibrinogen lacking FPB; α,β-fibrin, fibrinogen lacking FPA and FPB; Aα, Bβ, fibrinogen chains containing FPA and FPB, respectively; des-(Bβ1–42)-fibrinogen, fibrinogen lacking Bβ1–42; des-AB-NDSK, amino-terminal disulfide knot fragment lacking FPA and FPB.

binding to D dimer-Sepharose, which presumably contains the b site (16). Deletion of Bβ-9–72, as occurs in Fibrinogen New York I, results in impaired fibrin polymerization (17, 18). Furthermore, selective removal of Bβ1–42 by protease III from *Crotalus atrox* venom, resulted in a molecule (des-(Bβ1–42)-fibrinogen) lacking a cleavable B peptide whose rate of polymerization as α-fibrin, as assessed by clot turbidity, was 180-fold slower than that of native α,β-fibrin (19). Since development of clot turbidity is an exponential function of fiber width (20), these results suggest that thick fiber formation (*i.e.* lateral fibril association) is markedly impaired.

Fibrinogen contains two types of sites having demonstrable thrombin affinity. The first type, termed the "substrate site," involves regions concerned with thrombin-mediated release of FPA and FPB (21–23). Following fibrinopeptide release, a "secondary" anionic binding site on fibrin becomes measurable (24–27). There is less than one (0.4 mol/mol) high affinity site (K_a, 5 to 6 × 10^5 M^{-1}) per fibrin molecule (25, 28). It can be increased at low ionic strength, abolished at high ionic strength, and is located in the fibrin E domain (27, 29, 30). Studies by Liu *et al.* (17, 18) with fibrinogen New York I have suggested that at least a part of this site is located in the amino-termimal region of the fibrin β-chain, since this abnormal molecule lacks the Bβ9–72 sequence, and after conversion to fibrin, does not bind thrombin.

The present studies were initiated to assess the function of the Bβ1–42 segment of the molecule in fibrin polymerization. In order to do this, we compared the polymerization of native- and des-(β1–42)-fibrin formed from thrombin- or reptilase-treated fibrinogen, evaluated the ultrastructure of evolving fibrin matrices, and measured binding of thrombin to native and des-(Bβ1–42)-fibrin. Our findings offer new insights into the role played by this region of the molecule in the fibrinogen to fibrin conversion, both with respect to its thrombin-binding properties and its role in the fibrin polymerization process.

MATERIALS AND METHODS

Tris was obtained from Aldrich. Reptilase (Atroxin), phenylmethylsulfonyl fluoride, and Coomassie Brilliant Blue R-250 were purchased from Sigma. Trasylol (aprotinin) was obtained from Mobay Chemical Corp. Human thrombin (7100 units/ml, 2334 units/mg) was generously supplied by Dr. John W. Fenton II, New York Department of Health, Albany, NY. Common chemicals were reagent-grade or better.

Native human fibrinogen and des-(Bβ1–42)-fibrinogen were prepared as described (31) and stored freeze-dried at −20 °C. The lyophylized fibrinogens were redissolved with gentle stirring in 150 mM Tris-HCl buffer, pH 7.5–8.0, containing 0.1 mM phenylmethylsulfonyl fluoride and Trasylol, 10 kallikrein-inactivating unit/ml, and dialyzed against 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, buffer, containing Trasylol, 5 kallikrein-inactivating unit/ml. The subunit structure of these materials was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32) on 1.5-mm polyacrylamide slab gels (5% gels, nonreduced samples; 9% gels, reduced samples).

To express enzyme activity in standard units, thrombin or reptilase clotting activity was calibrated in a Fibrometer Precision Coagulation Timer (BBL) at room temperature by adding 100 μl of enzyme diluted in 50 mM Tris, 100 mM NaCl, pH 7.4, buffer to 300 μl of citrated plasma. One unit of activity was defined as the amount of enzyme that clotted pooled citrated human plasma within 15 ± 0.5 s.

The rate of fibrinopeptide release from fibrinogen was determined by incubating fibrinogen (3–5 mg/ml) in 0.15 M ammonium acetate buffer, pH 7.5, or in 0.05 M ammonium acetate buffer, pH 7.5, with thrombin (0.05 to 0.2 unit/ml) or reptilase (0.075–0.2 unit/ml) at room temperature for periods of up to 360 min. The reaction was terminated by heating the mixture in a boiling water bath for 3 min, the supernatant solution clarified by filtration, and FPA quantified by high performance liquid chromatography (33). Over the range of enzyme studied, the rate of FPA release ($t_{1/2}$, min) was proportional to the amount of enzyme added to the reaction mixture. Under physiological buffer conditions (pH 7.4; μ = 0.14), thrombin that had been calibrated against a plasma standard released FPA 30–38% (mean, 34%, n = 4) faster from purified native fibrinogen than did an equivalent number of units of reptilase.[2] Thus, in experiments comparing thrombin directly with reptilase, enzyme activity was adjusted to yield identical FPA release rates (*e.g.* 1.3 units of reptilase/unit of thrombin).

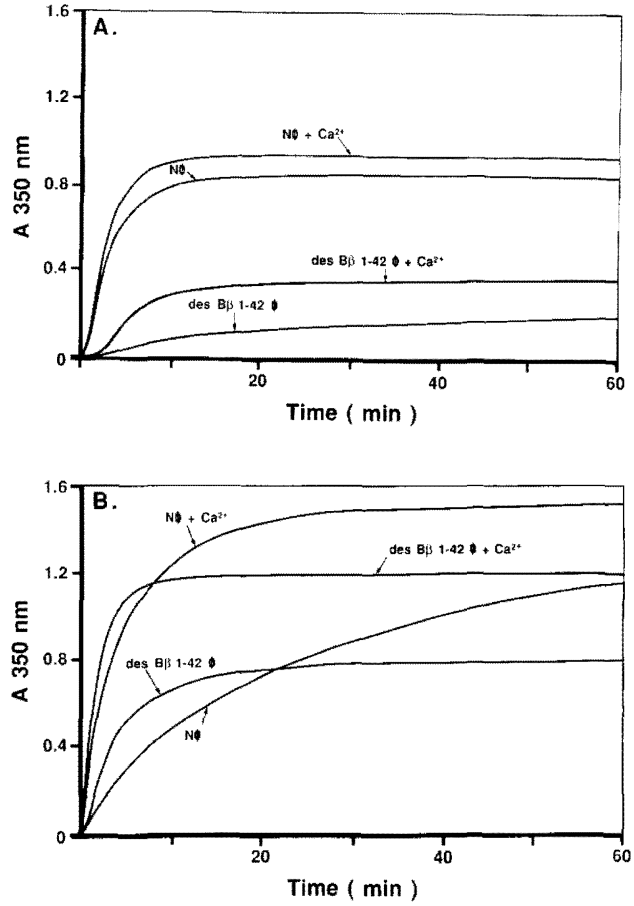

FIG. 1. **Thrombin-catalyzed polymerization of native and des-(Bβ1–42)-fibrinogen.** The reaction was carried out at room temperature in 50 mM Tris, 100 mM NaCl, pH 7.4 (μ, 0.14; *panel A*), or in 50 mM Tris, pH 7.4, buffer (μ, 0.04; *panel B*). Polymerization was initiated by adding thrombin (0.1 unit/ml, final concentration) to the fibrinogen solutions (500 μg/ml) and was monitored at 350 nm as a function of time.

Fibrin polymerization was monitored at 350 nm at room temperature in a Gilford Response recording spectrophotometer. Reaction mixtures contained fibrinogen (native or des-(Bβ1–42)-fibrinogen) at 0.5, 0.1, or 0.05 mg/ml in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, buffer (μ, 0.14) or in 50 mM Tris, pH 7.4, buffer (μ, 0.04). When included, $CaCl_2$ was present at a final concentration of 10 mM. Polymerization was initiated by the addition of thrombin or reptilase.

Thrombin binding by native and des-(Bβ1–42)-fibrin was measured in the 50 mM Tris buffer system (μ, 0.04 or 0.14) by incubating fibrinogen (0.50 or 1.85 mg/ml) with thrombin (1–25 units/ml, final concentration) at room temperature for 2 h. The resulting clots were synerized and thrombin activity remaining in the clot liquor and in the clot itself was assayed at room temperature in 50 mM Tris, 100 mM NaCl, pH 8.3, buffer using S-2238 (Kabi) as substrate (0.1 mM, final concentration). Control samples contained thrombin alone incubated under identical conditions.

Fibrin monomer repolymerization studies were performed as described by Gralnick *et al.* (34). Reaggregation of fibrin monomer from acetic acid solutions was initiated by diluting an aliquot of the fibrin solution into at least a 10-fold excess of 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, buffer (final fibrin concentration, 0.5 mg/ml).

Samples of native fibrin or des-(Bβ1–42)-fibrin for negative stain-

[2] The reason for the difference in the functional activities of thrombin and reptilase may be due to a relatively greater propensity of plasma to inhibit thrombin compared to reptilase.

ing electron microscopy were formed at room temperature (μ, 0.14) as 50-μl drops (0.1 mg/ml fibrinogen) in a Petri dish, incubated for 0–20 min with thrombin or reptilase (0.1 unit/ml, final), picked up on glow discharged carbon/formvar-coated grids, and then negatively contrasted with 2% (w/v) uranyl acetate. For processing by the critical point drying technique, the clots were formed overnight and critical point-dried as previously described (10).

Electron microscopy was performed in a Philips Model 400 transmission electron microscope at 80 or 120 kV. Fibrin fiber diameters were measured from 8 × 10 enlargements of micrographs. All fibers within randomly selected areas of each photograph were measured and averaged.

RESULTS AND DISCUSSION

Thrombin-catalyzed Polymerization of Native and Des-(Bβ1–42)-fibrin—Des-(Bβ1–42)-fibrinogen (0.5 mg/ml) at physiological pH and ionic strength (μ, 0.14) polymerized at a much reduced rate when compared to native fibrinogen (ΔA_{350}/min, des-(Bβ1–42)-fibrin, 0.01/min; native fibrin, 0.28/min) (Fig. 1*A*), even though the rate of FPA release did not differ significantly ($t_{1/2}$, des-(Bβ1–42)-fibrinogen, 20.2 min; native fibrinogen, 21.4 min at 0.2 unit/ml, thrombin). Addition of calcium to these mixtures resulted in a small increase in the polymerization rate of native fibrin and a more marked increase in that of des-(Bβ1–42)-fibrin (1.3- *versus* 6-fold). In each case, the lag period before the onset of turbidity was longer for des-(Bβ1–42)-fibrinogen than it was for native fibrinogen.

Compared with physiological conditions, at low ionic strength (μ, 0.04) there was a 2-fold decrease in the polymerization rate of native fibrin, whereas the polymerization rate of des-(Bβ1–42)-fibrinogen was increased 18-fold and was even more rapid than that of native fibrin (Fig. 1*B*). Addition of calcium resulted in augmented polymerization rates for both fibrins, but the polymerization rate of des-(Bβ1–42)-fibrin remained greater than that of its normal counterpart. The ultimate turbidity developed by des-(Bβ1–42)-fibrin in the absence or presence of calcium was lower than that for native fibrin under the same conditions. Similar polymerization behavior was observed at lower protein concentrations (0.1, 0.05 mg/ml; results not shown).

The prolonged lag period, slow polymerization rate, and

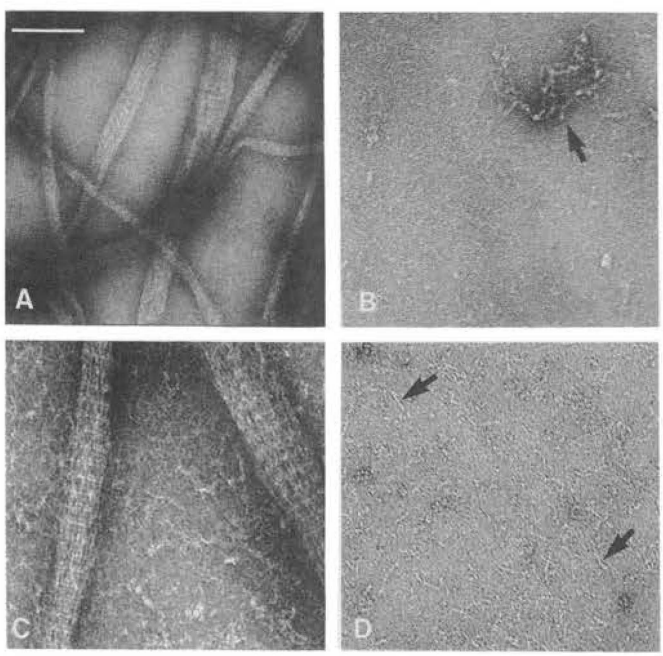

FIG. 2. **Electron micrographs of negatively stained native (*panels A* and *C*) and des-(Bβ1–42) (*panels B* and *D*) polymerizing fibrin.** Fibrinogen (0.1 mg/ml) was incubated at room temperature with thrombin or reptilase for up to 20 min. *Panels A* and *B*, 50 mM Tris, 100 mM NaCl, pH 7.4, buffer plus thrombin at 3 min; *panels C* and *D*, 50 mM Tris, 100 mM NaCl, pH 7.4, buffer plus reptilase at 2 min; the grid surface in *panel D* is covered with unpolymerized fibrin(ogen) molecules (*arrows*). (*Bar* in *panel A* represents 200 nm in *panels A* and *B* and 100 nm in *panels C* and *D*.)

TABLE I

Thickness of mature native and des-(Bβ1–42)-fibrin fibers

Mean fiber width (nm) ± S.D. (range, nm).

	Native fibrinogen	Des-(Bβ1–42)-fibrinogen
Thrombin	88 ± 48 (22–288)	61 ± 39 (11–211)
Thrombin + Ca^{2+}	116 ± 71 (25–357)	63 ± 30 (7–192)
Reptilase	77 ± 54 (14–220)	101 ± 61 (36–266)
Reptilase + Ca^{2+}	88 ± 52 (22–220)	129 ± 79 (26–317)

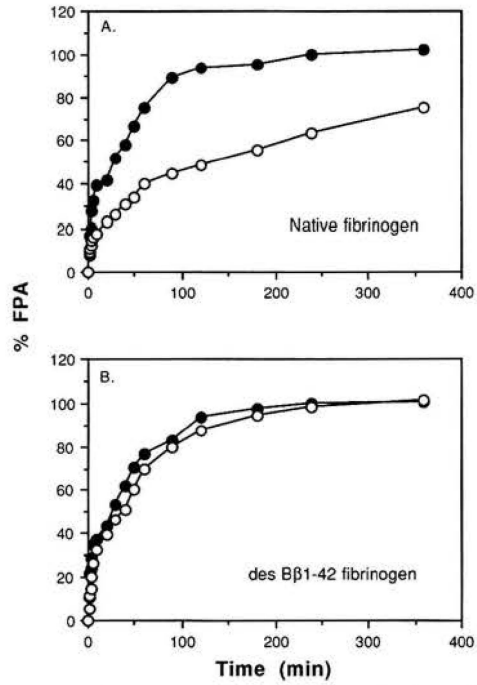

FIG. 3. **FPA release from native (*panel A*) and des-(Bβ1–42)-fibrinogen (*panel B*) at physiological ionic strength (μ, 0.14; *closed symbols*) and at low ionic strength (μ, 0.04; *open symbols*). Native and des-(Bβ1–42)-fibrinogen (3 mg/ml) were incubated with thrombin (0.2 unit/ml), and the FPA content determined at various time intervals.**

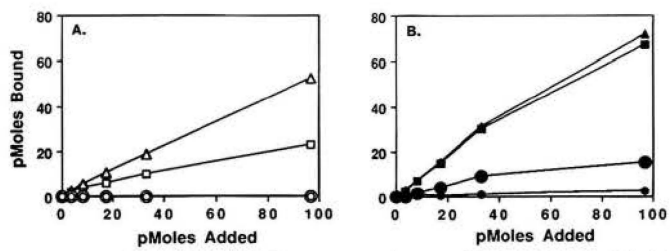

FIG. 4. **Thrombin binding to native and des-(Bβ1–42)-fibrin.** Fibrin clots were formed in 50 mM Tris, 100 mM NaCl, pH 7.4, buffer (μ, 0.14; *panel A*), or in 50 mM Tris, pH 7.4, buffer (μ, 0.04; *panel B*), with various levels of thrombin (1–25 units/ml, final) and incubated at room temperature for 2 h. The clots were removed and the clot liquor assayed for residual thrombin activity: □, ■, native fibrin, 0.50 mg/ml; △, ▲, native fibrin, 1.85 mg/ml; ○, ●, des-(Bβ1–42)-fibrin, 0.50 mg/ml; ○, ●, des-(Bβ1–42)-fibrin, 1.85 mg/ml. Assay of clot-bound thrombin yielded similar curves to those shown for the clot liquor. The recovery of bound and supernatant thrombin activity ranged from 82 to 94% of the amount added.

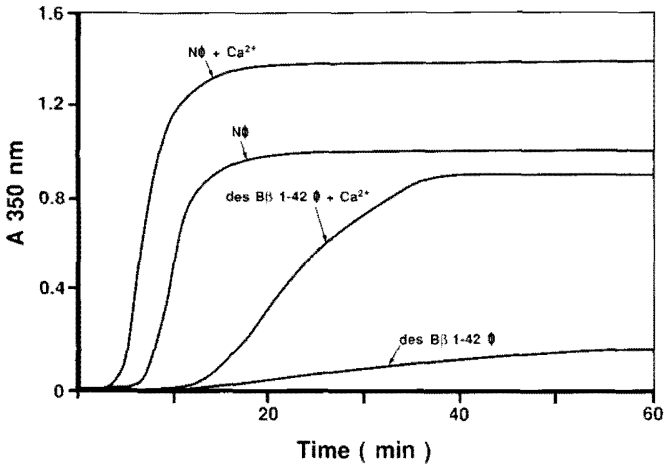

FIG. 5. **Reptilase-induced polymerization of native and des-(Bβ1–42)-fibrinogen.** The reaction was carried out at room temperature in 50 mM Tris, 100 mM NaCl, pH 7.4, buffer. Reptilase (0.13 unit/ml, final concentration) was added to fibrinogen solutions (500 μg/ml) to initiate the reaction and polymerization was monitored at 350 nm as a function of time.

TABLE II

Lag time, polymerization rate, and turbidity developed during repolymerization of native and des-(Bβ1–42)-fibrin monomer

Fibrin monomer preparation	Lag time[a]	Maximum rate	A_{350} at 120 min
	min	$\Delta A_{350}/min$	
Des-(Bβ1–42) (IIa)	2.6	0.017	0.262
Des-(Bβ1–42) (R)	2.3	0.014	0.251
Des-(Bβ1–42) (IIa) + Ca^{2+}	0.1	0.236	0.448
Des-(Bβ1–42) (R) + Ca^{2+}	0	0.254	0.471
Native (IIa)	2.1	0.354	1.066
Native (R)	3.8	0.192	0.759
Native (IIa) + Ca^{2+}	0	0.709	0.641
Native (R) + Ca^{2+}	0	0.624	0.725

[a] The lag time was measured from the time of fibrin monomer dilution to the onset of turbidity.

decreased final turbidity of des-(Bβ1–42)-fibrin could result from delayed fibril formation, retarded lateral association of fibrils into thicker cables, or both. To explore these possibilities, evolving fibrin clots were examined by electron microscopy to assess the rate of fibril and thick fiber formation (Fig. 2). In native fibrin (μ, 0.14), thin fibrils were plentiful after 2-min incubation with thrombin, either in the absence or presence of calcium ions. At 3 min the sample contained thick fibers as well as fibrils (Fig. 2, *panel A*). In contrast, the des-(Bβ1–42)-fibrin sample (*panel B*) contained only a few thin fibrils. Thus, the slow polymerization rate of des-(Bβ1–42)-fibrin is attributable to delayed formation of primary fibrillar structures, which may in turn contribute to delayed lateral fibril association.

Ultrastructural examination of critical point-dried mature native α,β-fibrin formed at μ 0.14, pH 7.4, in the presence or absence of calcium, yielded a typical clot matrix composed predominantly of thick branching striated fibers (Table I). The matrix formed from mature thrombin-treated des-(Bβ1–42)-fibrin displayed a similar structure, although under either condition the mean fiber width was less than that of the native fibrin fibers and the augmenting effect of calcium ions on fiber thickness was minimal. These foregoing results, demonstrating that thin fibril as well as thick fiber formation is impaired in the des-(Bβ1–42)-fibrin assembly process, indicate that the Bβ1–42 segment contributes significantly to the A polymerization domain in the native molecule.

Thrombin Binding to Des-(Bβ1–42)-fibrin—Previous studies with native fibrinogen showed that the accelerating effect of low ionic strength on polymerization is evidently offset by a decreased rate of FPA cleavage due to increased binding of thrombin to the secondary site on fibrin (35–37). In contrast to the slowed polymerization of native fibrin at low ionic strength (Fig. 1*B*), we observed an acceleratory effect of low ionic strength on des-(Bβ1–42)-fibrin polymerization. To investigate the possibility that this effect was attributable to decreased thrombin binding by des-(Bβ1–42)-fibrin, relative to that of native fibrin, we measured both the rate of thrombin-mediated FPA release as well as the binding of thrombin to these fibrin molecules. At physiological ionic strength, thrombin (0.2 unit/ml) released FPA from native fibrinogen with a $t_{½}$ of 22.2 min and from des-(Bβ1–42)-fibrinogen with a $t_{½}$ of 21.5 min (Fig. 3). At ionic strength μ 0.04, there was more than a 6-fold decrease in the FPA release rate from native fibrinogen ($t_{½}$, 138 min), but only a modest decrease in the release rate from des-(Bβ1–42)-fibrinogen ($t_{½}$, 36.5 min).

Under physiological buffer conditions, native fibrin (0.50 mg/ml) bound 20–60% of the added thrombin (Fig. 4), whereas at a higher fibrin concentration (1.85 mg/ml), 50–75% of the added thrombin was bound. At low ionic strength, 70–90% of the added thrombin bound to native fibrin. In contrast, thrombin binding to des-(Bβ1–42)-fibrin was undetectable under physiological buffer conditions at any fibrin concentration, and was very low (2–20%) at low ionic strength.

These findings suggest an explanation for the more rapid polymerization of des-(Bβ1–42)-fibrin at low ionic strength compared with native α,β-fibrin. Since secondary thrombin binding is only minimally expressed in des-(Bβ1–42)-fibrin, the acceleratory effect of low ionic strength, *per se*, on polymerization is augmented by an FPA cleavage rate that is only slightly less than that observed at physiological ionic strength. The addition of calcium, which further reduces the extent of secondary thrombin binding to fibrin (25, 29), enhances polymerization by increasing the thrombin effectively available for fibrinopeptide cleavage as well as by augmenting lateral fiber association (38, 39). Furthermore, the existence of a deficient A polymerization site and the possible absence or modification of the B polymerization site in des-(Bβ1–42)-fibrinogen, probably account for the finding that thrombin-activated des-(Bβ1–42)-fibrin forms thinner fibers than the corresponding native fibrin despite a greatly reduced polymerization rate, which by itself would favor thick fiber formation.

These observations on thrombin binding to des-(Bβ1–42)-fibrin clearly indicate that the Bβ-15–42 sequence in fibrin plays a major role in forming or providing at least a portion of the high affinity secondary thrombin-binding site, and are supported by the finding that Fibrinogen New York I, which lacks Bβ9–72 (18), fails to bind thrombin (17). Our conclusion as to the location of the secondary thrombin-binding site does not agree with that of Vali and Scheraga (30), who positioned this site within Aα17–51. Their conclusion was based on the finding that this sequence was common to all fragments or peptides derived from fibrinogen that bound to active site-inhibited thrombin (*i.e.* des-AB-NDSK, fibrin, or fibrinogen fragment E, and *S*-sulfonated Aα17–78). This interpretation is open to question, since as summarized below, it appears that these fragments may each have bound to thrombin at a site in their structure that did not involve the native Aα17–51 sequence. First, their *S*-sulfonated Aα17–78 peptide contained four negative charges inserted on cysteine residues at positions 28, 36, 45, and 49 (40) that are not present in the native Aα sequence. Furthermore, it has been shown that the

Aα17–27 sequence preceding the Aα chain disulfide bridges does not bind to thrombin (27). Therefore, it seems likely that sulfonation of cysteine residues in Aα17–78, rather than any intrinsic quality of this region of the native Aα chain, provided the type of anionic environment required for thrombin binding. Second, it seems highly improbable that the Aα chain sequence of the plasmic fibrinogen E fragment began, as they asserted, at Aα17 (*i.e.* lacking FPA and consequently the intact Aα chain thrombin substrate-binding site) because plasmin does not cleave at this position (40–42). Therefore, at least some E fragments contained an intact amino-terminal Aα sequence (*i.e.* a primary substrate-binding site) to which thrombin could bind. On the other hand, des-AB-NDSK and fibrin fragment E each lack a substrate-binding site for thrombin, but both contain the Bβ15–42 sequence, which we believe contributes to or constitutes the secondary thrombin-binding site in fibrin.

Kaczmarek and McDonagh (43) studied binding of thrombin to fibrinogen fragments D and E, or to the *S*-carboxymethyl derivatives of the fibrinogen Aα, Bβ, and γ chains. They concluded that the secondary thrombin-binding site(s) was situated on all three peptide components of the E fragment (α20–78, β54–120, γ1–53). Their differing conclusion seems attributable mainly to the fact that under their conditions of complexation and dissociation, they were not measuring a high affinity secondary binding site since the interactions they identified were abolished under physiological buffer conditions in the absence of glutaraldehyde fixation. At most, it appears they were assessing a "low" affinity thrombin binding activity (28).

The exact nature of the nonsubstrate thrombin-binding site is not well understood. It may be available in the fibrinogen molecule itself, or more likely, is expressed only after fibrinogen has been converted to fibrin. Data on the association constants for the substrate-binding sites and the secondary noncatalytic thrombin-binding site (25, 28, 44–46) indicate that these values are similar, a finding that would be consistent with the secondary site remaining cryptic until fibrinopeptide release had occurred. Our findings indicating that Bβ15–42 is a constituent of the secondary site, would place this site near to or superimposed upon the Bβ chain catalytic cleavage site. In this situation, thrombin would remain bound in the Bβ15–42 region after it had cleaved FPB. Additional studies will be required to resolve this interesting question.

Comparison of Reptilase- and Thrombin-catalyzed Fibrin Polymerization—To distinguish the contribution of the Bβ1–42 sequence to the A polymerization site in native fibrin from its contribution to the B site, turbidity measurements were made using reptilase for clot initiation, since only FPA and therefore only the A site is exposed in fibrinogen by reaction with reptilase (4). Under physiological buffer conditions in the presence of reptilase, des-(Bβ1–42)-fibrinogen displayed a longer lag period (11.7 *versus* 5.1 min) and a 28-fold slower polymerization rate than did native fibrinogen (Fig. 5). As was observed with thrombin, the addition of calcium shortened the lag period in both cases, and increased the polymerization rate of native and des-(Bβ1–42)-α-fibrin 2- and 9-fold, respectively. These observed differences between native and des-(Bβ1–42)-α-fibrin were not due to a variable enzymatic effect since FPA was released by reptilase from both (0.2 unit/ml) at nearly the same rate ($t_{½}$, native fibrinogen, 26.1 min; des-(Bβ1–42)-fibrinogen, 24.7 min).

As assessed by electron microscopy, native fibrinogen treated with reptilase formed fibrils within 60 s; at 2 min, coalescence into thick fibers was evident (Fig. 2, *panel C*). Under the same conditions, there were virtually no identifiable thin fibrils in the des-(Bβ1–42)-fibrin specimen (*panel D*); fibrils and thick fibers did not appear in this specimen until 20 min had elapsed (not shown).

When critical point-dried specimens of mature α-fibrin clots were examined, the matrix formed from the native molecule was similar to that of native α,β-fibrin (Table I). The addition of calcium ions to the reaction mixture resulted in somewhat thicker fibers. Reptilase-treated des-(Bβ1–42)-α-fibrin fibers were somewhat thicker than fibers obtained from thrombin-treated des-(Bβ1–42)-α-fibrin, an expected result, since fiber width in a mature clot is known to be inversely related to the rate of fibrin generation (8, 47, 48).

Under conditions normalized with respect to FPA release, both native and des-(Bβ1–42)-fibrinogen polymerized more slowly in the presence of reptilase than they did when they were activated with thrombin (*cf.* Figs. 1*A* and 5). This finding is especially remarkable for des-(Bβ1–42)-fibrinogen which yields identical molecules of α-fibrin with either enzyme.

In order to further explore the observed differences between thrombin- and reptilase-catalyzed polymerization of fibrinogen, the repolymerization of fibrin monomer that had been prepared from native or des-(Bβ1–42)-fibrinogen by treatment with either reptilase or thrombin was studied (Table II). Native α,β-fibrin monomer, no doubt due to the presence of an active B as well as an A polymerization site, repolymerized more rapidly than did the native reptilase α-fibrin preparation. However, when reptilase or thrombin des-(Bβ1–42)-α-fibrin monomer was repolymerized, there were no differences in polymerization under either buffer condition, indicating that observed differences between thrombin- and reptilase-catalyzed conversion of fibrinogen to fibrin are attributable to events occurring during FPA release.

Ferry and co-workers (49–51) calculated that thrombin catalyzes the release of the second FPA from a fibrinogen molecule 16 times faster than the first FPA, thus inferring that thrombin acts in a positively cooperative manner on fibrinogen, favoring production of divalent α-fibrin molecules. This idea is supported by the work of Wilf and Minton (52), who showed by size exclusion chromatography that fibrin/fibrinogen oligomers present early in the clotting reaction were predominantly trimers consisting of one divalent α-fibrin molecule in complex with two molecules of fibrinogen. Given the above evidence for concerted thrombin action on fibrinogen, our findings suggest that since the rate of FPA release is the same for both enzymes, delayed assembly of reptilase fibrin is related to slower evolution of fully functional divalent α-fibrin molecules, perhaps due to more random FPA release by reptilase.

In summary, these investigations have led to two conclusions regarding the function of the fibrinogen Bβ1–42 sequence. 1) The Bβ1–42 sequence contributes to formation of the A polymerization site that is exposed when FPA is released from the fibrinogen molecule. 2) The Bβ15–42 sequence plays a major role in forming or providing the secondary thrombin-binding site on the fibrin molecule.

Acknowledgments—We are most grateful to Diane M. Bartley and Carol A. Burns for their excellent technical assistance, to Angela M. Mallett for assistance in preparation of the manuscript, to Susan Schuder for graphic arts, and to William Semrad for photographic services.

REFERENCES

1. Siebenlist, K. R., DiOrio, J. P., Budzynski, A., and Mosesson, M. W. (1989) *Thromb. Haemostasis* **62**(1), 71
2. Laurent, T. C., and Blombäck, B. (1958) *Acta Chem. Scand.* **12**, 1875–1877

3. Blombäck, B., Hessel, B., Hogg, D., and Therkildsen, L. (1978) *Nature* **275,** 501–505
4. Blombäck, B., and Laurent, T. C. (1958) *Ark. Kemi.* **12,** 137–146
5. Shainoff, J. R., and Dardik, B. N. (1983) *Ann. N. Y. Acad. Sci.* **408,** 254–267
6. Shen, L. L., Hermans, J. McDonagh, J., and McDonagh, R. P. (1977) *Am. J. Physiol.* **232,** H629–H633
7. Hantgan, R., McDonagh, J., and Hermans, J. (1983) *Ann. N. Y. Acad. Sci.* **408,** 344–366
8. Wolfe, J. K., and Waugh, D. F. (1981) *Arch. Biochem. Biophys.* **211,** 125–142
9. Müller, M. F., Ris, H., and Ferry, J. D. (1984) *J. Mol. Biol.* **174,** 369–384
10. Mosesson, M. W., DiOrio, J. P., Müller, M. F., Shainoff, J. R., Siebenlist, K. R., Amrani, D. L., Homandberg, G. A., Soria, J., Soria, S., and Samama, M. (1987) *Blood* **69,** 1073–1081
11. Mosesson, M. W., Siebenlist, K. R., Amrani, D. L., and DiOrio, J. P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1113–1117
12. Kudryk, B., Collen, D., Woods, K. R., and Blombäck, B. (1974) *J. Biol. Chem.* **249,** 3322–3325
13. Olexa, S. A., and Budzynski, A. Z. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 1374–1378
14. Laudano, A. P., and Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 3085–3089
15. Laudano, A. P., and Doolittle, R. F. (1980) *Biochemistry* **19,** 1013–1019
16. Shimizu, A., Saito, Y., and Inada, Y. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 591–593
17. Liu, C. Y., Nossel, H. L., and Kaplan, K. L. (1979) *Thromb. Haemostasis* **42,** 79
18. Liu, C. Y., Koehn, J. A., and Morgan, F. J. (1985) *J. Biol. Chem.* **260,** 4390–4396
19. Pandya, B. V., Cierniewski, C. S., and Budzynski, A. Z. (1985) *J. Biol. Chem.* **260,** 2994–3000
20. Carr, M. E., Jr., and Hermans, J. (1978) *Macromolecules* **11,** 46–50
21. Nagy, J. A., Meinwald, Y. C., and Scheraga, H. A. (1985) *Biochemistry* **24,** 882–887
22. Ni, F., Konishi, Y., Bullock, L. D., Rivetna, M. N., and Scheraga, H. A. (1989) *Biochemistry* **28,** 3106–3119
23. Ni, F., Meinwald, Y. C., Vasquez, M., and Scheraga, H. A. (1989) *Biochemistry* **28,** 3094–3105
24. Wilner, G. D., Danitz, M. P., Mudd, M. S., Hsieh, K.-H., and Fenton, J. W., II (1981) *J. Lab. Clin. Med.* **97,** 403–411
25. Kaminski, M., and McDonagh, J. (1983) *J. Biol. Chem.* **258,** 10530–10535
26. Berliner, L. J., Sugawara, Y., and Fenton, J. W., II (1985) *Biochemistry* **24,** 7005–7009
27. Fenton, J. W., II, Olson, T. A., Zabinski, M. P., and Wilmer, G. D. (1988) *Biochemistry* **27,** 7106–7112
28. Liu, C. Y., Nossel, H. L., and Kaplan, K. L. (1979) *J. Biol. Chem.* **254,** 10421–10425
29. Kaminski, M., and McDonagh, J. (1987) *Biochem. J.* **242,** 881–887
30. Vali, Z., and Scheraga, H. A. (1988) *Biochemistry* **27,** 1956–1963
31. Pandya, B. V., and Budzynski, A. Z. (1984) *Biochemistry* **23,** 460–470
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685
33. Kehl, M., and Henschen, A. (1981) in *HPLC in Protein and Peptide Chemistry* (Lottspeich, F., Henschen, A., and Hupe, K.-P., eds) p. 339, Walter de Gruyter, New York
34. Gralnick, H. R., Givelber, H. M. Shainoff, J. R., and Finlayson, J. S. (1971) *J. Clin. Invest.* **50,** 1819–1830
35. Carr, M. E., Jr., Kaminski, M., McDonagh, J., and Gabriel, D. A. (1985) *Thromb. Haemostasis* **54,** 159
36. Kaminski, M., and McDonagh, J. (1985) *Thromb. Haemostasis* **54,** 293
37. Nilsen, D. W. T., Brosstad, F., Kierulf, P., and Godal, H. C. (1986) *Thromb. Haemostasis* **55,** 352–356
38. Boyer, M. H., Shainoff, J. R., and Ratnoff, O. D. (1972) *Blood* **39,** 382–387
39. Furlan, M., Rupp, C., Beck, E. A., and Svendsen, L. (1982) *Thromb. Haemostasis* **47,** 118–121
40. Watt, K. W. K., Cottrell, B. A., Strong, D. D., and Doolittle, R. F. (1979) *Biochemistry* **18,** 5410–5416
41. Budzynski, A. Z., Marder, V. J., and Shainoff, J. R. (1974) *J. Biol. Chem.* **249,** 2294–2302
42. Olexa, S. A., Budzynski, A. Z., Doolittle, R. F., Cottrell, B. A., and Greene, T. C. (1981) *Biochemistry* **21,** 6139–6145
43. Kaczmarek, E., and McDonagh, J. (1988) *J. Biol. Chem.* **263,** 13896–13900
44. Higgins, D. L., Lewis, S. D., and Shafer, J. A. (1983) *J. Biol. Chem.* **258,** 9276–9282
45. Hofsteenge, J., Taguchi, H., and Stone, S. R. (1986) *Biochem. J.* **237,** 243–251
46. Naski, M. C., and Schafer, J. A. (1990) *J. Biol. Chem.* **265,** 1401–1407
47. Rosser, R. W., Roberts, W. W., and Ferry, J. D. (1977) *Biophys. Chem.* **7,** 153–157
48. Shah, G. A., Nair, C. H., and Dhall, D. P. (1985) *Thromb. Res.* **40,** 181–188
49. Bale, M. D., Janmey, P. A., and Ferry, J. D. (1982) *Biopolymers* **21,** 2265–2277
50. Janmey, P. A., Bale, M. D., and Ferry, J. D. (1983) *Biopolymers* **22,** 2017–2019
51. Janmey, P. A., Erdile, L., Bale, M. D., and Ferry, J. D. (1983) *Biochemistry* **22,** 4336–4340
52. Wilf, J., and Minton, A. P. (1986) *Biochemistry* **25,** 3124–3133