Fibrinogen Stony Brook, a Heterozygous Aα16Arg → Cys Dysfibrinogenemia

Evaluation of Diminished Platelet Aggregation Support and of Enhanced Inhibition of Fibrin Assembly

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Abstract

Assessed by high performance liquid chromatographic and amino acid sequence determinations, approximately one half (n = 4) of A peptide in fibrinogen Stony Brook (ϕ SB) contained the Aα16Arg → Cys substitution. To examine its functional behavior, mutant molecule-rich soluble subfractions that partly or fully lacked their normal A peptide were obtained from cryoprecipitates or from incoagulable material, respectively. Such subfractions consistently induced a more pronounced decrease (n = 3) in the turbidity of normal polymerizing fibrin than that induced by normal fibrinogen, by whole ϕ SB (n = 4) or by fibrinogen from an unrelated homozygous proband. These subfractions also exhibited decreased (12–50% of normal controls, fibrinogen 30–590 nM, n = 5) ADP-induced aggregation support of gel-sieved platelets, a decrease not demonstrable by whole ϕ SB, by fibrinogen from the homozygous proband, or by enrichment of the latter with normal soluble fibrin. A single isolate displaying diminished platelet aggregation support was ^{125}I-labeled and examined further. It exhibited decreased binding to platelets, and Scatchard analysis indicated decreased binding affinity but normal maximum binding. We infer that ϕ SB contained heterodimers that exhibited these distinct functional properties when their normal A peptide had been cleaved.

Introduction

Dysfibrinogenemia of congenital origin (1, 2) provides valuable models for examining the structure–function relationships and the clinical importance of fibrinogen. After cleavage of amino-terminal peptides A and B from normal fibrinogen, two pairs of binding sites are exposed on its central of three domains, GlyProArg on the α and GlyHisArg on the β chains, respectively (3). These and possibly additional sequences on the same chains bind to yet undefined complementary sites on the outer or carboxy-terminal domains of juxtaposed molecules during fibrin polymerization. The initial polymer consists of a two-molecule-thick linear strand. This progresses to form variably thicker and branching strands resulting in the assembly of the gel matrix. Because the outer domains do not require activation to bind, fibrinogen also binds to fibrin by its outer domains but its inactive central domain limits further polymer growth. At sufficiently high fibrinogen/fibrin ratios, this limiting effect can result in soluble fibrin oligomers (4) and in fibrin gels containing predominantly fine fibril structures. Dysfibrinogens may enhance this effect by their partial or no peptide A or B release, their defective or incomplete polymerization, or a combination of these.

Of the 52 structurally identified dysfibrinogens (5–30) most are single amino acid substitutions (5–28), with a high frequency of substitutions at Arg positions Aα16 (7, 19–26), Aα19 (8, 27, 28), Bβ14 (5, 14), and γ275 (6, 16–18). All dysfibrinogens have been detected because of their functional defect. For example, in 31 of the families known to date, the substitution was either Aα16Arg → His or Aα16Arg → Cys (7, 14–26), and it resulted in prolonged clotting times. It is not known if the lack of titratable −SH in dysfibrinogens with Arg → Cys substitutions (19, 31) contributes to the functional defect. Moreover, most of the known substitutions occurred in heterozygous probands in that both mutant and normal sequences were found in their fibrinogen, and these probands lacked clearly related clinical disorders. By contrast, of the five known probands whose isolated fibrinogen contained only mutant molecules (2, 20, 21, 27, 28), there was an associated hemorrhagic diathesis in four, two Aα16Arg → His (2, 20), one Aα16Arg → Cys (21), and one Aα19Arg → Ser (27).

There is little known on whether affected molecules circulate in heterozygous probands as homodimers (i.e., both identical chains are mutant), heterodimers (i.e., only one chain is mutant), or both. Efforts to identify heterodimers have been described in two Aα16Arg → His families (7, 26), by sequential use of batroxobin which cleaves only the normal A and of thrombin which cleaves both normal and mutant A peptides (24, 26). Results were consistent with the presence of heterodimers in one (7) and the absence in the other (26) family. In preliminary reports (32, 33), we identified a partly coagulable dysfibrinogen, fibrinogen Stony Brook (ϕ SB),[1] characterized by incomplete release of A peptide and delayed fibrin polymerization not corrected by equimolar mixtures with normal fibrin. The present studies describe its Aα16Arg → Cys substitution, and examine its fibrin polymerization and its platelet aggregation support. The results imply the presence of heterodimers.

Methods

Clinical laboratory studies. Blood was obtained from normal volunteers and from the asymptomatic 48-yr-old black proposita by written consent according to the guidelines of the Helsinki Declaration. Prothrombin time (18 s, normal 9–11 s) and activated partial throm-

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1. *Abbreviations used in this paper:* A, normal peptide A; A*, mutant peptide A; desA, fibrin lacking one of its two A peptides; desAdesA, fibrin lacking both of its A peptides; desBdesB, fibrin lacking both of its B peptides; desArg B, peptide known to lack its carboxy-terminal Arg; ϕ SB, fibrinogen Stony Brook; GFP, gel-filtered platelets; SB-C, SB-R, and SB-T, ϕ SB subfractions produced respectively by centrifugation, or by clotting with batroxobin or thrombin.

boplastin time (56 s, normal 26–37 s) were prolonged but equal mixtures with normal plasma yielded only partial correction (e.g., activated partial thromboplastin time of 42 s). Bleeding time and platelet counts were normal. Immunoassayed serum fibrinogen/fibrin was 180–360 μg/ml and euglobulin lysis was normal. Fibrinogen concentrations of proposita plasma (32) were normal (210 mg/dl) by immunoassay, low (56 mg/dl) by thrombin time rate–dependent (34) assay, and intermediate (160 mg/dl) by a rate–independent (35) procedure. Thrombin times of equal mixtures of normal plasma and fibrin/fibrinogen-free (36) proband serum were normal.

Fibrinogen isolation and related studies. Proposita fibrinogen fraction I-2, termed φ SB, and normal fibrinogen I-2 were isolated and an absorbance coefficient, $A^{1\%}_{1cm}$ at 280 nm, of 15.5 was assumed (37) for measuring concentrations. To assess factors well known to influence fibrin polymerization behavior, initial assessment included sialic acid content (38), SDS-PAGE (39), and coagulability (37). No differences between normal and SB fibrinogen were observed by the first two parameters, thus excluding abnormal sialic acid content and proteolytic degradation. φ SB coagulability was 21–36% ($n = 4$) by batroxobin (Pentapharm, Basel, Switzerland) and 63–71% ($n = 4$) by human thrombin (a gift from Dr. J. Fenton, New York State Department of Health, Albany, NY), normal samples yielding ≥ 96% by either enzyme. When allowed to clot at 4°C overnight ($n = 2$) in the presence of 2 mM $CaCl_2$ and Factor XIII (≃ 15 nM, a gift from Dr. S. I. Chung, National Institute of Dental Research, Bethesda, MD), thrombin coagulability was increased to ≥ 95%. Such φ SB clots showed complete cross-linking (40) of their γ and α chains.

Isolation of φ SB subfractions. Attempts were made to separate normal from mutant molecules so that the functional behavior of the latter could be examined more closely, and this resulted in isolation of three different types of φ SB subfractions, SB-R, SB-T, and SB-C. Subfractions SB-R and SB-T were batroxobin or thrombin incoagulable, respectively, harvested from the following clot supernatants. φ SB (11–27 mg/ml) solutions (10 mM PO_4, pH 7, 150 mM NaCl) were clotted with thrombin (1–2 U/ml) or batroxobin (10–30 μl/ml) containing 200 U/ml Kunitz pancreatic trypsin inhibitor (FBA Pharmaceuticals, New York), at ambient temperature for 4–6 h. Thrombin or batroxobin was then inactivated as described (36). Clots were synerized and removed, and material in clot supernatants was precipitated with 25% saturated ammonium sulfate, dissolved, and dialyzed in 300 mM NaCl. The third subfraction, SB-C, was harvested from precipitates formed in φ SB isolates,[2] during freeze-thawing (at 37°C, 11–25 mg/ml in 300 mM NaCl, $n = 4$). This was obtained by centrifugation (ambient temperature) immediately after thawing, resuspended, and washed (4°C, 1–2 h) twice with excess buffer, redissolved in 300 mM NaCl (37°C), and amounted to 26–43% ($n = 4$) of the total I-2 fibrinogen. Enrichment of SB-C with fibrin, suggested by its cryoprecipitability, was also indicated by its amino-terminal (41) Gly content of 0.22 M/2 M Tyr or moles of fibrinogen ($n = 1$) and by its decreased normal peptide A content (*vide infra*). SDS-PAGE analyses of reduced samples ($n = 4$) from all three subfractions were routinely performed to ascertain that no proteolytic degradation had occurred, and for some samples electrophoretic bands were quantified by gel densitometry (42). For example, the distribution of α, β, and γ chains of one batroxobin clot preparation was 34%, 38%, and 28% of the total, respectively, and this compared with 36%, 36%, and 28% of its clot supernatant. SB-C isolates yielded similar results as did thrombin-induced clots and their (SB-T) supernatants (32).

Fibrin preparation and polymerization. To evaluate the time course of polymerizing fibrin, polymerization was induced by ancrod (a gift from Mr. Grant Barlow, Abbott Laboratories, North Chicago, IL), by human thrombin, by batroxobin, or by repolymerization of solubilized fibrin as described (36). To obtain fibrin enriched fibrinogen, thrombin (0.001 U/ml) or batroxobin (1–3 μl/ml) added to fraction I-2 (15–33 mg/ml in 0.3 M NaCl, Kunitz pancreatic trypsin inhibitor 200 U/ml,) was inactivated (34) after 2 h and any insoluble fibrin formed was removed. Fibrin enrichment was indicated by cryoprecipitate formation at 4°C overnight, which was relatively minor or absent in untreated controls. The fibrin-enriched, normal or SB fibrinogen employed had been either thrombin treated in this way or obtained from redissolved I-2 cryoprecipitates. Another fibrin-rich isolate, fraction I-1 (37), was used in some experiments and this was electrophoretically pure consisting of band I with trace amounts of band II (42) as described.

Platelet preparation and aggregation. For obtaining platelet preparations, the anticoagulant, 0.1 vol of 3.2% sodium citrate also contained 0.01 vol of 1 mM acetylsalicylic acid (Mallinckrodt, Inc., Paris, KY) to inhibit the release reaction. Platelet-rich plasma was prepared by centrifugation (280 *g*, 15 min, 22°C). Platelets were gel filtered as described (43), using Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.01 M Hepes-modified Tyrode's solution, pH 7.5. Gel-filtered platelets (GFP) were stimulated with 10 μM ADP in the presence of increasing fibrinogen concentrations (30–600 nM). Platelet response was measured in a Chronolog Corp. (Haverton, PA) dual-channel aggregometer. Recorder baselines were set at 10% light transmission using GFP suspensions and 90% light transmission using Hepes-modified Tyrode's buffer. Data were expressed as the percentage of light transmission relative to that of buffer (100%).

Fibrinogen binding to platelets. ^{125}I-fibrinogen was prepared as described (44). Aliquots of GFP, stimulated with 10 μM ADP in the presence of increasing concentrations (0.03–3.0 μM) of labeled fibrinogen for 2 min at 22°C, were transferred on to silicone oil (*d* 1.040) and centrifuged (12,000 *g*, 4 min) in a microfuge (Beckman Instruments, Inc., Palo Alto, CA) to separate bound from unbound fibrinogen. Platelet-associated radioactivity was measured in a gamma counter. The data were analyzed according to Scatchard (45) and plotted with the aid of a Hewlett-Packard Co. (Corvallis, OR) curve-fitting program.

Size exclusion chromatography. To compare the amounts of fibrin/fibrinogen complexes in comparable normal and proposita fibrinogen isolates, 500-μg samples were subjected to gel permeation, using a 240 × 8-mm Sepharose 4B column, ambient temperature, flow rate 6 ml/h, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, monitored at 280 nm. In two pilot experiments, first, both SB-C and normal fibrinogen solutions displayed a similar, early ascending peak shoulder followed by the symmetrical protein peak. Second, addition of up to 20 μg of normal soluble (36) fibrin resulted in a similar increase of the early peaks of normal and of SB fibrinogen, respectively. In further experiments, the column was equilibrated with 0.2% BSA, and ~ 0.5 ng of ^{125}I-labeled fibrinogen with or without 100 μg of unlabeled fibrinogen was applied. Fractions (0.5 ml) were collected and counted in a gamma counter.

Fibrinopeptide analyses. Separation and isolation of A and B peptides (46) was performed by HPLC as described. To obtain maximum release of peptides from S-aminoethyl chains (22), 400 μg of chains was suspended in 200 μl of 150 mM ammonium acetate buffer, pH 8.5, and incubated with thrombin (2 U/ml) for 18 h. For peptide release from unmodified fibrinogen a 4-h incubation of 1–3 μM fibrinogen with 2–4 U of thrombin/ml in the same buffer was sufficient, in that further incubation yielded no additional A peptide. For rate of release

2. Two of six φ SB preparations lacked major amounts of this precipitate, but displayed it after thrombin treatment as described in Methods. Variable amounts of precipitates also formed in some freeze-thawed previously untreated normal I-2, in all ($n = 3$) thrombin-treated normal (I-2) and in untreated band I fractions of which A peptide content was decreased (34). In these normal isolates precipitates were coarse strands and some could be only partly redissolved and were discarded. Precipitates from φ SB (I-2) were finely granular and always redissolved. Thrombin times of one redissolved normal cryoprecipitate (1 μM, pH 7, $I = 0.16$) were 18 s (17.2–19.5, $n = 3$) as compared to 57.2 s (55.4–57.9) of SB-C. Under conditions detailed in Table II, thrombin times of SB-C were 83.6 s (81.8–85.4, $n = 3$). Comparison by electrophoresis (SDS-PAGE) of these SB subfractions and normal fibrinogen disclosed no differences.

comparisons, 300 μg of fibrinogen was mixed with thrombin (0.05–0.1 U/ml) in 200 μl of buffer (22), 37°C, and the reaction was stopped by brief immersion (45–60 s) in boiling water. In some experiments the resulting protein precipitates were repeatedly washed with deionized water to extract and correct for occluded A and B peptides. Supernatants were passed through a 0.45-μm filter before HPLC and protein (206 or 210 nm) peaks were measured by planimetry.

Amino acid and related analyses. During the amino acid analyses (47), elution conditions (22) permitting separation of S-aminoethyl Cys from Lys and His were employed. Sequence analyses (48) were performed as described. Measurements of −SH groups were performed by use of the 4-vinylpyridine procedure (49) as follows. Fibrinogen was dissolved in 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.6, immediately mixed with vinyl pyridine (2% vol/vol), and the mixture incubated for 90 min at room temperature. After extensive dialysis the samples were hydrolyzed for amino acid analysis. S-β-(4-pyridylethyl) cysteine content was computed using standards as described.

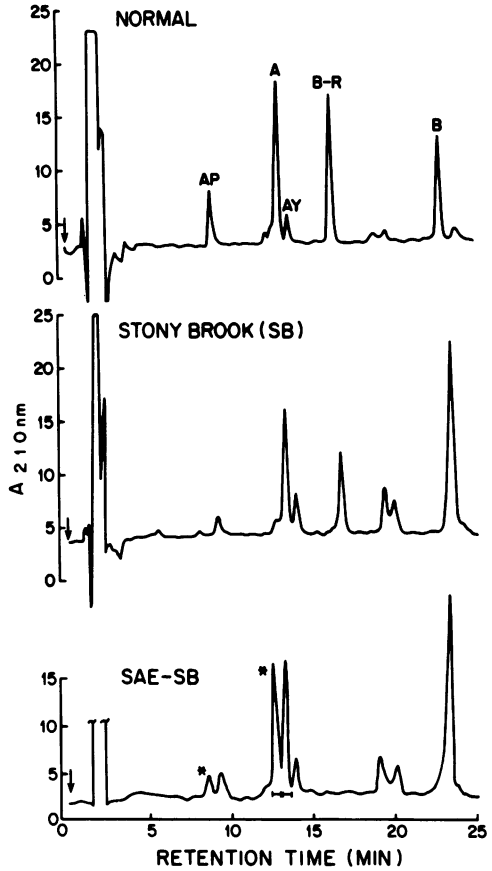

Figure 1. Comparison by HPLC of the maximum amounts of fibrinopeptides released from normal and SB fibrinogen. Ordinate ($A_{210\,nm}$): absorbance scales are shown $\times 10^3$. AP, peptide A containing a phosphorus group on its Aα3Ser; AY, peptide A lacking its amino terminal Ala; B, peptide B lacking its carboxy-terminal Arg; SAE, S-aminoethylated chains. These peptides had been released from ~ 400 μg of normal (*upper graph*), φ SB (*middle graph*), and SAE φ SB (*bottom graph*), respectively. Bracketed horizontal lines indicate peaks harvested for amino acid and sequence analyses. Bottom graph shows that among peptides released are abnormal peptides A* and AP*. Normal SAE control (not shown) did not differ from the upper graph shown with the exception that like all SAE preparations it also lacked the B-R (desArg B) peptide, a lack presumed to reflect loss of contaminant endopeptidase(s) during the aminoethylation procedure. Vertical arrows indicate initiation of gradient preceded by sample application.

Table I. Results of Amino Acid Analysis

	φ SB		φ normal	
Amino acid	A*	A	A	A expected
			mol/mol	
Asp	2.1	2.1	2.2	2.0
Ser	1.1	1.0	1.1	1.0
Glu	2.1	2.1	2.1	2.0
Gly	4.8	4.8	5.0	5.0
Ala	2.0	1.9	2.0	2.0
Val	0.9	0.9	0.8	1.0
Leu	1.0	1.0	0.9	1.0
Phe	1.0	1.1	0.9	1.0
Cys	0.7	0.2	0.1	0
Arg	0.2	0.8	0.7	1.0

The expected A peptide values are entered from the known composition of normal A peptide for comparison. Minor amounts of Cys in normal A peptide and of Arg in mutant A* peptide are attributable to the minor peak overlap (Fig. 1, *bottom graph*) during the HPLC peptide separation. A, normal A peptide; A*, mutant A peptide.

Results

Identification of the abnormal structure. Measurements of released fibrinopeptides (Fig. 1) disclosed that approximately half of peptide A could not be released from φ SB. The known failure of Aα16Arg → Cys dysfibrinogens (22, 23) to release their mutant A peptide led us to explore release of the mutant A peptide after reduction and aminoethylation. This resulted in release of an abnormal peptide (Fig. 1, *bottom graph*). Isolation of this peptide and amino acid analyses (Table 1) established that this was the remainder of peptide A, and disclosed in addition that it contained Cys and lacked Arg. Amino acid sequence analyses confirmed the substitution of Aα16Arg by Cys. In addition, assay of titratable −SH disclosed < 0.05 mol/mol of φ SB. Thus, the presence of both normal and mutant peptide A demonstrated the heterozygous expression of this substitution.

Analyses of polymerization and rates of peptide release. Studies were directed at the characterization of polymerization of SB fibrin lacking either its normal A of both its normal A and B peptides. Prolonged thrombin clotting times (Table II) were shown and were consistent with the delayed fibrin formation disclosed by turbidity measurements (Fig. 2 *A*). In addition, decreased turbidity could be shown whether SB fibrin

Table II. Comparison of Thrombin Times of φ SB and Normal Single Donor Fibrinogen

	φ SB (6)	Normal (4)
	s	
Mean	69.9	25.3
Range	58.4–72.0	25.1–25.5

The number of determinations is shown in parentheses. Clot mixtures contained 1.3 μM fibrinogen and 0.3 U/ml thrombin, pH 7.4, I = 0.15. Reagents were kept on ice and prewarmed 3 min (37°C) before testing.

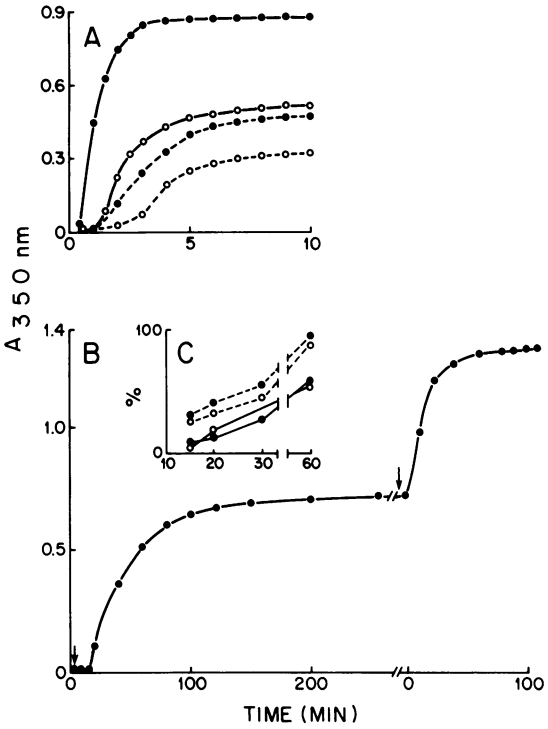

Figure 2. Turbidimetric comparisons of SB and normal fibrin polymerization. (*A*) Solid lines (normal, •; SB, ○): thrombin (0.5 U/ml) was added to fibrinogen (1.7 μM) solutions in Tris-HCl, pH 7.4 (I = 0.16) buffer containing 2 mM $CaCl_2$. Dashed lines (normal, •; SB, ○): repolymerization of 1 μM fibrin, 50 mM PO_4, pH 7, and 50 mM NaCl (one of three experiments showing similar results by use of different buffer and fibrin concentration conditions). (*B*) Polymerization of φ SB induced by batroxobin (*left arrow*) and by subsequent addition of thrombin (*right arrow*). Dry batroxobin, 50 μg, was dissolved in 700 μl of fibrinogen (7 μM, pH 7.5, I = 0.15) and at ~ 280 min the clot was synerized and removed. Thrombin (3 U/ml) was then added to the clot liquor and the further rise in turbidity was plotted by adding the absorbance increments to the maximum absorbance of the preceding clot. Two aliquots of clot supernatant, one obtained before addition of thrombin and the other after syneresis and removal of the thrombin clot, were analyzed by HPLC, and are shown in Fig. 3, graphs *I* and *II*, respectively. (*C*) Time course comparisons of normal A (– – –) and B (——) peptides released from unmodified normal (•) and SB (○) fibrinogen as detailed in methods. Amounts released are plotted as percentage of total releasable A or B, respectively.

was solubilized and repolymerized (Fig. 2 *A, lower set of graphs*) or φ SB was polymerized by exposure to thrombin (Fig. 2 *A, upper graphs*). Moreover, the decrease in turbidity was always greater in batroxobin-induced than in thrombin-induced SB fibrin clots (not shown). This was consistent with the coagulability results (see Methods) showing that between one third and one half of φ SB contained molecules which were incoagulable with batroxobin but coagulable with thrombin. Also, a major increase in turbidity was demonstrated (Fig. 2 *B*, one of two experiments) when thrombin was added after the polymerization of φ SB by batroxobin. These results indicated that φ SB contained molecules which polymerized or incorporated in polymerizing fibrin only after release of their B peptides. In a related series of experiments, the release rates of its normal A and B peptides were examined. Time course comparisons (Fig. 2 *C*) disclosed that release rates of both its normal A and B peptides did not differ from those of normal fibrinogen.

Polymerization comparisons of φ SB and its subfractions. Differences in coagulability between φ SB isolates lacking their normal A and B and those lacking only their normal A (*vide supra*) were explored further by use of isolated subfractions. Subfraction SB-C was characterized by decreased normal peptide A (27% and 31%, n = 2, Fig. 3, *graph III*), its enrichment with soluble fibrin (see Methods), and its otherwise intact subunit polypeptide chains (see Methods). Also, its B peptide content was not diminished (Fig. 3), indicating that its increased amino-terminal Gly reflected prior release of normal peptide A. Together these two measurements accounted for approximately one half of its peptide A, indicating its mutant/normal A peptide ratio was similar to that of the parent φ SB. In other comparisons, SB-C displayed longer clotting times,[2] lower thrombin coagulability (Table III), and a more marked decrease in its clot turbidity (Fig. 4 *A*) than that of parent φ SB. Also, inspite of its fibrin enrichment, increased fibrin/fibrinogen oligomers could not be shown (Fig. 4 *B*). In addition, SB-C displayed a more marked inhibition of polymerization of normal (desAdesA/desBdesB) fibrin, i.e., fibrin lacking both its A and B peptide pairs (Fig. 4, *C* and *D*) than that of its parent φ SB. In control experiments not shown, a comparable normal fibrin-rich isolate[2] and its parent fibrinogen fraction displayed

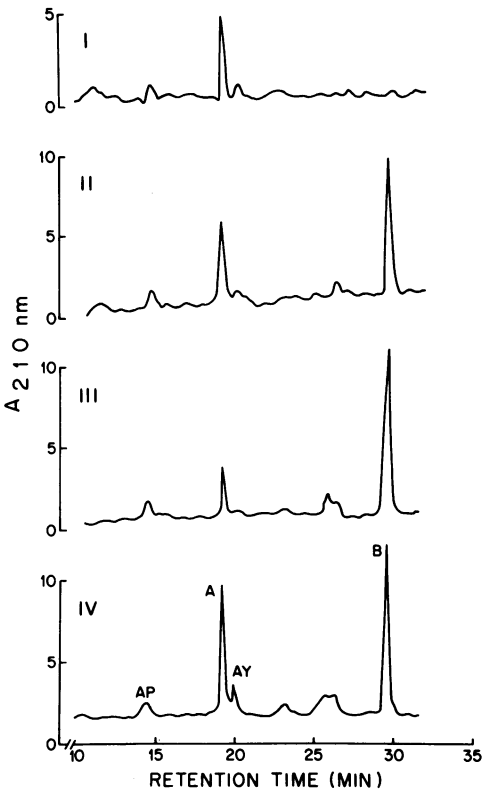

Figure 3. HPLC comparison of fibrinopeptides released from φ SB, SB-C, and from normal fibrinogen. Absorbance ($A_{210\,nm}$) scale shown was as described in Fig. 1. Chromatograms *I* and *II*: fibrinopeptides contained in 100-μl aliquots of batroxobin (*I*) and thrombin (*II*) SB clot supernatants during the experiment shown in Fig. 2 *B*. Chromatograms *III* and *IV*: fibrinopeptides released by thrombin from 400 μg each of subfraction SB-C and a normal control, respectively.

Table III. Thrombin Coagulabilities of ϕ SB and Its SB-C Subfraction

Preparation	ϕ SB (5)	SB-C (3)
	%	
Mean	65	29
Range	54–72	23–36

Normal controls were ≥ 96% coagulable (see Methods). (Batroxobin coagulability was 27% and 34% for ϕ SB, and negligible for SB-C; two preparations of each were tested.)

minor or negligible differences in clotting times and in maximum turbidity. These results indicated that the mutant molecules in SB-C were more inhibitory against polymerizing normal desAdesA/desBdesB fibrin than were those in the parent ϕ SB.

Two other subfractions, SB-R and SB-T (see Methods), were also examined. Subfraction SB-R displayed functional similarity to SB-C by its enhanced inhibition of the polymerization of normal fibrin (Table IV and Fig. 4, *C* and *D*). Subfraction SB-T, however, lacked this enhanced inhibition of normal fibrin shown by SB-C, SB-R, and whole ϕ SB.

Investigation of platelet aggregation support. In view of a possible role by the Aα chain in the platelet fibrinogen interaction (50–54), we explored the support of platelet aggregation by ϕ SB and its subfractions. SB-C (n = 3) displayed a diminished support of ADP-induced platelet aggregation, at a range of fibrinogen concentrations (Figs. 5 and 6), an effect also displayed by subfractions SB-R and SB-T (Table V). By contrast, whole ϕ SB (n = 3) and fibrinogen from a homozygous proband[3] did not differ from normal controls. A single SB-C isolate was examined further in binding experiments, on three different occasions using platelets from three different donors. The results showed decreased binding to platelets (Fig. 7, *left graph*). Comparison of Scatchard plot analyses (Fig. 7, *right graph*) confirmed the decrease in normal fibrinogen binding affinity as receptor occupancy increased (43). In addition, this comparison indicated a significant reduction in the high-affinity binding of SB-C to platelets (K_d = 0.196±0.06 μM, n = 3), relative to that of normal single-donor fibrinogen (K_d = 0.040±0.02 μM, $P < 0.05$). The binding maxima, however, were similar indicating 50,741±16,103 and 55,242±15,247 molecules per platelet for SB-C and normal fibrinogen, respectively.

Discussion

Two important findings emerged from this investigation, the diminished platelet aggregation support by isolates enriched with structurally defined mutant molecules, the first report of such an association,[4] and evidence for the presence of heterodimeric molecules. The latter is deduced from a series of foregoing observations. First, this was a heterozygously expressed dysfibrinogenemia, and in order to evaluate the functional behavior of mutant molecules, isolation of subfractions enriched with them was necessary. To achieve this, advantage was taken of the characteristically partial coagulability of whole ϕ SB at ambient or higher temperatures. Secondly, these subfractions (Table VI) displayed diminished platelet aggregation support, an effect not demonstrated by the parent ϕ SB or by an identical dysfibrinogen from an unrelated homozygous proband.[3] Thirdly, there was no proteolytic degradation or other subunit chain anomaly[5] demonstrable to explain this behavior. Fourthly, inhibition of fibrin polymerization, more pronounced by the parent ϕ SB than by normal fibrinogen, was most pronounced when subfractions SB-C or SB-R were employed. Lastly, the unique incoagulability of these subfractions also supports the conclusion of heterodimer presence as follows. It is well known that normal desAdesA/BB fibrin (61) is coagulable while normal AA/desBdesB fibrin is incoagulable (4) at ambient or higher temperatures. We thus conclude that of the molecules in the thrombin incoagulable subfraction, SB-T, most were mutant homodimers, i.e., A*A*/desBdesB. By contrast, the undiminished amounts of B peptide in subfractions SB-C and SB-R and their solubility at ambient temperatures left only one other possible explanation, enrichment with heterodimeric molecules of which all (i.e., SB-R) or a major subgroup (i.e., 22% of SB-C showing amino-terminal Gly) lacked their normal peptide A. That normal fibrinogen, even when saturated with soluble fibrin (see Methods), yielded only minor amounts of cryoprecipitate compared with those of ϕ SB lends support to this conclusion.

The conclusion on heterodimers raises two possible explanations regarding the diminished platelet aggregation support. We favor the assumption that diminished platelet aggregation support resulted from steric hindrance during binding to platelets, owing to the presence of soluble complexes containing A*desA/BB heterodimers. Our failure to detect increased soluble fibrin complexes does not exclude their presence as the known dissociability of normal desAdesA/BB oligomers (4) implies. Assuming such complexes in concentrations higher than those attainable in normal fibrin-saturated fibrinogen, it is possible that fibrinogen binding sites were partly inaccessible to the platelet receptors. For example, if such an oligomer

3. Galanakis, D. K., J. C. Soria, and E. I. Peerschke. Unpublished observations. A single Metz (fibrinogen Metz, references 21 and 55) preparation displayed no differences from the normal single-donor control in ADP-induced platelet aggregation support used in concentrations shown in Fig. 5. Similarly, equimolar mixtures of fibrinogen Metz and either fibrin-enriched normal I-2 or I-1 (see Methods, single experiments) disclosed no decrease in platelet aggregation support. In fibrin polymerization experiments, equimolar mixtures of normal and of ϕ Metz (1.5 M, pH 7.4, I = 0.15, 37°C, 60 min) yielded a maximum batroxobin-induced clot turbidity 78% that of the normal control, the fibrinogen Metz control showing no turbidity rise as expected. By contrast, mixtures of ϕ SB and normal fibrinogen resulted in maximum turbidity 20% and 14% of control, for the mixture and ϕ SB alone, respectively.

4. Fibrinogen Oslo I (56, 57) reportedly displayed enhanced platelet aggregation support, increased binding to platelets (56), and abnormally short clotting times. An association with thrombotic disorders (57) was shown only in some dysfibrinogenemic probands. To date, identification of its structural defect has not been described.

5. A minor subfraction (i.e., ~ 15%) of normal fibrinogen contains a γ-chain variant (58) of which the carboxy-terminal sequence is extended by 16 amino acids (i.e., the last four amino acids are replaced by a 20-residue peptide). These molecules display diminished platelet aggregation support (50, 59, 60) particularly when lacking carboxy-terminal segments of their Aα chains (50). We measured (42) this normal variant and no differences between ϕ SB (n = 2), normal fibrinogen could be shown.

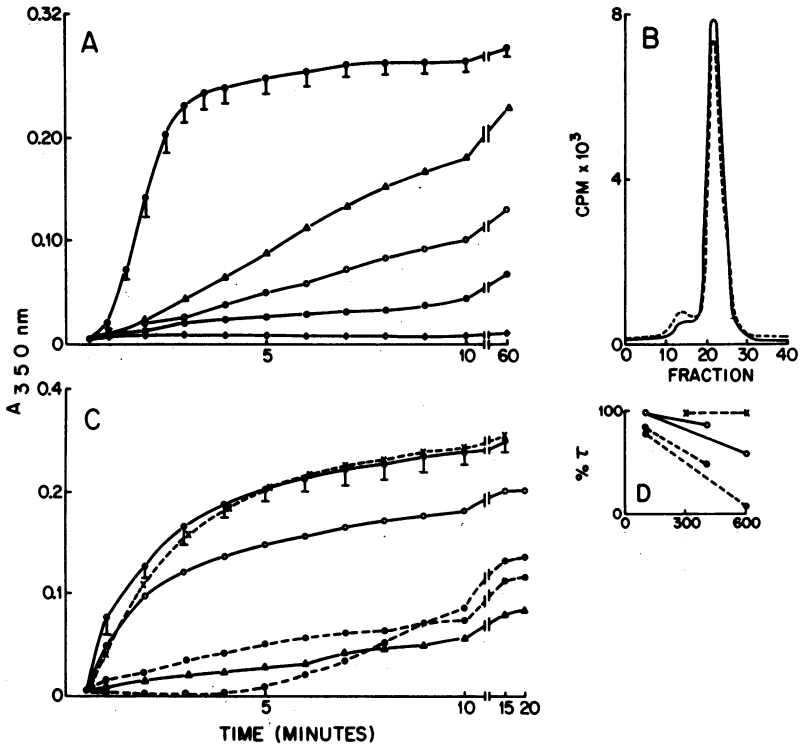

Figure 4. (*A, C,* and *D*) Turbidimetric and (*B*) chromatographic comparisons of ϕ SB subfractions. (*A*) Ancrod-induced fibrin polymerization. Normal (●; range of triplicates shown by vertical lines), ϕ SB containing (○) and lacking (△) SB-C, and SB-C before (●) and after (◇) washing with buffer at 4°C (see Methods). Polymerization conditions were buffer as in Fig. 2*A*, I = 0.17, 23°C, fibrinogen 1 μM, and ancrod 5 U/ml. (*B*) Comparison of SB-C (– – –) with normal (——) fibrinogen by gel permeation chromatography. ^{125}I-labeled fibrinogen in unlabeled fibrinogen excess (as described in Methods) was applied to the column at room temperature. (*C*) Effect of ϕ SB subfractions (0.4 μM) on the repolymerization of normal fibrin (0.4 μM, pH 7.4, I = 0.15). Results shown are from two experiments using an SB-R (●-----●) isolate and from single experiments using SB-T (× – – – ×), SB-C (△ —— △), parent ϕ SB (○ —— ○), and normal fibrinogen (● —— ●) triplicates; range shown by bracketed vertical lines. (*D*) Effect of ϕ SB and of two of its subfractions on turbidity maxima of 0.6 μM normal fibrin. Two preparations each of ϕ SB (○) and SB-C (●) and a single preparation of SB-T (×) are compared at the concentrations shown. Results are plotted as percentage of buffer control containing the corresponding concentrations of normal fibrinogen.

bound to a receptor, the receptor-free molecules in the oligomer may have been inaccessible to neighboring receptors. Since a major fraction of the molecules could form such potential oligomers, however transiently, this can explain the observed decrease in platelet binding and aggregation support. The clearly high proportion of soluble fibrin in ϕ SB-C (i.e., A*desA/BB heterodimers, *vide supra*) and in SB-R lends support to this assumption. That this effect was not displayed by the parent ϕ SB (Table VI) implies that the amounts of molecules other than A*desA/BB heterodimers were in sufficient excess to interact with platelets normally. A similar fibrinogen excess can explain the normal platelet aggregation support displayed by redissolved normal fibrin/fibrinogen solutions.[3] That is, we assume that the fibrinogen/fibrin ratio was much greater in normal fibrinogen/fibrin than in SB-R or SB-C solutions.

Table IV. Comparison of the Effects of ϕ SB Subfractions SB-R and SB-T on the Maximum Turbidity of Normal Repolymerized desAA/desBB (0.6 μM) Fibrin

	Stony Brook		
	SB-R	SB-T	Normal
μM		%	
0.2	88	102	97
0.4	37	96	95
0.6	2	104	84

Values reflect percent turbidity relative to single buffer (PO_4, pH 7, I = 0.15, ambient temperature) controls determined for each inhibitor concentration. Normal fibrinogen controls are shown on the right.

The second possible explanation presumes that the A*desA/BB heterodimer reacted with the platelet receptor in an altered way perhaps owing to a perturbed conformation in its platelet recognition sequences. Platelet receptor recognition sequences (52–54, 62) are not contiguous with the present defect. For example, the Aα95–97 sequence, the closest structure possibly playing a role in normal binding to platelets (52), is 78 residues from the present mutant site. The distance between the present defect and the platelet recognition sequences notwithstanding, evidence for conformational perturbations distant to altered fibrinogen sites has been described (63).

The distribution of the three dimeric forms in ϕ SB cannot be precisely ascertained. We postulate from our results and conclusions, however, that approximately one quarter of ϕ SB molecules were normal homodimers, one quarter were mutant homodimers, and one half were mutant/normal heterodimers. The partial thrombin (i.e., 63–71%) coagulability is attributable to mutant homodimers accounting for most of the thrombin-incoagulable species. Even with correction for other molecular forms present (i.e., this is a crude form of separation), the mutant homodimer amount in SB-T could account for approximately one quarter of the total in ϕ SB. Similarly probable is that batroxobin (i.e., 21–36%) coagulability reflected normal homodimers in amounts approximately one quarter of ϕ SB molecules. By extension, heterodimers seem to

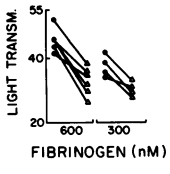

Figure 5. Effect of a single SB-C isolate on ADP-induced platelet aggregation. Light transmission values (see Methods, single determinations) of SB-C (△) are compared with those of a normal fibrinogen control (●), at the two fibrinogen concentrations shown. Lines are drawn through the respective test and control points to guide the eye.

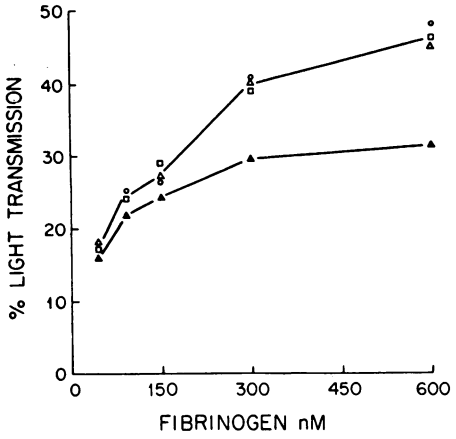

Figure 6. Comparison of the effects on ADP-induced aggregation of aspirin-treated platelets by ϕ SB and SB-C. The extend of aggregation response is expressed in light transmission units as defined in methods. GF platelets were ADP stimulated in the presence of increasing fibrinogen concentrations of single isolates of SB-C (▲), normal single-donor fibrinogen (○), and ϕ SB containing (□) and lacking (△) SB-C.

Table V. Effect of Single Isolates of Subfractions SB-R and SB-T on the ADP-induced Aggregation of Aspirin-treated GFP

	Light transmission (decrease from normal)	
Fibrinogen	SB-R	SB-T
nM	%	
30	25	22
73	48	
145	42	
220	44	
290	50	13
440	37	
580	43	

Values reflect decrease in light transmission as percentage of single-donor control (100%). Experimental conditions were as described in Methods.

have accounted for the remainder one half of ϕ SB. Consistent with this is that SB-R represented 66–79% of ϕ SB. Since one third of SB-R contained thrombin-incoagulable molecules (*vide supra*), the remaining two thirds of (thrombin coagulable) molecules in SB-R represented one half of ϕ SB. An apparent discrepancy emerges when SB-C (i.e., up to 43% of ϕ SB, see Methods) is considered, since most, but not all, of SB-C is concluded to have consisted of heterodimers (*vide infra*). These fibrin heterodimers were highly soluble and presumed losses into the fibrinogen solution during isolation of SB-C could not be estimated. Also, the maximum possible amounts of SB-C which could be formed by thrombin exposure of SB were not investigated. Nevertheless, the amino Gly content and the estimated amounts of A*desA/BB + A*A/BB heterodimers in SB-C approached one third of ϕ SB, in general agreement with our assumption that these molecules represented a greater fraction of ϕ SB than that of normal or that of mutant homodimers. The distribution of the three possible molecular forms that we postulate is consistent with a random mutant/normal Aα chain incorporation during the assembly of ϕ SB molecules, also suggested for a heterozygously expressed Aα16Arg → His dysfibrinogen (7) in which a similar distribution was estimated.

Enrichment of SB-C subfractions with A*A/BB (fibrinogen) heterodimers (Table VI) can also be reasoned from foregoing observations. It is clear that ϕ SB yielded this major subfraction only when some of its normal A peptide had been cleaved.[2] That is, measurements of amino terminal Gly and normal peptide A content in SB-C (*vide supra*) easily accounted for one half of its total A* + A peptides. Random cleavage of A from heterodimers and from normal homodimers can be expected to result in similar amounts of fibrinogen associated with each. Thus, by random association and assuming the distribution proposed above, approximately one half of these fibrinogen molecules in SB-C were A*A/BB heterodimers, the remainder being mutant and normal homodimers. The expected result is enrichment of SB-C with fibrinogen heterodimers.

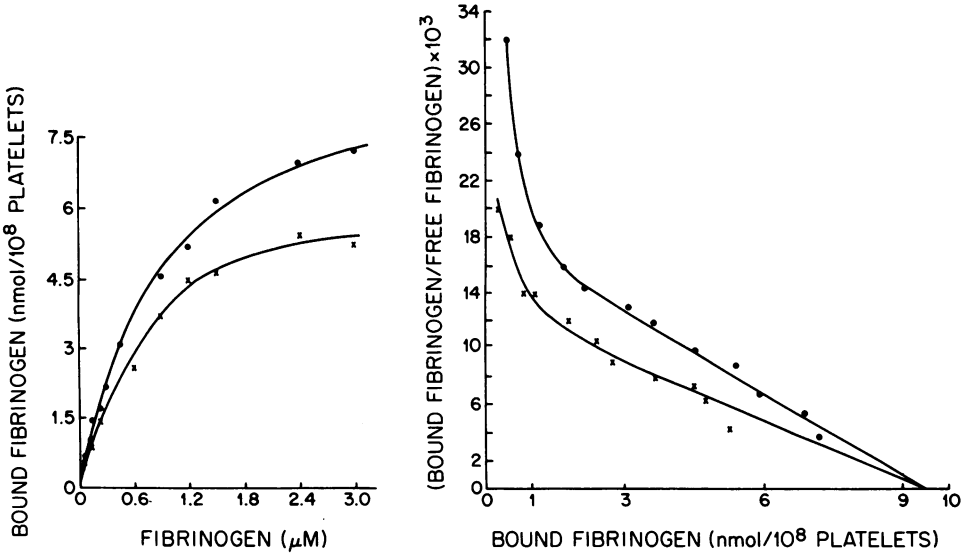

Figure 7. Platelet-binding comparison of single isolates of normal fibrinogen (●) and of SB-C (×). (*Left*) Binding to ADP-stimulated GF platelets. Platelets were stimulated with 10 μM ADP in the presence of increasing concentrations of ^{125}I-labeled fibrinogen as shown. (*Right*) Comparison by Scatchard analysis of ^{125}I-labeled fibrinogen binding to ADP-stimulated platelets.

Table VI. Summary of the Proposed Molecular Species and of the Functional Properties of ϕ SB and Its Three Subfractions

Isolate	SB-C	SB-R	SB-T	Parent ϕ SB
Molecular species	A*A/BB A* desA/BB	A* desA/BB	A*A*/desBdesB	AA/BB (¼) A*/A/BB (½) A*A*/BB (¼)
Platelet aggregation support	Decreased	Decreased	Decreased	Normal
Fibrin assembly inhibition	Enhanced	Enhanced	Not enhanced	Enhanced

Molecular species listed under each subfraction represent only the major or predominant molecules (i.e., amounting to at least one half of the population in each subfraction). Those listed under Parent ϕ SB represent only fibrinogen species, and shown in parentheses is their postulated distribution (i.e., this includes any amounts of the corresponding fibrin forms present, see Discussion).

A unique solubility of mutant/normal (A*desA/BB) heterodimers is also implied by the foregoing results. It is clear that such forms were readily soluble at ambient or higher temperatures, in contrast to the known solubility behavior of other fibrin forms. This conclusion is consistent with the well known slower release of peptide B compared to that of A peptides (4), and with the known coagulability of desAdesA/BB fibrin at ambient or higher temperatures (61). It therefore assumes that in rewarming the cryoprecipitate, the desAdesA/BB fibrin would tend to form insoluble polymers and be removed. Moreover, it is in general agreement with the known dissociability of normal desAdesA/BB fibrin being greater than that of normal desAdesA/desBdesB fibrin (4). The cryoprecipitation procedure, therefore, served to separate these fibrin heterodimers from most of the excess fibrinogen and their presumed weak association permitted their resolubilization on rewarming. There is no information on normal AdesA/BB heterodimers, but our results raise the possibility that such normal fibrin molecules display properties similar to those we have ascribed to ϕ SB heterodimers.

Absence of hemostatic disorders in our and in other reported heterozygous probands with this anomaly (2, 8) is consistent with the foregoing results. The delayed clotting times of ϕ SB clearly reflect incomplete A release. Evidence that major amounts of B peptides are released during spontaneous blood clotting (64), and that B peptide release rate is unaffected by the foregoing anomaly, suggests that sufficient B is released during in vivo thrombogenesis to offset the fibrin polymerization inhibitory effect by the transiently formed A*desA/BB heterodimers. Primary hemostasis was also unaffected since the platelet aggregation inhibition was not demonstrable by whole ϕ SB, presumably owing to the presence of insufficient amounts of A*desA/BB heterodimers relative to the mutant and/or normal fibrinogen.

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