Nonclottable Fibrin Obtained from Partially Reduced Fibrinogen: Characterization and Tissue Plasminogen Activator Stimulation†

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ABSTRACT: Out of 29 disulfide bonds in human fibrinogen, 7 were cleaved during limited reduction under nondenaturing conditions in calcium-free buffer: 2 Aα442Cys–Aα472Cys and 2 γ326Cys–γ339Cys intrachain disulfide bonds in the carboxy-terminal ends of the Aα- and γ-chains and the symmetrical disulfide bonds at γ8Cys, γ9Cys, and Aα28Cys. We studied the loss of thrombin clottability that followed limited reduction and the increase in the susceptibility of the fibrinogen Aα19–Aα20 bond to hydrolysis by thrombin. Using differential scanning calorimetry, we show that the extent of unfolding and denaturation of specific domains following limited reduction is small. Heat absorption peaks corresponding to the melting of the major regions of compact structure give high calorimetric enthalpies, as in untreated nonreduced fibrinogen, indicating that substantial regions of native structure are still present in partially reduced fibrinogen. Thrombin releases fibrinopeptide A at an identical rate as in nonreduced fibrinogen while fibrinopeptide B release is slower. Sedimentation velocity studies show that thrombin treatment leads to complex formation; however, gelation does not occur. Amino-terminal analysis indicates that the second thrombin cleavage in the Aα-chain at Aα19–Aα20 takes place only after fibrinopeptide A release. Thus, the loss of clottability appears to result from perturbation of carboxy-terminal polymerization sites, probably a consequence of γ326Cys–γ339Cys intrachain disulfide bond cleavage. The thrombin-treated partially reduced fibrinogen remains soluble in buffered saline and fully expresses at least one epitope, Bβ15–21, unique to fibrin. Furthermore, this nonclottable form accelerates the tissue plasminogen activator dependent conversion of plasminogen to plasmin.

Human fibrinogen has a molecular weight of 340 000 and contains six polypeptide chains: two Aα-, two Bβ-, and two γ-chains [for a review of the structure, see Furlan (1988) and Doolittle (1984)]. There are 17 interchain disulfide bonds located in clusters in the amino-terminal and middle regions of the chains and 12 intrachain disulfide bonds in the middle and carboxy-terminal regions. Reduction under mild conditions with a low concentration of dithiothreitol (DTT)[1] leads to cleavage of the two Aα442Cys–Aα472Cys intrachain disulfide bonds in the carboxy-terminal region of the Aα-chains, the two γ326Cys–γ339Cys intrachain disulfide bonds in the carboxy-terminal end of the γ-chains, and the symmetrical Aα28Cys–Aα28Cys bond which links the two Aα-chains in the central globular region of the molecule. Free chains of fibrinogen are not released during or following the limited reduction (Procyk & Blombäck, 1990). A loss of clottability with thrombin occurs as a consequence of the limited reduction. Thrombin cleaves the partially reduced fibrinogen at the normal Aα16–Aα17 site to release fibrinopeptide A; however, a second cleavage site at Aα19–Aα20 results in release of the Aα17Gly–18Pro–19Arg segment (Procyk & Blombäck, 1990), a peptide that can block fibrin polymerization (Laudano & Doolittle, 1978). The sequence of cleavage events in partially reduced fibrinogen and the effect on clotting have not been previously investigated.

The loss of clottability does not occur in fibrinogen that is subjected to limited reduction in buffer containing calcium ions (Blombäck et al., 1985). Calcium prevents reduction of the γ326Cys–γ339Cys intrachain disulfide bonds, and thrombin cleavage occurs only at the normal Aα16–Aα17 site (Procyk & Blombäck, 1990). These observations suggest that perturbation of tertiary structure supported by the γ326Cys–γ339Cys intrachain disulfide bond may somehow be related to the loss of clottability and susceptibility of the Aα19–Aα20 bond to thrombin cleavage. The present study was conducted to determine the relationship between disulfide bond reduction, the stability of compact structures, thrombin cleavage at Aα19–Aα20, and the loss of clottability. In addition, the expression of epitopes in partially reduced fibrinogen and in thrombin-treated material and also the ability of these

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[1] Abbreviations: desAA-fibrin (or fibrin I), fibrin with fibrinopeptides A removed; desAABB-fibrin (or fibrin II), fibrin with both fibrinopeptides A and B removed; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; HT, high temperature; IC_{50}, concentration of fibrinogen (or its derivatives) in chain equivalents causing 50% inhibition of antibody binding; LT, low temperature; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; PTH, phenylthiohydantoin; R-FBG, fibrinogen following limited reduction in calcium-free buffer (see Materials and Methods); R-FBG-Ca, fibrinogen following limited reduction in buffer containing calcium (see Materials and Methods); R-FBG-Th, fibrinogen subjected to limited reduction in calcium-free buffer and subsequently treated with thrombin (see Materials and Methods); SDS, sodium dodecyl sulfate; TNE-buffer, Tris–saline–EDTA buffer (0.05 M Tris, 0.1 M NaCl, and 1 mM EDTA, pH 7.4); t-PA, tissue plasminogen activator; Tris, tris(hydroxymethyl)aminomethane.

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modified forms of fibrin(ogen) to stimulate the tissue plasminogen activator (t-PA)-dependent conversion of plasminogen to plasmin are reported.

Materials and Methods

Proteins and Reagents. Human fibrinogen and plasminogen-free fibrinogen (Imco, Stockholm, Sweden), obtained from American Diagnostica, Inc. (Greenwich, CT), were processed as previously described (Procyk & Blombäck, 1990) and stored at −70 °C in Tris–saline–EDTA buffer (0.05 M Tris, 0.1 M NaCl, and 1 mM EDTA, pH 7.4; TNE-buffer). Plasminogen, t-PA, and the chromogenic substrate S-2251 were obtained from Kabi Vitrum (Stockholm, Sweden). Human thrombin and goat anti factor XIII antiserum were obtained from the Department of Blood Coagulation Research, Karolinska Institutet (Stockholm, Sweden). Iodoacetic acid (Sigma, St. Louis, MO) was recrystallized 3 times from petroleum ether before use. Hirudin was obtained from Pentapharm (Basel, Switzerland).

Factor XIII Free Fibrinogen. Goat anti-factor XIII IgG–Sepharose 4B was prepared by coupling the IgG fraction [obtained by precipitation of the serum with 33% (v/v) saturated ammonium sulfate] to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ). Fibrinogen (12 mg/mL) in TNE-buffer was mixed with goat anti-factor XIII immunoadsorbent (3 mL wet volume/mL of fibrinogen) overnight at 4 °C in sealed Centrex disposable centrifugal microfilters (Schleicher & Schuell, Keene, NH). The fibrinogen was recovered by centrifugation.

Fibrin Monomer. Fibrinogen (0.3 μM) was incubated with thrombin (2 units/mL) in TNE-buffer containing 10 mM calcium and 6 mM Gly-Pro-Arg-Pro peptide (Sigma, St. Louis, MO) overnight at room temperature, conditions that led to complete release of fibrinopeptides A and B.

Antibodies. Preparation and characterization of the monoclonal antibodies T2G1, 1-8C6, 1D4/xl-f, and 4-2/xl-f were described (Procyk et al., 1991). Briefly, antibody T2G1 (IgG_1 κ) recognizes the epitope Bβ15–21 found in fibrin II (des-AABB-fibrin) (Kudryk et al., 1984) and does not react with either fibrinogen or fibrin I (desAA-fibrin). It was kindly supplied by Dr. John M. Brown of Centocor, Inc. (Malvern, PA). Antibody 1-8C6 (IgG_{2a} κ), directed against an epitope present in or around the thrombin-susceptible Bβ14Arg–15Gly bond, reacts with fibrinogen, fibrin I, or derivatives of either which contain at least the Bβ1–21 segment (Kudryk et al., 1983). Antibody 1-8C6 does not react with fibrin II. Antibody 1D4/xl-f (IgG_1 κ) is directed to an epitope located in the carboxy-terminal region of the Aα-chain and reacts completely with fibrinogen, purified Aα-chain, and intact or trypsin-digested CNBr fragment Aα241–476. Antibody 4-2/xl-f reacts with isolated γ-chain, peptide γ392–411, and the tryptic fragment γ392–406 but shows poor reactivity with fibrinogen in solution.

Limited Reduction of Fibrinogen. Fibrinogen was subjected to limited reduction as described by Procyk and Blombäck (1990), except that alkylation was performed without removal of the reducing reagent. This was found to prevent reoxidation of cleaved disulfide bonds at positions γ8Cys and γ9Cys (see Results and Discussion). Briefly, a fibrinogen stock solution was diluted with TNE-buffer to a final concentration of 7 mg/mL, and DTT was added (from a concentrated stock solution in water) to 5 mM. Nitrogen was blown over the sample and the tube sealed and placed in a water bath at 37 °C for 45 min. Afterward, the pH of the sample was adjusted to 8.1 by the addition of 2 M Tris, pH 8.5, and iodoacetic acid (500 mM in 0.1 N NaOH) was added to a final molarity of 25 mM. Nitrogen was blown over the sample and the tube sealed and kept in the dark at room temperature for 45 min. Afterward, the reagents were removed by dialysis against TNE-buffer during 4–20 h at 4 °C. The final material is referred to as R-FBG.

Limited reduction in the presence of calcium was done as described above, except that fibrinogen was incubated with 20 mM calcium (5 min, 37 °C) prior to addition of reducing reagent. The final material is referred to as R-FBG-Ca.

Thrombin Treatment. Partially reduced and alkylated fibrinogen prepared in calcium-free buffer (R-FBG) was diluted to 3 mg/mL with TNE-buffer and treated with thrombin (0.5 NIH unit/mL) for 2 h at room temperature. These conditions were sufficient to remove >97% of the fibrinopeptide B, as determined by ELISA using the anti-fibrinogen antibody 1-8C6 (not shown). SDS–PAGE analysis indicated that all of the Aα- and Bβ-chains were converted to the lower molecular weight α- and β-chains (not shown). The thrombin reaction was stopped by addition of hirudin to a concentration of 2–7 ATU/mL. Alternatively, thrombin was removed from the reaction mixture by elution through a hirudin–Sepharose column prepared by coupling hirudin to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ). The final material is called R-FBG-Th.

Fibrinopeptide Release. Samples from incubations with thrombin (or clot liquors where a clot formed) were applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA). The cartridge was washed with water, and fibrinopeptides were eluted with 50% acetonitrile. Eluate was concentrated (Speed-Vac Model SVC-100 HT; Savant Instruments, Farmingdale, NY) and separated by FPLC using a RPC PEP 5/5 column (Pharmacia) equilibrated with 0.2% trifluoroacetic acid–9% aceotnitrile. Fibrinopeptides were eluted with a gradient of 9–11% acetonitrile in 0.2% trifluoroacetic acid and quantitated by weighing excised peaks from the chart record.

Gel Filtration. Chromatography on Sephacryl S-300 HR in a XK 16/100 column (Pharmacia) was done using a bed height of 90 cm, 16 mL/h flow rate, and 0.8-mL samples containing 5 mg/mL protein.

Calorimetry. Calorimetric studies were performed on differential scanning calorimeters DASM-1M and DASM-4 (Privalov & Potekhin, 1986) at a 1 °C/min heating rate with 1–2 mg/mL protein. An excess pressure of 1.5 atm was applied to the calorimetric cell during measurements in order to expand the heating range above 100 °C. Each experiment was reproduced 4 times. The molar heat capacity of the proteins and the molar calorimetric enthalpy (ΔH_m^{cal}) were determined manually as described earlier (Privalov & Medved', 1982) and also using software available from MicroCal (Northampton, MA). Measurements were performed at pH 3.5 to avoid aggregation after denaturation.

Indirect Competition ELISA. The procedure described by Kudryk et al. (1984) was followed. Fibrinogen or acid-solubilized fibrin (4 mg/mL fibrin stock solution in 10% acetic acid) were used to coat the wells of polyvinyl microtiter plates. Dilutions of the monoclonal antibodies and sample containing antigen (i.e., fibrinogen, fibrin monomer standards, R-FBG, or R-FBG-Th) were mixed and allowed to come to equilibrium (usually for 15–60 min, room temperature) and then added to the plate. Specifically bound antibody was subsequently detected with a peroxidase-conjugated rabbit antibody directed against mouse immunoglobulins (DAKO Corp., Santa Barbara, CA).

Electrophoresis. Polyacrylamide slab gel electrophoresis was performed on 5 or 7% gels in SDS–phosphate buffer

(McDonagh et al., 1972), SDS–Tris–glycine (Laemmli, 1970), or acid–urea (Brummel & Montgomery, 1970).

Amino Acid Sequencing. Isolation of polypeptide chains from fibrinogen subjected to limited reduction and amino acid sequencing was performed as described by Procyk and Blombäck (1990).

Analytical Ultracentrifugation. Sedimentation velocity studies were performed with a Beckman Model E ultracentrifuge equipped with a cylindrical lens, mirrored optics, and a photoelectric scanner. The photomultiplier carriage position and absorbance output signals were acquired and stored through an ISAAC Model 41A data acquisition system (Cyborg Corp., Newton, MA) interfaced to an Apple IIe microcomputer (Apple Computer, Inc., Cupertino, CA) (Pryzdial, 1991). All measurements were obtained using 4° double-sector cells containing 12-mm charcoal-filled Epon centerpieces and sapphire windows. Prior to ultracentrifugation, samples were extensively dialyzed against 0.1 M arginine, 0.1 M sodium phosphate, and 0.01% Tween 20 at 4 °C. The samples were scanned at 280 nm to follow the protein sample.

Sedimentation velocity experiments were carried out using sample volumes of 0.38 mL at a temperature of 25 °C. In all experiments, the rotor was accelerated to a speed of either 48 000 rpm (208000*g*) or 60 000 rpm (260000*g*) (as noted), and scanning was initiated after the meniscus was depleted of protein. Scans were of 4-min intervals. Sedimentation coefficients were calculated from linear least-squares regression analysis of the natural logarithm of the equivalent boundary position versus time. Boundary positions were calculated by both midpoint and second-moment analysis [for background information, see Canter and Schimmel (1980)]. Second-moment determinations ($\bar{r}^2$), which yield average sedimentation coefficients, were calculated according to the equation (Goldberg, 1953):

$$\bar{r}^2 = (1/A_p)\int_{r_m}^{r_p}(dA/dr)r^2\,dr$$

where A is the absorbance, A_p is the absorbance of the plateau region, r is the radial position, r_p is the radial position at an arbitrary point within the plateau region, and r_m is the radial position at a point that has been depleted of solute. The square root of the second moment was used to obtain average boundary positions. When it was necessary to determine the sedimentation coefficient of a single species (e.g., the sedimentation coefficients of R-FBG), boundary positions were calculated by determining the radial position of the particular boundary's absorbance midpoint. In all cases, the observed sedimentation coefficients are reported.

Results and Discussion

Assignment of Reduced Disulfide Bonds. Limited reduction of fibrinogen in calcium-free buffers leads to cleavage of two Aα442Cys–Aα472Cys and two γ326Cys–γ339Cys intrachain disulfide bonds and the symmetrical Aα28Cys disulfide bond (Procyk & Blombäck, 1990). In the present work, the alkylation step following reduction was done without removal of DTT (see Materials and Methods) since this was found to prevent re-formation of cleaved disulfide bonds at positions γ8Cys and γ9Cys. Cleavage of the symmetrical disulfide bonds was verified by amino acid sequencing. Following limited reduction and alkylation with iodoacetic acid, samples were subjected to total reduction, and free cysteines were blocked with 4-vinylpyridine (Procyk & Blombäck, 1990). Sequencing of the isolated polypeptide chains showed that 91–96% of the PTH-cysteine derivatives detected at positions

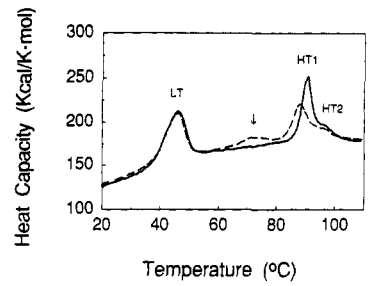

FIGURE 1: Differential scanning calorimetric curves for (—) fibrinogen and (--) R-FBG. Low- (LT) and high (HT)-temperature peaks are marked. The arrow indicates an additional low-intensity peak found in fibrinogen subjected to limited reduction (see text). Measurements were performed in 0.05 M glycine, pH 3.5.

Aα28Cys, γ8Cys, and γ9Cys in fibrinogen subjected to limited reduction in calcium-free buffers (R-FBG) were PTH-(carboxymethyl)cysteine (retention time of 7.1 min) with the remainder being PTH-(pyridylethyl)cysteine (retention time of 21 min). The same results were obtained with fibrinogen subjected to limited reduction in buffer containing calcium (R-FBG-Ca). These results indicated that the disulfide bonds at Aα28Cys, γ8Cys, and γ9Cys were cleaved during the limited reductions.

Compact Structure. Half-molecules of fibrinogen of 170000 molecular weight (one set of Aα-, Bβ-, and γ-chains) were not observed in R-FBG or R-FBG-Ca preparations, even during gel filtration at 60 °C in buffer containing 6 M guanidine hydrochloride or in 5% acetic acid (not shown), or during electrophoretic analysis according to Laemmli (1970), Brummel and Montgomery (1970), or McDonagh et al. (1972) (not shown). Thus, the integrity of the whole molecule appears to be stabilized by strong noncovalent interactions in addition to the symmetrical disulfide bonds at Aα28Cys, γ8Cys, and γ9Cys (see below).

We used differential scanning calorimetry, a method that measures the amount of heat required to alter or destroy protein native structure, to determine how limited reduction affected the stability of fibrinogen's overall conformation and domains containing the susceptible bonds. As shown in Figure 1 (solid line), heat absorption peaks in two temperature ranges were obtained for human fibrinogen: peak LT1 at T_m = 45.5 °C with enthalpy ΔH_m^{cal} = 565 ± 25 kcal/mol in the low-temperature range and a sharp peak, HT1, at T_m = 90.5 °C with an overlapping second peak, HT2, visible as a broad shoulder in the high-temperature region. The sum of the enthalpies for the HT1 and HT2 peaks was equal to 535 ± 25 kcal/mol. Assignments deduced on the basis of earlier studies of bovine fibrinogen (Donovan & Mihalyi, 1974; Privalov & Medved', 1982; Mihalyi & Donovan, 1985; Medved' et al., 1986) indicate that the HT1 peak in Figure 1 relates to melting of the central part of the molecule (i.e., the E region including the amino termini of all six chains) and the HT2 peak to melting of the terminal parts of the coiled-coil connectors located at the amino-terminal part of the D regions. The LT1 peak represents melting of domains in the D region encompassing the carboxy-terminal parts of the Bβ- and γ-chains and also the carboxy-terminal Aα-chain regions.

Analysis of R-FBG (Figure 1, dashed line) or R-FBG-Ca (not shown) indicated that substantial regions of native structure were still present in the samples subjected to limited reduction and cleavage of the susceptible disulfide bonds destabilized only a part of the overall structure of fibrinogen. Specifically, the melting curve displayed an LT1 peak at the same temperature as nonreduced fibrinogen, but with slightly lower intensity. The enthalpy of this peak for R-FBG was

about 12% lower than for control nonreduced fibrinogen and about 6% lower for R-FBG-Ca (not shown). Since the melting of about eight individually folded domains is represented in this peak (Privalov & Medved', 1982), the observed decrease in enthalpy in the samples subjected to limited reduction is too small to indicate domain denaturation and rather reflects minor conformational changes accompanying limited reduction.

The HT1 peak for both R-FBG and R-FBG-Ca (not shown) was shifted by 2.5 °C to a lower temperature region and had only about half of the intensity of the control HT1 peak. In addition, a new highly reproducible low-intensity peak (Figure 1, arrow) appeared in the 65–80 °C region for both partially reduced samples, probably representing the destabilization of a structure that had originally melted in the HT1 peak region in nonreduced fibrinogen. The sum of the enthalpies for the high-temperature peaks of R-FBG was about 20% lower than for control nonreduced fibrinogen. The same was found with R-FBG-Ca (not shown). These results show that the domains which compose the central part of the molecule and melt in the HT1 peak are destabilized to some extent following reduction of the three symmetrical disulfide bonds at Aα28Cys, γ8Cys, and γ9Cys, although the relatively large enthalpy remaining after cleavage of these bonds indicates that compact structure and interactions between domains are largely preserved.

Studies of the immunoreactivity of R-FBG were done to determine if epitope expression was affected by changes in the stability of the domains containing the cleaved disulfide bonds. We found no change in expression of two out of three epitopes examined. Epitopes associated with segments Bβ1–21 and Aα241–476 were similarly expressed on control nonreduced fibrinogen, R-FBG, and R-FBG-Ca (not shown). The inhibition curves and IC_{50} values for these samples were similar in competition ELISA using fibrinogen-coated microtiter plates (i.e., ~0.01 μM for antiβ1–21 and ~0.02 μM for antiAα241–476). By contrast, fibrinogen and R-FBG-Ca gave IC_{50} values of ~30 μM and were poor inhibitors in assays using plates coated with γ-chain and antibody 4-2/xl-f that recognizes an epitope containing γ392–411. R-FBG appeared to be better (IC_{50} = ~7 μM). Although γ326Cys–γ339Cys disulfide bond cleavage helped modulate expression of the γ392–406 epitope, an approximately 20-fold molar excess of R-FBG was needed to obtain a level of inhibition comparable to the free peptide γ392–411, which is a very potent inhibitor (IC_{50} ~0.3 μM). These results suggest that a small conformational change occurred in the D region of R-FBG following γ326Cys–γ339Cys interchain disulfide bond cleavage.

Thrombin Cleavage and Polymerization. Thrombin-catalyzed activation of R-FBG was studied to determine how limited reduction affected both the rate of fibrinopeptide release and the site of thrombin-catalyzed cleavages. Fibrinopeptide A was rapidly released from R-FBG even though the sample did not clot during the 10 min of incubation (Figure 2). Fibrinopeptide A release in control nonreduced fibrinogen occurred to a similar extent except that the sample clotted by 2 min. The retention time of the fibrinopeptide A peak in the FPLC analysis was identical in all samples (not shown). Fibrinopeptide B release was detected by 2 min in both samples but then appeared to proceed more slowly from R-FBG.

The site of α-chain cleavage was determined from the identity of the amino-terminal amino acid (obtained through amino acid sequencing). For R-FBG treated with thrombin for up to 10 min (0.09 unit of thrombin/mg of R-FBG, room temperature), PTH-Gly was the major amino acid recovered

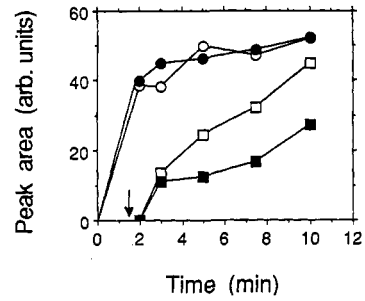

FIGURE 2: Release of fibrinopeptides. Samples containing fibrinogen in TNE-buffer (1.38 mg/mL) with calcium (20 mM) were mixed with thrombin (0.19 unit/mL). The clotting reaction was stopped at specific time intervals by addition of PPACK (1.3 mM), and supernatant/clot liquor was analyzed by FPLC (see Materials and Methods). (●) Fibrinopeptides A and (■) B of R-FBG; (○) fibrinopeptides A and (□) B of control nonreduced fibrinogen. Final concentrations are denoted in parentheses. The clotting time of control nonreduced fibrinogen is indicated by an arrow.

Table I: Observed Sedimentation Coefficients (s_{obs}) of R-FBG-Th[a]

species	s_{obs} (S)	species	s_{obs} (S)
1	5.8	4	14.4
2	6.5	5	17.6
3	10.9		

[a] From Figure 4; see Materials and Methods for details.

in the first cycle of α-chain sequencing, indicating cleavage at the normal Aα16–Aα17 site. About 85% of the total fibrinopeptide A was released from R-FBG by this time, and PTH-Val was not detected (not shown). After 30 min of incubation with thrombin, the proportion of α-chains beginning with Val20 was about 18% of the total population of α-chains. The remainder of the α-chains showed Gly17 as the amino terminus. The proportion of α-chains beginning with Val20 increased to 50% by 2 h of incubation. These results indicate that Aα19–Aα20 cleavage was occurring only after the normal Aα16–Aα17 cleavage and that it was a slow event. Under normal conditions, fibrinogen would rapidly polymerize following fibrinopeptide A release, and steric factors related to the packing of the fibrin monomers in a protofibril would probably prevent subsequent access of thrombin to the Aα19–Aα20 site. This is consistent with what is known about the Aα19–Aα20 site cleavage and the occasions when it occurs, such as in Aα-chain fragments (Ni et al., 1989; Lord & Fowlkes, 1989) or fibrinogen fragments (Blombäck et al., 1967). It is likely that R-FBG-Th did not form polymers or that it formed abnormal complexes, thereby allowing thrombin access to the second cleavage site.

Sedimentation velocity measurements were performed to determine directly the particulate nature of R-FBG and R-FBG-Th preparations. Control, nonreduced fibrinogen when analyzed at 48 000 rpm showed sample homogeneity with a sedimentation value of 7.7 S (Figure 3A). When analyzed in a similar fashion, R-FBG also showed sample homogeneity and had an s value of 7.4 S (Figure 3B). However, when R-FBG-Th was analyzed, it displayed sample nonhomogeneity with discernible complexes ranging in sedimentation coefficients from 5.8 to 17.6 S (Figure 4 and Table I). This indicated that thrombin treatment of R-FBG led to the formation of different molecular size species of R-FBG-Th, possibly up to octamers; however, the complexes failed to assemble into fibers or an insoluble gel network.

Since R-FBG-Th remains in solution and its β-chain amino terminal is identical to fibrin's, inhibition binding experiments using R-FBG-Th were performed with fibrin-coated plates and

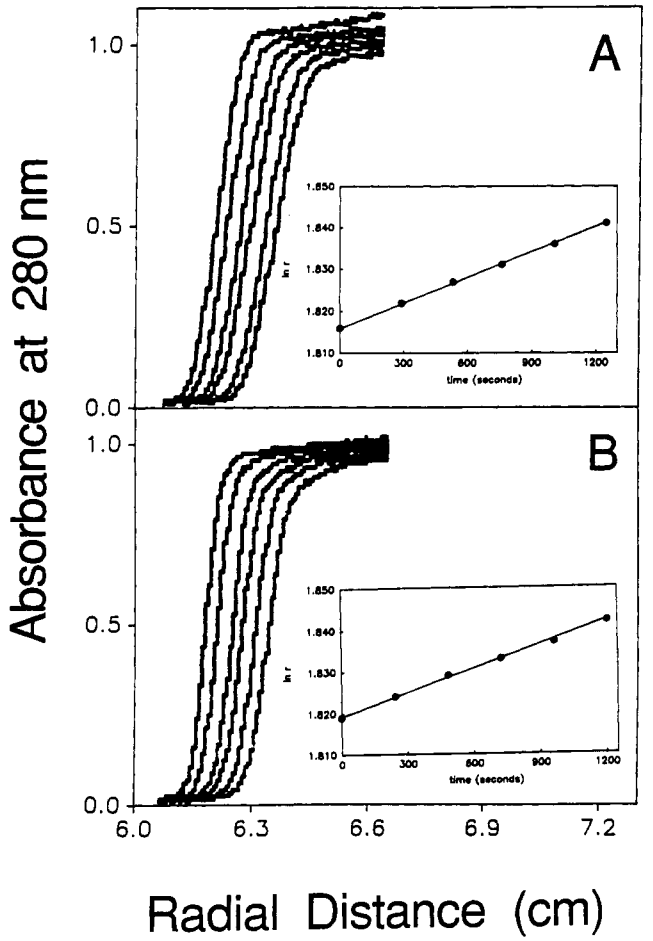

FIGURE 3: Sedimentation of fibrinogen at 48 000 rpm monitored at 280 nm. Insets: average sedimentation coefficients determined by linearizing radial positions that were calculated by the second moments of absorbance scans acquired at 4-min intervals. Samples in 0.1 M arginine, 0.1 M sodium phosphate, and 0.01% Tween 20 at 25 °C. Panel A, 2 μM normal human fibrinogen; panel B, 2 μM R-FBG.

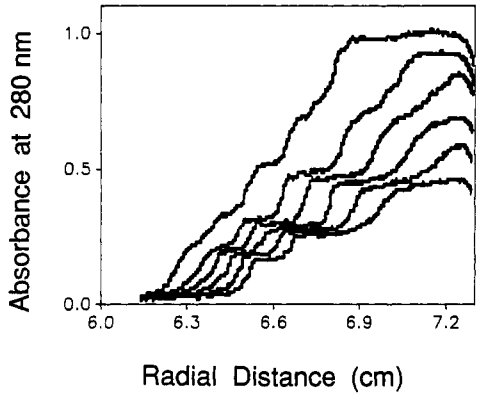

FIGURE 4: Sedimentation of 2 μM R-FBG-Th at 60 000 rpm monitored at 280 nm. Sample was in 0.1 M arginine, 0.1 M sodium phosphate, and 0.01% Tween 20 at 25 °C.

the fibrin-specific antibody T2G1 (antiBβ15–21). R-FBG and control nonreduced fibrinogen did not react with antibody T2G1 as seen by a lack of inhibition of antibody binding to the plate (Figure 5). By contrast, effective inhibition occurred with R-FBG-Th at dilutions of 7.5–75 pmol (calculated in chain equivalents, i.e., per β-chain). Fibrin monomer (prepared in the presence of the polymerization inhibitor Gly-Pro-Arg-Pro; see Materials and Methods) gave an almost identical result, which indicated that the monomer and R-FBG-Th displayed the T2G1 antigenic site in a similar fashion. Thus,

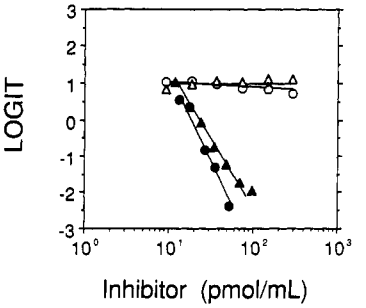

FIGURE 5: Competitive inhibition curves obtained with different antigens and the anti-fibrin antibody T2G1. Microtiter plate wells were coated with acid-soluble fibrin (see Materials and Methods). Indirect binding ELISA was done using 9.5 pmol/mL T2G1 antibody competing with serially diluted (●) R-FBG-Th, (▲) soluble fibrin monomer, (△) fibrinogen, and (○) R-FBG. Preparation of antigens described under Materials and Methods.

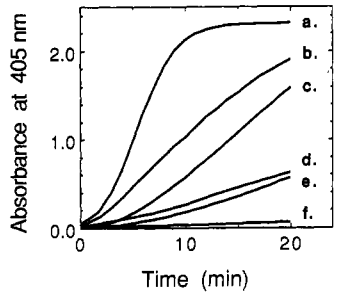

FIGURE 6: Plasminogen activation in the presence of different t-PA stimulators. Final concentrations of components in assay: plasminogen (0.22 μM); S-2251 (0.45 mM); t-PA (33 units/mL); stimulator (0.1 mg/mL). Assay buffer: 63 mM Tris, pH 8.5, and 0.01% Tween 80. (a) R-FBG-Th; (b) fibrin monomer standard supplied with the COA-SET fibrin monomer kit (Kabi Diagnostica, Molndal, Sweden); (c) R-FBG; (d) R-FBG-Ca prepared from factor XIII free fibrinogen (see Materials and Methods); (e) fibrinogen; (f) no stimulator.

R-FBG-Th is useful as a soluble fibrin-like standard for competition ELISA experiments employing antibody T2G1 because its use does not require fibrin polymerization inhibitors needed to keep conventional fibrin monomers in solution.

Interaction with t-PA. Fibrin monomers accelerate t-PA-mediated plasminogen activation (Wallen, 1977; Camilio et al., 1971; Rånby, 1982). We studied the influence of R-FBG-Th on the plasminogen activation reaction using a chromogenic substrate assay for plasmin activity (Figure 6). A plot of the absorbance as a function of time indicated that R-FBG-Th was an effective accelerator, comparable to various types of fibrin monomers. R-FBG also accelerated the t-PA-mediated conversion of plasminogen, although at a slower rate. The extent of stimulation obtained with R-FBG-Ca, however, was much lower and approximately equaled the extent obtained with control nonreduced fibrinogen.

The stimulation obtained with fibrinogen alone in Figure 6 was not due to trace amounts of fibrin monomers (Hoylaerts et al., 1982) since ELISA testing with the anti-fibrin antibody T2G1 did not reveal any fibrin monomer in our fibrinogen preparation (detection limit 0.3 μg/mg of fibrinogen; not shown). However, fibrinogen can interact more readily with t-PA and/or plasminogen and stimulate the generation of plasmin when cross-linked into oligomers or denatured with 10 M urea (unpublished observations), or if treated with 2.5–6 M urea (deSerrano et al., 1989), or when bound to plastic surfaces (Adelman & Quynn, 1989). These treatments apparently induce conformational changes in fibrinogen that expose or align latent interaction sites. Two sites have been characterized: one is at Aα148–160 (Voskuilen et al., 1987)

and a second at γ311–379 (Yonekawa et al., 1990). Limited reduction in calcium-free buffer probably leads to a conformational change in fibrinogen that favors interaction with t-PA and/or plasminogen, as seen from the increase in the capacity of R-FBG alone to stimulate the t-PA-mediated reaction (Figure 6). Thrombin treatment of R-FBG with subsequent formation of oligomeric complexes further enhances the stimulatory activity. By contrast, R-FBG-Ca has little accelerating ability since the γ326Cys–γ339Cys intrachain disulfide bond is protected from reduction in the presence of calcium (Procyk & Blombäck, 1990) and cleavage of the other disulfide bonds (i.e., Aα442Cys–Aα472Cys and the symmetrical Aα28Cys, γ8Cys, and γ9Cys disulfide bonds) does not lead to denaturation of the molecule or to the necessary favorable conformational changes.

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Registry No. Thrombin, 9002-04-4; plasminogen activator, 105913-11-9; plasminogen, 9001-91-6; plasmin, 9001-90-5.

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