Isolation and Characterization of the Fibrin Intermediate Arising from Cleavage of One Fibrinopeptide A from Fibrinogen*

(Received for publication, June 5, 1996, and in revised form, July 1, 1996)

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The thrombin-catalyzed cleavage of N-terminal fibrinopeptide A (FPA) from the two Aα-chains of fibrinogen exposes aggregation sites with the critical sequence GPR located just behind FPA. It is well known that exposure of this site is a bifunctional process forming fibrin monomers, self-aggregating, fully coagulable A-fibrin monomers, but the fibrin precursor with one site exposed and one FPA intact has eluded description. The formation of this “α-profibrin” in the course of thrombin reactions and its distribution among both the aggregating and non-aggregating components of the reactions are characterized here by immunoprobing electrophoretic and gel chromatographic separations using monoclonal antibodies specific for FPA and for exposed GPR sites. These analyses show α-profibrin to be a non-aggregating derivative indistinguishable from fibrinogen in solutions that are rich in fibrinogen relative to dissolved fibrin. But α-profibrin forms soluble complexes with A-fibrin monomer under conditions in which it and fibrin predominate over fibrinogen. It was isolated as a complex with fibrin by gel chromatography of cryoprecipitates and then separated from the fibrin either by electrophoretic gel shifts induced with a peptide analog of the GPR aggregation site or by chromatographic gel shifts induced with monoclonal anti-FPA antibody. The weak aggregation of α-profibrin with itself and with fibrinogen conforms with prior indications that coupled interactions through the paired GPR sites on fibrin monomers are pivotal to their aggregation. It is suggested that α-profibrin may be a hypercoagulable fibrin precursor because it is converted to A-fibrin monomer faster than fibrinogen converts to monomer.

The discovery that proteolytic cleavage of N-terminal fibrinopeptides A and B from fibrinogen underlies its conversion to fibrin by thrombin (1–3) and the demonstration that the fibrinogen macro-molecule is a bifunctional assembly of two sets of self-aggregating, fully coagulable A-fibrin monomers, but the fibrin precursor with one site exposed and one FPA intact has eluded description. The formation of this “α-profibrin” in the course of thrombin reactions and its distribution among both the aggregating and non-aggregating components of the reactions are characterized here by immunoprobing electrophoretic and gel chromatographic separations using monoclonal antibodies specific for FPA and for exposed GPR sites. These analyses show α-profibrin to be a non-aggregating derivative indistinguishable from fibrinogen in solutions that are rich in fibrinogen relative to dissolved fibrin. But α-profibrin forms soluble complexes with A-fibrin monomer under conditions in which it and fibrin predominate over fibrinogen. It was isolated as a complex with fibrin by gel chromatography of cryoprecipitates and then separated from the fibrin either by electrophoretic gel shifts induced with a peptide analog of the GPR aggregation site or by chromatographic gel shifts induced with monoclonal anti-FPA antibody. The weak aggregation of α-profibrin with itself and with fibrinogen conforms with prior indications that coupled interactions through the paired GPR sites on fibrin monomers are pivotal to their aggregation. It is suggested that α-profibrin may be a hypercoagulable fibrin precursor because it is converted to A-fibrin monomer faster than fibrinogen converts to monomer.

* This work was supported in part by Grant HL-16361 from NHLBI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: FPA, fibrinopeptide A; mAb, monoclonal antibody; GPRP-NH₂, Gly-Pro-Arg-Pro-amide; PPACK, phenylalanine prolylarginine chloromethyl ketone; HPLC, high pressure liquid chromatography; serpin, serine protease inhibitor; Bicine, N,N-bis(2-hydroxyethyl)glycine; PBS, phosphate-buffered saline; TAME, p-tosyl-1-arginine methyl ester; GPRphoresis, agarose electrophoresis of fibrinogen modulated with GPR-peptides.

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thrombin reactions. We view the observations as helping to reconcile some of the disparate perspectives on the significance of FPA release, the kinetics of fibrin formation, and the relationship between FPA release and fibrin aggregation.

EXPERIMENTAL PROCEDURES

Materials—Commercial sources of chemicals and biologicals as follows: Peptide Synthetix, Ltd., Moscow (Gly-Pro-Arg-Pro-amine (GPR-PNH2)); Calbiochem (phenylprolalarginine chloromethyl ketone (PPACK)); Eastman Kodak Co. (acrylamide); Hisapanag S. A., Madrid, Spain (agarose); Research Organics, Cleveland, OH (buffer components); Bio-Rad (Bio-Gel® A-5m); Sigma (Atroxin®, Lubrol, Tween, SDS molecular weight markers (Mn = 200,000), and horseshid peroxidase-conjugated horse IgG and United States Biochemical Corp. (bovine thrombin, hemin, and thimerosal). Otherwise, chemicals were specified as reagent-grade or better.

Protein and Antibody Preparations—Fibrinogen was prepared from citrated human plasma by sequential precipitations with ethanol, glycine, and ammonium sulfate/α-aminoacapoic acid as described (31). Portions were converted to α-fibrin monomer for use as a standard by rehydration of dried samples with SDS-PAGE resolving gel and subsequently ultrafiltered prior to HPLC. To do this, 0.1-ml portions were used to measure FPA by HPLC. Addition of p-nitrophenyl-p′-guanidinobenzoate (0.18 mM) produced a temporary blockade of activity lasting enough to perform GPRphoresis when initiated within 1 h, but its derivatives were fixed in place by heat denaturation as described (31). Bovine thrombin was purified according to the chromatographic procedure of Prentice et al. (32), but human thrombin was used as the commercially supplied Fibrinex® (Ortho Diagnostics, Raritan, NJ). For some experiments, the fibrinogen was radiiodinated, and in others, the antibodies were radiiodinated for quantitation of the derivatives with the PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Livers were performed by the iodine monochloride method of McFarlane (33).

Anti-FPA mAb was affinity-purified using FPA linked to glyoxylagarose as described previously (29, 34). Anti-FPA mAb binds both the free peptide and the intact segment at the N terminus of the Aα-chain of fibrinogen (α1–16). Anti-fibrin α17–23 mAb was affinity-purified as described (30) and binds to fibrin lacking FPA, but not to fibrinogen with FPA intact. The binding of anti-fibrin α17–23 mAb to fibrin occurs only after denaturation of the fibrin (30). Cellulose nitrocellulose membranes induced fibrin monomer denaturation to a degree enabling antibody uptake equal to that observed with fibrin denatured with urea. Retention of the antibodies by antigens on Western blots and directly immunoprobed gels was determined by immunoprophoresis with either peroxidase- or 125I-labeled goat anti-mouse IgG antibodies.

Kinetic Studies—Thrombin at either 0.4 or 9 mg/ml was incubated for the indicated times with fibrinogen at either 3 or 9 mg/ml, respectively, in Tris-buffered saline/EDTA (0.1 mM) (TBSE) containing GPRP-NH2, and the reactions were stopped by adding PPACK (20 mM). The reaction mixtures (0.5-ml samples) were analyzed for fibrinogen derivatives by GPPhoresis and for release of fibrinopeptides by HPLC.

Conversion of fibrinogen to α-profibrin and fibrin was also studied with the venom procoagulant Atroxin (0.5 μg/ml). Atroxin proved difficult to inactivate by known serpins for periods long enough to allow processing of the samples for HPLC. Addition of p-nitrophenyl p′-guanidinobenzoate (0.18 mM) produced a temporary blockade of activity long enough to perform GPPhoresis when initiated within 1 h, but its activity eventually returned. An irreversible inactivation with minimal processing was needed to measure FPA by HPLC. To do this, 0.1-ml portions were acidified with 4 μl of 1 N HCl, neutralized using 6 μl of 1 N NaOH, and subsequently ultrafiltered prior to HPLC.

Measurements of fibrinopeptides released in thrombin reactions were made by isocratic HPLC essentially as described (35), but with 17% acetonitrile and 0.09% trifluoroacetic acid and a 30 × 150-mm Nova-Fak C18 column. Samples were deproteinized by centrifugal filtration through Ultrafree™ 10,000 nominal molecular weight limit filter units (Millipore Corp., Bedford, MA). Fibrinopeptide standards were prepared by coagulating fibrinogen (3 mg/ml) with chromatographically purified bovine thrombin (5 units/ml, 1 h) and ultrafiltering fluid extruded from the clot.

GPPhoresis—Gel shifts induced with GPR peptides admixed in the reaction mixtures were used to distinguish self-aggregating from non-aggregating components in thrombin/fibrinogen reaction mixtures. The equipment and procedure used were the same as described previously (36), except that we substituted GPRP-NH2 for the tripeptide GPR. Because of relatively fast cathodic migration of the amide, we were able to cast a single uniform gel instead of the binary GPR-rich/GPR-poor gel required previously to stage precipitation of the fibrin band in a desirable location, ~4 mm anodal to the sample application point. From experiments tracking the mobility of the GPRP-NH2 electrophoresis buffer, the aggregation of the monomers into immobile fibrin strands occurred precisely as the anodal migrating band of fibrinogen and monomer traversed the cathodal moving band of GPRP-NH2 bound to the gel. Also, because of a much greater inhibitory effect on fibrin aggregation (37), the use of GPRP-NH2 enabled us to analyze samples with a much higher content of fibrin monomer. Yet regardless of the concentrations of GPRP-NH2 added to the reaction mixtures, we were unable to suppress fibrin aggregation toward the end-stage thrombin reactions due to release of fibrinopeptide B in addition to FPA. To ensure full application of protein onto the gels for quantitation of all derivatives, the samples were admixed with an equal volume of 0.5 μl urea just prior to sample application. Results obtained with the urea added were the same as the sample obtained with the urea withheld for early, non-viscous samples where FPA release was <30% of the total; however, when FPA release was >30%, the urea was required for complete transfer of the fibrin to the gels.

For preparation of 30 ml of gel, 0.75 ml of low electroendosmosis agarose and 0.3 g of SepaPrep™ agarose were dissolved in 27 ml of 0.1 mM HCl by heating and then admixed with 3 ml of 0.01% buffer (0.2 citric acid, 0.18 M Tris, 0.01 % Bicine, 2.5 mM GPRP-NH2, 7.5 mM EDTA) delivered from a peristaltic pump. Electrodes contained 1 × buffer without GPRP-NH2. The gels were cast in a pressure chamber at 1 atm, incubated for 1 h at a constant 1000 V (8 cm), and the temperature maintained at 27°C. To obtain a sharp interface with buffer lacking GPRP-NH2 at the anodal edge of the gel, a thick slab (5 mm thick by ~2 cm wide) of “contact gel” was cast from 1% agarose in buffer lacking GPRP-NH2, butt against the edge of the specimen gel for the first 30 min, and then placed ~1 cm over the edge to assure continued contact for the remainder of the run.

Gels that were to be immunoprobbed directly were fixed by immersion in hot PBS/Tween at a denaturing temperature of 53°C for 30 min. For general protein staining, gels were fixed for 30 min with 10% trichloroacetic acid, stained with colloidal Coomassie violet R-250 (39), and dried on an overlay dialedysis membrane. For stoichiometric quantitation, the parent fibrinogen was radiiodinated, and radioactivities of components (the fibrin and the fibrinogen/α-profibrin bands) in the electropherograms were measured with the PhosphorImager. Results are expressed as percentages of total radioactivity.

Immunoprophoring—Three different methods were used for immunoprophoring: 1) immunoperoxidase staining when the fibrinogen was radiiodinated; 2) direct immunoprophoring of the gels with 125I-labeled anti-fibrin antibody; and 3) Western blots followed by 125I-labeled antibodies. Direct immunoprophoring: 1) immunoperoxidasestaining when the fibrinogen was radioiodinated, and 2) direct immunoprophoring of the gels with 125I-labeled antibody. Samples were soaked in 125I-labeled antibody, and the coating was performed with direct immunoprophoring, which is the more conventional of the three procedures, but required corrections due to incomplete blot transfers of the fibrin. Except for the second procedure using 125I-labeled antibodies, the immunoprophoring served only to back up the assessments of α-profibrin and fibrin made by the stoichiometric assessment of α-profibrin from the differences between the conversion of 125I-fibrinogen to fibrin and the release of FPA.

To immunoprophore the gels directly (no blot transfer), fibrinogen and its derivatives were fixed in place by heat denaturation as described, and after washing, the gels were immersed overnight in PBS/Tween containing 5 μg/ml primary antibody, 0.1% NaNO3, and 1 mg/ml albumin. For quantitation of FPA and fibrin α17–23 epitope in the electrophoretically separated components, nonradioactive fibrinogen was used in the thrombin reactions, and the gels were immunoprophored with radiiodinated antibody. Antibody retention was quantified directly with the PhosphorImager. For analyses of reactions based on fibrin production in relation to released FPA, the fibrinogen was radiiodinated so that fibrin monomer production could be precisely measured by GPRphoresis using the PhosphorImager, and the immunoreactivities of components resolved in these gels were probed in duplicate gels using anti-FPA and anti-fibrin α17–23 mAbs and secondary staining with peroxidase-labeled anti-mouse IgG antibodies. With either procedure, removal of excess primary antibody was performed by suctioning through the gel 1.30 g volumes (wash time of ~40 min) PBS/Tween containing 0.1% albumin, and the wash was precisely monitored (±0.1-mm gel thickness) using a described wash-limiting device (38, 40). The retained primary antibody was then immediately fixed in place (34) by immersing the gel in 0.25% glutaraldehyde for 30 min, followed by extensive washing with TBSE. This fixation prevents dissociative loss of primary antibody during long exposures of the gel to secondary antibody (34, 41). Peroxidase-labeled goat anti-mouse IgG second
antibody (Sigma) was applied at 1:500 dilution overnight, and washout of unbound antibody was effected by suctioning 1.30 gel volumes of PBS/Tween/albumin, as described for the primary antibody.

For Western blotting, which we selected to illustrate the early production of α-profibrin because of good contrast of low levels of product (see Fig. 3), the gels were laid over cellulose nitrate blotting membranes, and protein was transferred onto the membranes by suctioning through the gel and membrane 4 volumes of 5 m guanidine HCl at pH 8.6 via a vacuum applied through a gel dryer as described (34). Blotting membranes were blocked with 1% nonfat dry milk prior to immunoprobing. Primary antibody was applied to the specimens at 5 μg/ml in PBS/Tween containing 0.1% NaNO2 and 1 mg/ml albumin overnight and then given a 2-min rinse. Peroxidase-labeled goat anti-mouse IgG secondary antibody was applied at 1:500 dilution for 1 h and given a 2-min rinse and washed prior to staining (0.05% diaminobenzidine and 0.005% H2O2, in PBS). However, we did not rely on blotting for critical measurements because we were never able to get a full transfer of all components, particularly fibrin, onto the membranes in numerous trials using a wide range of transfer solvents. For thrombin reactions using 131-I-fibrinogen for precise assessment of fibrin formation, we were able to back-up our measurements by immunoperoxidase staining of the fibrin and fibrin/fibrinogen by Western blotting of anti-fibrin a17–23 mAb. Maximal levels of α-profibrin determined from the immunochromatographic back-ups coincided within 10% of the levels determined from the stoichiometry.

Cryoprecipitations—These were performed mainly to enrich the relative concentrations of α-profibrin/fibrinogen prior to gel filtration. Thrombin (0.68 unit/ml) reactions with fibrinogen (9 mg/ml) were stopped after 40 s by adding PPACK (20 μM) to produce solutions rich in both fibrin monomer and α-profibrin, each comprising ~20% of the starting fibrinogen after 30% release of FPA. The fibrin remained soluble in these concentrated solutions over this time frame. The solution was immediately chilled in an ice bath for 30 s to promote α-profibrin/fibrin interaction and then admixed with 0.05 volume of freshly neutralized 0.15 M TAME to lower solubility of the complexes (12, 42). After 2 h, the cryoprecipitate was washed with cold TBSE, dissolved in 0.3 M TBSE at one-fifth the original volume at 42 °C, and subsequently diluted with an equal volume of water. The solution was then held at 25 °C for 2 h, during which time much of the fibrin separated as a clot, which was spun onto a glass rod. The solution was then chilled at 4 °C for 1 h (no TAME added), and complexes that precipitated were removed prior to chromatography at 4 °C. This procedure yielded solutions enriched in α-profibrin (~50% total protein) and relatively poor in α-fibrin (~8% total protein).

Gel Chromatography—The illustrated analytical scale separations were performed at a flow rate of 1.56 cm/h through a jacketed column (0.7 × 24 cm bed) of 6% agarose (Bio-Gel A-5m) equilibrated with TBSE at 4 °C, and effluent was collected in 0.5-ml fractions. Samples usually comprised 0.8 mg of protein in 0.4 ml. Elution patterns were characterized by absorbance at 280 nm and by GPRphoresis. Proteins in the fractions analyzed by electrophoresis were concentrated by precipitation with 0.14 volume of rapidly admixed ethanol at 0 °C and redissolved in 20 μl of GPR-P-NH2 electrophoresis buffer admixed with 10 μl of 9 M urea. Electrophoresis was performed within a few hours of concentrating the samples. Columns were freed of any precipitated fibrin by washing with 3 ml urea followed by 4 ml guanidine HCl prior to re-equilibrating.

Affinity Chromatography with Immobilized Anti-FPA mAb—To attempt separation of fibrinogen derivatives according to FPA content, we prepared an immunoabsorbent consisting of anti-FPA mAb that was initially adsorbed to protein A/G-agarose (Schleicher & Schuell) and subsequently cross-linked with dimethyl pimelimidate, as described (43). The immunoabsorbent contained 6 mg of anti-FPA mAb linked to 2 ml (bed) of protein A/G-agarose in a 5-mm diameter column. Specimens applied to the column contained maximally 0.5 mg of fibrinogen-related antigen. The mobile phase consisted simply of TBSE, and linear flow rates ranged from 1.6 to 9 cm/h. Fibrin precipitates formed within the columns were removed by washing with 2 ml of 2 M guanidine HCl, pH 7.4, followed by re-equilibration with TBSE.

Plasma Samples—With informed consent, plasma samples were obtained from 22 normal subjects and 23 patients with non-acute occlusive peripheral vascular disease. The blood was drawn into EDTA-admixed with PPACK/protein solution as described (44), but with higher 1.6 μM PPACK. Portions of the plasma samples were admixed with 0.1 volume of 2% GPRP-NH2 and stored at −70 °C until thawed (42 °C) for electrophoresis.

Data Processing—Chromatographic, densitometric, and Phosphor Imager data were analyzed using PeakFit software (Jandel Scientific, San Rafael, CA). Curve fitting of kinetic data was carried out by non-linear least-square correlations using SlideWrite™ Plus Version 3 software (Advanced Graphics Software, Inc., Carlsbad, CA). Equations 1 and 2 were adapted from Ref. 45. The relative rates of the two reactions (k1/k2), were also independently estimated from the ratios of maximal concentrations of α-profibrin/initial fibrinogen ([p]max/[f]0) observed over the course of the reactions (Equation 3),

$$\frac{[p]}{[f]} = \frac{k_1}{k_2} \cdot \exp \left( -k_1 t - \exp \left( -k_2 t \right) \right)$$ (Eq. 1)

$$\frac{[f]}{[p]} = \frac{k_1}{k_2} \cdot \left( \exp \left( -k_1 t \right) - \exp \left( -k_2 t \right) \right)$$ (Eq. 2)

expressing concentrations of α-profibrin ([p]) and fibrin ([f]) relative to the initial fibrinogen ([f]0) over the course of a catenating reaction sequence (Sequence 1),

$$k_1 \cdot k_2 \cdot \frac{\phi}{p} = f$$

SEQUENCE 1

except at k1 = k2, where [p]max/[f]0 = 1/e = 0.37.

RESULTS

The "GPRphoresis" electrophoretic method, on which many of the findings are based, staged gel shifts with synthetic GPRP-NH2 as an aggregation inhibitor added to the resolving gel buffer. The peptide-rich buffer was bounded by electrode buffer lacking the peptide. On commencing electrophoresis, this boundary migrated cathodally, leaving behind it a sharp front of buffer deficient in the peptide, while fibrin monomer and fibrinogen in the samples migrated anodally toward the buffer lacking the peptide, while fibrin monomer and fibrinogen in the samples migrated cathodally, leaving behind it a sharp front of buffer deficient in the peptide, while fibrin monomer and fibrinogen in the samples migrated anodally toward the peptide-poor boundary. As illustrated previously (36), fibrin monomers at concentrations below ~1 mg/ml migrated in a non-aggregated form at a rate equal to fibrinogen while the inhibitory peptide was present, but as they passed into buffer lacking GPRP-NH2, they sharply aggregated into immobile fibrin strands. At high concentrations (>1 mg/ml), portions of fibrin monomer exceeding solubility in GPRP-NH2 remained behind, near the application point. As tested with mixtures of fibrinogen and purified α-fibrin monomer, the method resulted in clear separations of the monomer from fibrinogen and enabled detection of the monomer at levels down to 1% of the 3 mg/ml fibrinogen (Fig. 1).

Fibrin as the Principal Self-aggregating Derivative of FPA

FIG. 1. GPRphoresis of mixtures of fibrinogen (ϕ) and purified α-fibrin monomer (p). The illustration shows Coomassie-stained electropherograms of mixtures with varying percentage content of α-fibrin monomer. Immunostaining of a duplicate gel probed with anti-fibrin a17–23 mAb showed that none of the added fibrin comigrated in the fibrinogen band (not shown). p, α-fibrin.
The results illustrated in Fig. 3 show thrombin reactions that were carried out in the presence of 1 mM GPRP-NH₂, which minimizes release of fibrinopeptide B by suppressing fibrin aggregation (46). Similar results were obtained when reactions were carried out under a variety of conditions (data not shown). These conditions included reactions 1) without added GPRP-NH₂, 2) with 2.5 mM added calcium, 3) with either low or high thrombin concentrations (0.1–10 units/ml), 4) using both bovine and human thrombin, and 5) at ambient or physiological temperature.

Identification of α-Profibrin as a Component Comigrating with Fibrinogen—Immunoprobing Western blots with the fibrin-specific (30) anti-fibrin α17–23 mAb (anti-GPRVVE) revealed the early appearance of a component lacking FPA but coprecipitated with fibrinogen (Fig. 3, lower panel). This component appeared in small quantities prior to detectable fibrin (Fig. 3, 15S versus lane 15P). The small amounts of FPA that were carried out in the presence of 1 mM GPRP-NH₂, so that reaction products could be quantified on the PhosphorImager with greater precision than staining, which tends to be saturable. Duplicate gels were immunoblotted with anti-FPA mAb and anti-fibrin α17–23 mAb and are illustrated in Fig. 3, lower panel. The small amounts of FPA associated with the fibrin monomer band and was fully separable from it. As determined by densitometry of immunostained gels, the apparent concentrations of α-profibrin found in the course of the reactions reached maximally 20–25% of the terminal fibrin concentration observed in six separate reactions, three with fibrinogen at 3 mg/ml and three at 9 mg/ml.

There was some background staining of the electropherograms in the region between the fibrinogen and fibrin bands by anti-fibrin α17–23 mAb at the late, but not the early, stages of thrombin reactions (Fig. 3, lower panel). This background staining did not arise from α-profibrin because it was not observed with anti-FPA mAb (Fig. 3, upper panel). Similar backgrounds were observed with high loads of purified α-fibrin monomer and were accordingly deemed to be due to trailing of slow aggregating components of the fibrin, which do not precipitate as sharply as the bulk.

Summarily, the electrophoresis provided good separation of fibrin monomer from fibrinogen and α-profibrin, but no separation of α-profibrin from fibrinogen. Consequently, a method was sought to physically separate α-profibrin from fibrinogen.

Enrichment of α-Profibrin by Cryoprecipitation with Fibrin—Cryoprecipitates from reaction mixtures were examined to determine whether low temperatures could drive (47) α-profibrin into forming aggregates. The cryoprecipitates were produced with TAME added as a solubility-lowering agent, previously shown to facilitate precipitation of fibrin complexes (12, 42). GPRPhoresis showed that the cryoprecipitation drove all but trace levels of fibrin monomer from solution, and it coprecipitated with a nearly equal amount of fibrinogen/α-profibrin (Fig. 4). Immunostaining indicated that α-profibrin coprecipitated with the fibrin to a greater extent than did the fibrinogen. As verified by subsequent gel filtration studies (Fig. 5), the cryoprecipitates generally contained nearly equal amounts of fibrinogen and α-profibrin associated with the fibrin, while α-profibrin comprised <20% of the fibrinogen prior to the cryoprecipitation. The α-profibrin did not undergo cryoprecipitation by itself since 1) most of it remained soluble when fibrin levels were low compared with α-profibrin levels (Fig. 4, left panel, lane 15S versus lane 15P), and 2) the amounts of α-profibrin precipitated were always smaller than those of fibrin (lane 15P). The cryoprecipitation of α-profibrin was accordingly deemed to be dependent on its complexing with fibrin monomer.

The preferential binding of α-profibrin to fibrin was most evident in cryoprecipitates from reaction mixtures containing large amounts of fibrin monomer (40% of Coomassie staining) (Fig. 4, right panel). With these solutions, coprecipitation of α-profibrin by the large amount of fibrin monomer nearly depleted α-profibrin from the solution (Fig. 4, left panel, lane 60S versus lane 60P), while only 20% of the fibrinogen cryoprecipitated as estimated from Coomassie staining of the supernatant solution (right panel, lane 60S versus lane 60R). After dissolving the cryoprecipitates at 42 °C and adjusting the temperature back to 20 °C, much of the redisolved fibrin monomer aggregated into a clot. We could drive more of the fibrin monomer out of solution by lowering the temperature further, and all could be precipitated by also adding 8 mM TAME, but losses of the α-profibrin through coprecipitation became unacceptably high. Subsequent attempts to separate the α-profibrin from the fibrinogen by gel chromatography proved difficult with the fibrin fully removed.

Gel Chromatography—Full separations of α-profibrin from fibrinogen were achieved by chromatography of redisolved cryoprecipitate on 6% agarose gel at 4 °C (Fig. 5). However, the separations were deceptive in that they were dependent on the α-profibrin/fibrin interaction and were not due to an intrinsic separability of α-profibrin from fibrinogen. No separation of
with TAME (7.5 mM) to drive the precipitation of fibrin monomer. The specimens were chilled to 0°C and admixed of fibrin monomer. There was partial coprecipitation of precipitated fully. There was partial coprecipitation of precipitates. The supernatant solutions showed that fibrin monomer pre-

The transport of the fibrin in a soluble form through the columns in the lead fractions and its persistent solubility on storage of the lead fractions at low temperature surprised us because it is usually very insoluble at low temperatures. The enhanced solubility of the fibrin in the chromatographic fractions was due in part to the absence of fibrinogen. We could remove the fibrin from the lead fractions by GPRphoresis, but a higher capacity method was needed.

Fibrin Removal with Anti-FPA mAb—Immobile anti-FPA mAb (covalently linked to protein A/G-agarose) was examined as an affinity gel to attempt separation of components according to their FPA content, but it yielded only marginal separations of α-profibrin from fibrinogen, which were retarded but not retained in the columns. However, the affinity gel did have a useful effect in promoting precipitation of all of the fibrin out of the applied samples near the top of the column. As illustrated in Fig. 6, when an α-profibrin/fibrin sample that had coeluted free of fibrinogen on gel filtration was applied to the anti-FPA mAb column, no coelution of the fibrin occurred. Thus, we were able to obtain α-profibrin free of both fibrinogen and fibrin by first separating the α-profibrin/fibrin from fibrinogen by gel filtration chromatography and then removing contaminating fibrin from the α-profibrin by passing the fractions through the anti-FPA mAb affinity gel.

We reasoned that the antibody was partitioning the α-profibrin away from the fibrin, which was largely restricted to the void volume, and was also shielding the exposed GPR domain on α-profibrin, which in turn diminished its inhibitory effect on fibrin aggregation. This latter prospect was supported by the observation that adding soluble anti-FPA mAb (2:1 molar proportion) to an α-profibrin/fibrin solution resulted in aggregation of 75% of the dissolved fibrin into a precipitate.

Kinetic Aspects of Formation of α-Profibrin and α-Fibrin Monomer—Two approaches were taken to quantify the levels of α-profibrin and α-fibrin monomer over the course of the reactions with thrombin: 1) stoichiometrically by calculating the α-profibrin levels from the differences between the percentage FPA release determined by HPLC and the percentage conversion of 125I-fibrinogen to fibrin determined from radioactivities

α-profibrin from fibrinogen was obtained when the fibrin content of the preparations was reduced below 3% of the total protein. Poor separations were also obtained when chromatography was performed at ambient instead of low temperature. Separations similar to that shown were regularly obtained with redissolved cryoprecipitate containing fibrin monomer at levels amounting to 8–12% of the protein, the portion remaining soluble after two-thirds of the fibrin in the cryoprecipitate were removed by its coagulation at room temperature. Most of the fibrin eluted near the leading edge of the α-profibrin peak (Fig. 5).

The transport of the fibrin in a soluble form through the

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FIG. 3. Western blots of thrombin/fibrinogen reactions immunoprobed with anti-FPA mAb (upper panel) and anti-fibrin α17–23 mAb (lower panel), with antibody retention reported with peroxidase-labeled anti-mouse secondary antibody. f, fibrin; ϕ, fibrinogen; p, α-profibrin.

FIG. 4. GPRphoresis showing enrichment of α-profibrin in cryoprecipitates from thrombin/fibrinogen reaction mixtures. Reactions were limited by adding PPACK at either 15 or 60 s to produce specimens with either low (lane 15R) or high (lane 60R) concentrations of fibrin monomer. The specimens were chilled to 0 °C and admixed with TAME (7.5 mM) to drive the precipitation of fibrin monomer to completion (35) and were analyzed by GPRphoresis. Direct immunoprobing of the gel with 125I-labeled anti-fibrin α17–23 mAb (left panel) by the PhosphorImager showed the relative concentrations of fibrin monomer (f) and α-profibrin (p) in the reaction mixtures (Rxn, R) and in the supernatant solutions (Sup, S) and pellets (Pel, P) of the cryoprecipitates. The supernatant solutions showed that fibrin monomer precipitated fully. There was partial coprecipitation of α-profibrin (lane 15S versus lane 15P) by fibrin at low concentrations, but nearly complete coprecipitation by high fibrin concentrations. The right panel shows Coomassie staining of the samples from the 60-s reaction. The relative intensities of staining of the fibrin and α-profibrin/fibrinogen (ϕ) bands in lane 60P (right panel) did not differ greatly from the immunostaining with anti-fibrin α17–23 mAb (left panel, lane 60P), an indication that α-profibrin comprised much of the protein in the α-profibrin/fibrinogen band.
**FIG. 5.** Low temperature gel chromatography of redissolved cryoprecipitate (A) and immunoprobed electropherograms of chromatographic fractions (B). The applied sample consisted of 1 mg (0.4 ml) of redissolved cryoprecipitate from which the fibrin was partially removed as a clot that formed on standing 2 h at 20°C. The plots in A profile the portions of total UV-absorbing material occurring in each of the fractions (●) and the distributions of fibrinogen (+), α-profibrin (□), and α-fibrin (○). The elution of fibrinogen (+) was tracked from radioactivity from 125I-fibrinogen added to the sample as a tracer and was profiled in relation to the total protein by setting the radioactivity equivalent to the UV absorbance in fractions 15 and 16, which were found by GPRegrophoresis (B) to be free of α-profibrin and fibrin. The relative amounts of α-profibrin and fibrin in the fractions were determined by immunoprobing electropherograms with anti-fibrin α17–23 mAb (upper panel), which binds equivalently to α-profibrin (p) and α-fibrin monomer (f), and anti-FPA mAb (lower panel), which binds equivalently to α-profibrin and fibrinogen (φ).

We also examined the production of α-profibrin and fibrin monomer by the venom enzyme Atroxin. This enzyme yielded high peak levels of α-profibrin that ranged between 50 and 60% [φ], in three experiments. This high maximum indicated, according to Equation 3, that the rate of conversion of α-profibrin to α-fibrin by this enzyme proceeded two times slower than the conversion of fibrinogen to α-profibrin (k1/k2 = 2.2), the converse of the relative rates determined for thrombin reactions, where k1/k2 ranged from 0.6 to 0.2.

**FIG. 6.** Electropherograms confirming removal of fibrin monomers from a partially purified α-profibrin/fibrin mixture. Lane 1 shows the fibrin content of redissolved cryoprecipitate applied to a gel chromatography column to separate the α-profibrin (p) and fibrin (f) from fibrinogen (φ), as in Fig. 5. Lane 2 shows the fibrin content of fibrinogen-free α-profibrin/fibrin fractions pooled from the column, as in fractions 10–12 of Fig. 5. Lane 3 depicts the presence of α-profibrin alone in the eluate from a column containing anti-FPA mAb affinity gel in which the fibrin underwent aggregation and precipitated. Eluted protein was concentrated by precipitation with 0.14 volume of cold ethanol and redissolved in TBSE.

As anticipated by many investigations spanning 3 decades, the first derivative in the fibrinogen/fibrin transformation does, indeed, consist of a discrete intermediate lacking one of the two residues of FPA that are cleaved from fibrinogen A chain, almost indistinguishable from fibrinogen as typically illustrated for the first subjects analyzed (Fig. 8). More important, fibrin monomer was also immeasurably small (<5% of the α-profibrin) in these subjects, as well.

**DISCUSSION**

As anticipated by many investigations spanning 3 decades, the first derivative in the fibrinogen/fibrin transformation does, indeed, consist of a discrete intermediate lacking one of the two residues of FPA that are cleaved from fibrinogen A-chains in the initial production of fibrin. This elusive precursor to α-fibrin, which had not been isolated or clearly characterized previously, is shown here to be an extremely weak aggregating derivative, almost indistinguishable from fibrinogen. It does not undergo perceptible aggregation with itself and does not co-aggregate with fibrin much more tightly than fibrinogen does during the initial stages of thrombin reactions when fibrinogen levels are high compared with fibrin levels. The similarity of the physical properties of α-profibrin and fibrinogen conforms with previous indications (12, 14–20, 22) that the fully converted α-fibrin monomer lacking both FPAs is the initial self-aggregating derivative produced under ordinary cir-
the levels of both electrophoresis and gel chromatography. Results of the present study indicate that the complexes are consists of high molecular weight complexes (48, 49). The re-

ever, electron microscope studies indicate that the lead peak eluted well ahead of fibrinogen. Smith (23) concluded that they 
tunes in many preceding studies had separated complexes that eluted well ahead of fibrinogen. Smith (23) concluded that they consisted of dimers formed by the fibrin intermediate. However, electron microscope studies indicate that the lead peak consists of high molecular weight complexes (48, 49). The results of the present study indicate that the complexes are principally mixtures of α-profibrin and α-fibrin. When fibrin monomer is absent, α-profibrin comigrates with fibrinogen on both electrophoresis and gel chromatography.

Methods were not previously available to distinguish the α-profibrin from fibrinogen because there is only a 0.5% difference in the molecular weights of the proteins. Meh et al. (28, 50) examined plasmin-derived and CNBr-derived N-terminal frag-
ments comprising one-sixth of the mass of the intact molecules and succeeded in partially resolving derivatives with mass and isolectric points in between those of the reference fragments from fibrinogen and α-fibrin monomer. The identification of α-profibrin in the present study was made by a two-step procedure in which 1) gel shifts induced with a peptide analog (GPRP-NH₂) of the fibrin aggregation site separated fibrin from a mixed fibrinogen/α-profibrin band, and 2) the α-profi-

brin in the mixed band was revealed by immunoprobing with a monoclonal antibody directed to the N-terminal segment of fibrin that becomes exposed on release of FPA (30).

The gel chromatography and electrophoresis applied in this study complemented each other for the isolation of α-profibrin free of both fibrinogen and fibrin. In electrophoresis, the self-aggregating fibrin complexes were sieved away and left permanently behind the fibrinogen and α-profibrin. With the fibrin removed, the α-profibrin and fibrinogen became inseparable. Conversely, in gel chromatography, the fibrin complexes moved ahead and aggregated into insoluble strands in front of the fibrinogen and α-profibrin, and the fibrin underwent continual redistribution in the trailing fibrinogen and α-profibrin. The tighter interaction of α-profibrin with the fibrin monomers promoted transport of the α-profibrin ahead of the fibrinogen. This continual transport of α-profibrin ahead of fibrinogen promoted full separation of α-profibrin from fibrinogen when fibrin concentrations were adequately high, but yielded mixtures of α-profibrin and fibrin. We were able to isolate fractional milligram quantities of α-profibrin by electrophoretically removing the contaminating fibrin from the chromatographic fractions, but the separations involved a lot of work for low yields. A more productive method was devised by using anti-FPA mAb to shield the α-profibrin against interacting with the fibrin, which in turn left the fibrin free to aggregate into a clot. With immobilized antibody, the fibrin formed insoluble aggregates as the α-profibrin was partitioned away from it into the gel matrix. This preferential partitioning of the α-profibrin into the gel matrix and concurrent aggregation of the fibrin in the excluded volume caused the fibrin to be retained while the α-profibrin moved ahead, retarded but not retained in the gel, a “reverse affinity chromatography.”

Basis for the Weak Aggregation of α-Profibrin—The release of FPA from the Aα-chains unMASKS aggregation sites with the essential sequence GPR located immediately behind FPA (8, 10), and one would expect that interactions through the single GPR site on α-profibrin would result in some dimerization. However, ultracentrifugation studies failed to detect any dimer formation in the course of thrombin reactions (14), and α-profibrin was not separable from fibrinogen by methods used here except as complexes with fibrin monomer. The interactions...
through single GPR sites seem to be quite weak by themselves. Otherwise, concentrations of GPR peptides on the order of 20 μM and not dissociate fibrin(ogen) monomers (8, 46, 51). The aggregation of fibrin is probably supported by additional coupling interactions that enable its two GPR sites to function cooperatively (52, 53). This was indicated by ultracentrifugation studies on β-fibrin (52, 54) and α-fibrin (55) monomers at dissociating temperatures, where trimers were the principal precursors of larger oligomers and dimers could not be found. From considerations with model structures (56, 57), illustrated as Structures A–D in Fig. 9, it can be seen how trimers and larger assemblies (Structures B and C) can become reinforced by coupling interactions that would not prevail in dimers formed through single GPR interactions (Structure A).

The two GPR sites on fibrin molecules would not produce reinforced assemblies if they functioned independently. Structure D in Fig. 9 illustrates a dimeric assembly reinforced by reciprocal single GPR site interactions on each of two molecules. Since purified α-profibrin was electrophoretically indistinguishable from fibrinogen (Fig. 5B, lanes 12–13), it does not seem likely that such coupling occurs to a substantial degree. However, we suspect that a partial expression of such coupling may be contributing to the tendency of fibrin monomer to interact more strongly with α-profibrin than with fibrinogen, about five times more strongly judging from the degree of enrichment of α-profibrin above fibrinogen in cryoprecipitates.

We believe that the principal coupling interaction that reinforces fibrin assembly probably arises from an intrinsic interaction that exists between fibrin(ogen) molecules through their D-domains. The contribution of this D ↔ D interaction was deduced previously (52), supported by the observation (31, 58) that the D-domains of fibrinogen undergo cross-linking as rapidly as the prealigned D-domains in fibrin at high, but not low, concentrations. The $K_D$ for the D ↔ D interaction is estimated to be on the order of $10^{-7} \text{ M}$ (31). It is too weak to be sensed except at high concentrations, but would operate cooperatively with the paired GPR interactions of fibrin monomers because these interactions place the D-domains in close apposition within the aggregates (Structures B and C in Fig. 9). Direct dissociation of α-fibrin trimers into monomers occurs at 41°C, with $K_D = 10^{-8.6} \text{ M}$, which corresponds to a $K_D$ on the order of $10^{-9.5} \text{ M}$ at 25°C based on an enthalpy of 20 kcal/mol (52). The $K_D = 10^{-4} \text{ M}$ for the D ↔ D interaction combined with the GPR site interaction on the order of $10^{-5} \text{ M}$ would explain the $K_D = 10^{-8} \text{ M}$ for the trimer. Large oligomers (Structure C) are probably stabilized somewhat further because of the reciprocal coupling that further reinforces the trimeric units within the oligomers (52), depicted by the molecules with duplicated coupling of interactions (\(\land \lor\)) in Structure C. These reinforcing interactions are viewed as intrinsic to the aggregation process and differ from the “b” aggregation sites that Olexa and Budzynski (59) suggested to arise as a product of aggregation. They are viewed as occurring within, rather than between, protomers.

Kinetics of α-Profibrin Formation—Our appraisal of the kinetics of formation of α-profibrin and its conversion to fibrin indicate that FPA is released from α-profibrin several times faster than it is released from fibrinogen by thrombin. Conversely, the venom procoagulant Atroxin cleaves FPA from fibrinogen and α-profibrin at equivalent rates. The 2-fold difference in rate constants ($k_r/k_a = 2$) in the Atroxin-catalyzed reactions reflects the fact that fibrinogen contains two FPAs whereas α-profibrin contains one. The underlying reason for the more rapid release of the second FPA by thrombin is not known. Numerous prior studies (12, 14–22) on the stoichiometry between the release of FPA and fibrin production had indicated that the loss of the second FPA proceeds even more rapidly than estimated here or in the studies by Meh et al. (28, 50) based on measuring the production of α-profibrin. It should be emphasized, however, that direct measurements of the release of FPA have generally coincided with the production of fibrin monomer within percentages on the order of 10%. A 10% disparity allows for a 20% production of α-profibrin, near the maximal level of production found here.

Studies by Naski and Shafer (60, 61) on the effects of fibrin on the enzymatic activity of thrombin toward other substrates demonstrated that thrombin binds to fibrin through an exosite that is independent of the binding through its active site. Furthermore, the exosite binding is enhanced in fibrin compared with fibrinogen. Whether this exosite binding might facilitate the release of the second FPA remains to be determined. The PPACK used to stop reactions in this study inactivates both free and fibrin-bound thrombin. We plan to determine whether use of thrombin inhibitors that act more preferentially with free thrombin might yield lower relative levels of α-profibrin/fibrin throughout the course of FPA release.

Direct measurement of FPA release from purified α-profibrin also needs study to determine whether it might be a better substrate than fibrinogen as suggested by the faster release of FPA. Furthermore, only one FPA needs to be released from α-profibrin for its conversion to fibrin, while two must be released from fibrinogen. Whether these two aspects of the conversion of α-profibrin to fibrin contribute significantly to hypercoagulable states predisposing thrombosis would depend on the levels occurring in blood. It is too early to comment on how high levels might rise. We found α-profibrin comprising only ~0.6% of the fibrinogen in the small group of patients with inactive peripheral vascular disease examined thus far, but we anticipate that much higher levels will be found in other groups. As depicted previously (62), patients undergoing coronary bypass surgery present presurgical levels of fibrin monomer as high as 5% of the fibrinogen, and we suspect that the levels of α-profibrin were probably much higher. More important, in the subjects presented here, the fibrin monomer levels were immeasurably small compared with α-profibrin levels. Many of the tests currently being used clinically to assess fibrin monomer levels in blood probably measure α-profibrin as well, without distinction. Whether it is important to distinguish between α-profibrin and fibrin monomer will depend in part on whether α-profibrin is cleared from the circulation at a rate very much slower than that of fibrin monomer (63).
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α-Profibrin and Its Conversion to Fibrin
