An Integrated Study of Fibrinogen during Blood Coagulation*

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Kathleen E. Brummel, Saulius Butenas, and Kenneth G. Mann‡

From the Department of Biochemistry, Given Building, Health Science Complex, University of Vermont, College of Medicine, Burlington, Vermont 05405-0068

The rate of conversion of fibrinogen (Fg) to the insoluble product fibrin (Fn) is a key factor in hemostasis. We have developed methods to quantitate fibrinopeptides (FPs) and soluble and insoluble Fg/Fn products during the tissue factor-induced clotting of whole blood. Significant FPA generation (>50%) occurs prior to visible clotting (4 ± 0.2 min) coincident with factor XIII activation. At this time Fg is mostly in solution along with high molecular weight cross-linked products. Cross-linking of γ-chains is virtually complete (5 min) prior to the release of FPB, a process that does not occur until after clot formation. FPB is detected still attached to the β-chain throughout the time course demonstrating release of only low levels of FPB from the clot. After release of FPB a carboxypeptidase-B-like enzyme removes the carboxyl-terminal arginine resulting exclusively in des-Arg FPB by the 20-min time point. This process is inhibited by ε-aminocaproic acid. These results demonstrate that transglutaminase and carboxypeptidase enzymes are activated simultaneously with Fn formation. The initial clot is a composite of Fn I and Fg already displaying γ-γ cross-linking prior to the formation of Fn II with Bj-chain remaining mostly intact followed by the selective degradation of FPB to des-Arg FPB.

Blood coagulation proceeds through a cascade of protein activation's that ultimately lead to the catalytic cleavage of fibrinogen (Fg) by thrombin to the product fibrin (Fn). Fn is generated from plasma Fg (M, 340,000) which is found in blood plasma at ~3 mg/ml and exists as a symmetrical dimer consisting of Aα, Bβ, and γ polypeptidic chains linked by noncovalent and disulfide bonds (1–4). The two carboxyl-terminal domains of the Bβ and γ-chains of Fg are designated “D” while the central domain which contains the amino termini of all the chains is designated “E.” Clot formation which has been extensively studied in anticoagulated plasma and purified Fg occurs in a series of steps (4) initiated by thrombin cleavage of the Aα and Bβ-chains of Fg. Cleavage at Aα-16 releases fibrinopeptide (FP) A to form Fn I. The release of two FPA peptides exposes a site in the E domain that aligns with a complementary site in the D domain to form overlapping fibrils (5). This is followed by cleavage at Bβ-14 releasing the two FPB peptides to form Fn II. FPB release appears to allow for lateral aggregation of the protofibrils (6, 7). The degree of lateral strand association contributes to the tensile strength of the clot, but its resistance to plasmin degradation is influenced mainly by covalent cross-linking. Cross-links are formed by the action of factor XIIIa (XIIIa), a transglutaminase enzyme whose formation from zymogen XIIIa (plasma concentration 90 nmol/liter) is also catalyzed by thrombin (8, 9). FXIII consists of an Aβ2 tetramer where the A subunit is acted upon by thrombin releasing an NH2-terminal activation peptide. Covalent isopeptide cross-links are formed between certain adjacent γ-carboxamido and ε-amino groups of glutamyl and lysyl residues within the extreme carboxyl-terminal γ-chains rapidly forming γ-γ dimers (10, 11). The carboxyl-terminal α-chains are also cross-linked but these isopeptide bonds form more slowly (12, 13). However, a recent study conducted in human plasma observed that α-polymers and Aα-polymers are already present at the point of gelation (14). The overall stability of the clot appears to be dependent upon the formation and orientation of the Fn monomers (15).

In vitro, Fn formation in native whole blood proceeds principally by the activation of thrombin by the intrinsic (contact) pathway of coagulation (16). However, the pathway relevant to physiological hemostasis is the extrinsic (tissue factor, TF) pathway (17) that proceeds through assembly of three membrane surface and vitamin K-dependent enzyme cofactor complexes (18, 19). The initiating complex, is formed when circulating blood containing factor VIIa comes in contact with TF membrane (20). The resulting complex activates zymogens factor X and factor IX (21–23) to the proteases which with their cofactors factor VIIIa and factor Va form the complexes which propagate the formation of thrombin. Studies of the relevant TF pathway in blood in vitro are only possible by suppression of the contact pathway. We have developed techniques by which selective inhibition of factor XIIa is accomplished by corn trypsin inhibitor (CTI) (24) permitting TF pathway analysis (25). Fg activation products have been studied extensively (2, 15, 26) utilizing a variety of methods including high performance liquid chromatography (HPLC) (27–30). Both in purified systems (31, 32) and plasma that was treated with thrombin (33) FPs isolated by HPLC methods showed Fg acted upon by thrombin releases first FPA followed by a delayed release of FPB. Previous studies undertaken to quantitate FPs generated in whole blood (25, 34–37) have utilized immunooassay techniques for FPA determination. Fg, FPA, thrombin levels, and platelet activation have been studied in whole blood in normal...
(25) and hemophilia patients’ (34). Studies on normal donors showed that significant amounts of Fg (80%) and FPA (45%) were incorporated into the initial clot at low levels of thrombin (−15 nmol/liter) (25). Comparative studies on a factor VIII-deficient patient (hemophilia A) showed increased clot time with decreased thrombin levels (1.9 nmol/liter/min versus normal 55 nmol/liter/min) characterized by a slower rate (−30% of the normal) of Fn formation (34). Normal levels of FPA were reached by 20 min even with decreased thrombin generation. Therefore, the level of FPA generation in hemophiliacs does not explain why clots are more friable in these patients. Hence, we wanted to develop a system that can be used to observe other Fg degradation products (i.e. FPB) involved in clot formation and possibly explain why clots are unstable in hemophiliacs. Characterizing other FP products (i.e. FPB) have been attempted (38) but little information is available regarding FPB detection in whole blood. The results obtained present a somewhat different sense of observations from those observed with purified Fg and anticoagulated plasma.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human Fg, >95% clotable (a gift from Dr. Laszlo Bajzar), was isolated by the procedure of Straughn and Wagner (39) followed by Lys-Phosphatase and α-XXIII chromato graphy to remove traces of naspinogen and IXIII. Thrombin was prepared from prothrombin by the general methods of Landblad et al. (40). Murine monoclonal α-Fg (a Fbng 2E) specifically binds the α-chain of Fg. Monoclonal α-FPB (P10/1) was a gift from B. Kudryk (NY Blood Center). The antibody reacts with both FBP (Bj1-14), des-Arg FBP (Bj1-13), and peptides containing FBP. Rabbit polyclonal α-XXIII (D4679) was a gift from Dr. Gerry Lasser (ZymoGenetics, Seattle, WA). Monoclonal α-TAFI number 16 was prepared as described previously (41) and shown to inhibit TAFI activation. δ-Phenylalanyl-d-prolyl-arginyl-prolyl-glycyl-L-lysine (derived from Fbng 2E) χIII, and thrombin activatable fibrinolysis inhibitor (TAFI) were gifts from Dr. Richard Jenny (Haematologic Technologies, Essex Junction, VT). Recombinant human TF was provided as a gift by Drs. Roger Lundblad and Shu-Liu Lin (Baxter Healthcare Corp., Duarte, CA). Recombinant soluble thrombomodulin (Solulin) was provided as a gift from Dr. J. Morser, Berlex (Richmond, CA). CTI was prepared according to modified methods (34) of Hjima et al. (24). Goat anti-mouse IgG horseradish peroxidase and goat anti-rabbit (H-L) IgG-horseradish peroxidase were purchased from Southern Biotech (Birmingham, AL). Assercharon enzyme-linked immunosorbent assay (ELISA) kits for FPA were purchased from Diagnostic Stago (American Bioproducts, NJ). Thrombin-AT-III ELISA kits were purchased from Enzymogen (TAT Behring, CA). HPLC grade Acetonitrile, CH3CN, 0.1% trifluoroacetic acid were purchased from VWR (Bridgeport, NJ). HEPES, KOH, ε-aminoacaproic acid (EACA), potato carboxypeptidase inhibitor, 1-palmitoyl-2-oleoyl-phosphatidylcholine, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine, chicken albumin, PEG 8000, and EDTA were purchased from Sigma.

**Methods**

Normal donors (age range 21–37) with no history of blood disorders, regular aspirin, or drug use were recruited and advised according to a protocol approved by the University of Vermont Human Subjects Committee. All individuals exhibited normal ranges for plasma Fg (147–340 mg/dl). Blood was collected by venipuncture and rapidly distributed to a series of test and control tubes containing 12.5 pmol/liter of TF eluted in 25 ml/liter of phosphatidylcholine/phosphatidylethanolamine (75% phosphatidylcholine/25% phosphatidylethanolamine as described previously (42)) and CTI (100 μg/ml) (25). When used, EACA (50 μmol/ml), was added to the tubes prior to the addition of blood. Tubes were quenched over time with a mixture of coagulation inhibitors, 50 mmol/liter EDTA with 20 mmol/liter benzamidine-HCl in HBS, pH 7.4, and 5 μmol/liter PFRck ± 50 μmol/liter EACA. Aliquots were collected either every minute from 0 to 10 or 2 to then 15 with an interval of 20 min. Samples were quenched by mixing with neutralizing CTI and no TF was added as a control to determine the quality of the phlebotomy and the extent of contact pathway inhibition. Clot time was determined visually. Solid material was removed by centrifugation (15 min at 2000 rpm) and stored at −80 °C. The fluid material was analyzed directly or aliquoted and stored at −80 °C for further analysis.

**Analysis of Whole Blood—SDS-PAGE (4–12%) was performed according to the modified (25) Laemmli (43) procedure. High molecular weight standard mixtures (4–200 kDa) were loaded along with Fg standards (300 ng/lane), XIXIII (50 ng/ml) were activated with 80% XIIIIXIII (XiIIA) to allow for comparison on the immunoblots. The gels were transferred to nitrocellulose membranes (Bio-Rad) and subjected to semi-dry transfer for 3 h at 250 mAMP as described by Towbin et al. (44). The primary antibody was either a-Fgpm2E or polyclonal α-XIIIIXIII used at 5 μg/ml, the secondary antibody (goat α-mouse IgG horseradish peroxidase or goat α-rabbit IgG horseradish peroxidase) at 1:5000 dilution. The substrate for emitting light was Lumilow (NEL Life Science Products Inc.). The blots were developed as described previously (25). Comparisons of XIIXIII conversion to XIIaIIa, and Fg levels in solution were analyzed on the immunoblots. A relative fraction was calculated from Fg or XIIaIIa present at each time point divided by total Fg or total XIIaIIa chain (XIIXIII = XIIaIIa) detectable.

FP standards were prepared from Fg (2.5 mg/ml) in HBS treated with thrombin (5 nmol/liter), 37 °C, 1 h. The reaction was stopped by the addition of HClO4 (0.2 mol/liter final) and the precipitated proteins removed by centrifugation (10 min at 14,000 rpm). The supernatant was subsequently treated with equimolar KOH and allowed to sit on ice for 30 min, removing the salt precipitate by centrifugation. The soluble material from the whole blood quenched sample time points were also treated with HClO4 (0.2 mol/liter final) and neutralized with equimolar KOH. The HPLC analyses of the FPs were conducted without further separation. The identities of the resolved fractions were confirmed by mass spectrometry, amino acid composition, and amino acid sequence analyses. Fractions corresponding to phosphorylated FPA (F-PFA), FPA, des-Ala FPA, FPB, and des-Arg FPA were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (linear model; PE Applied Biosystems, Foster City, CA). The matrix used was α-cyano-4-hydroxycinnamic acid, 70–300, H2O, CH3CN, 0.1% trifluoroacetic acid at a 1:1 ratio with the sample. Amino acid sequencing and composition were performed on the samples (Dr. Alex Kurosky University of Texas, Medical Branch, Galveston, TX). The concentrations of stock FPs were assessed by amino acid composition analysis.

To prepare des-Arg FPB standard analyses and the process, the reaction (2.5 mg/ml) was incubated for 35 min at ambient temperatures with TAFI (70 nmol/liter), thrombomodulin (4 nmol/liter), and thrombin (10 nmol/liter) in 20 mmol/liter HEPES, 150 nmol/liter NaCl, 5 nmol/liter CaCl2, 400 ng/liter human albumin (0.1% 80% TAFI), and the reaction was killed by the addition of HClO4 (0.2 mol/liter final), centrifuged (14,000 rpm, 10 min), supernatant neutralized with equimolar KOH, and the resultant solution analyzed by HPLC methods as described above. FPA and FPB stocks (1.5 μmol/liter) in 0.04 mol/liter NaH2PO4, 0.084 mol/liter NaCl, 0.01% PEG 8000, were also incubated with TAFI (70 nmol/liter) activated with thrombomodulin (40 nmol/liter) and thrombin (80 nmol/liter) in 20 mmol/liter HEPES, 150 nmol/liter NaCl, 5 nmol/liter CaCl2, and human albumin (0.1%). The reaction was carried out at 22 °C for 1 h then stopped by HClO4 (0.2 mol/liter final) neutralized with equimolar KOH and analyzed by HPLC methods.

FPA and FPB standard calibration curves of micrograms of injected versus area were developed. Correlation coefficient values were calculated to be as follows: FPA r2 = 0.986, FPB r2 = 0.940. Whole blood samples were spiked at the zero time point with a known amount of FPs and percent recovery determined. Recovery of spiked whole blood samples was calculated to be 57%. Therefore, all samples were corrected for by this factor along with corrections for whole blood sample dilution from 1.0 ml of added quench reagents as described previously (34).

Commercial FPA and thrombin-AT-III (TAT) ELISAs were conducted according to the manufacturer’s directions that have been previously described (34). Results were obtained and analyzed using a Vmax microtiter plate reader ( Molecular Devices, Menlo Park, CA) equipped with SOFTmax version 2.0 software and an IBM Personal System 2 Model 30/286 PC. Samples and standards (minimum of 5 standard concentrations) were run in duplicate or triplicate. The concentration was determined by log-logit fit of concentration of stand-
The clots were solubilized in 4 mol/liter urea, 10% SDS, 10% inhibitors (50 mmol/liter EDTA with 20 mmol/liter benzamidine-HCl) were quenched as a function of time with a mixture of coagulation inhibitors (50 mmol/liter EDTA with 20 mmol/liter benzamidine-HCl and 50 μmol/liter FFPrick). Soluble and insoluble material were separated by centrifugation (15 min at 2000 rpm). The soluble material was subsequently treated with HClO4 (0.2 mol/liter), centrifuged to remove any precipitate, then neutralized with equimolar KOH. A 10-min time point that was isolated by the same methods as described in A is illustrated in B. Combined FPA is seen along with FPB and a second peak that was identified as des-Arg FPB.

ard versus optical density as described by the manufacturer (Molecular Devices).

The clots were analyzed according to previously published procedures (45) as modified by our laboratory. The insoluble clotted samples were washed 2–3 times with 1 ml of 0.15 mol/liter NaCl and then allowed to sit in the salt solution (1 ml) for 12–15 h so that additional soluble material within the clots could diffuse into solution. The clots were rinsed with H2O to remove salt, lyophilized, and weighed. The dry clots were solubilized in 4 mol/liter urea, 10% SDS, 10% β-mercaptoethanol and analyzed by 5–15% SDS-PAGE. Products were visualized using a Coomassie Blue source or an α-FPB monoclonal antibody. Densitometry was performed on a Hewlett-Packard Scanjet 4C/T. Cross-linking results were expressed as a relative fraction of the dimer at each time point over the total (monomer + dimer).

RESULTS

Isolation, Characterization, and Quantitation of FPs—Purified Fg treated with thrombin releases three major forms of FPA: an unmodified form ADSGEGDFLAEGGGVR (FPA) which constitutes ∼70%, an NH2-terminal truncated form (des-Ala FPA) ∼10%, and a product in which Ser-3 is phosphorylated (P-FPA) ∼20%. This is followed by release of FPB (ZGVNDEEGGFSSR) which should exist in this unmodified form ∼95%. Fig. 1A displays an HPLC chromatogram depicting the forms of P-FPA, FPA, and FPB. The three forms of FPA while not completely resolved are identified by MALDI-TOF mass spectrometry (Fig. 2A). A whole blood assay performed as described (“Experimental Procedures”) was analyzed for FPs (Fig. 1B) following the procedure used for purified Fg. In contrast to the purified system, an additional peak, subsequently identified as des-Arg FPB was present. Approximately 22 studies on blood from multiple individuals confirmed these results although quantitative variability was observed from individual to individual. Des-Arg FPB was formed at different rates depending upon the individual blood being tested.

The identities of all Fg products was confirmed by MALDI-TOF mass spectrometry (Fig. 2) and amino acid analyses. Des-Ala FPA (Fig. 2A, a) shows a mass m/z = 1471.75, FPA (b) m/z = 1540.56, and P-FPA (c) m/z = 1621.68. Des-Arg FPB is seen in panel B (d) with a mass m/z = 1422.37 and its double ion at m/z = 2867.85 (d²). FPB in seen in panel C (e) as a molecular ion of 1556.48. The differences in the values compared with the true molecular ion mass are due to the addition of Na+ present. Since MALDI-TOF is not a quantitative tool the relative areas of the peaks cannot be used for quantitation.

Whole Blood Analysis of FPs—Coagulation of blood from normal volunteers after initiation with 12.5 pmol/liter TF was quenched at time points from 0 to 20 min. In Fig. 3, for a typical individual the time points of overlaid HPLC chromatograms are illustrated as 0 (I), 5 (II), 10 (III), and 20 (IV) min. Clotting was visualized at 4 ± 0.2 min while the CTI control (no TF) clotted at 13.25 min. FPA release was complete by 5 min, FPB was observed at lesser amounts, with des-Arg FPB already observed by 5 min. Over time FPB levels decreased and des-Arg FPB levels increased, resulting only in des-Arg FPB by 20 min. Similar results were obtained with des-Arg FPB levels in 15 other whole blood analyses on normal donors initiated with 12.5 pmol/liter TF, where the CTI controls ranged from 12 to 24 min and clot times were between 3 and 4.2 min.

The FPs were quantitated based upon calculated areas and standard curves were developed for FPA and FPB (Fig. 4). Clot time (CT) is represented by an arrow shown at 4 ± 0.2 min. The maximum levels (15.8 μmol/liter) (∼∼) of FPs which could be expected were calculated from the patients Fg levels (268 mg/dl) at the time of the blood draw. From this figure it is easy to see that (−50%, 7 μmol/liter) FPA (A) was present prior to clot time. This result is comparable to what has previously been seen in whole blood assays using commercial ELISA kits (25, 34). FPB (B) just starts to appear at clot time (<1 μmol/liter) and is quickly cleaved to des-Arg FPB (C). Maximum FPB and des-Arg FPB levels are equivalent (∼5 μmol/liter). Both the final levels of FPA (∼10 μmol/liter) and FPB (∼5 μmol/liter) at 20 min are below the expected FP levels (∼16 μmol/liter), possibly an indication of other degradation processes not yet identified. FPA levels appear to reach a maximum at clot time (∼14 μmol/liter) and begin to decrease with time to a final level of ∼10 μmol/liter. Overall, combined levels of released FPB are not equivalent to FPA levels. The delayed release of FPB after clot formation along with decreased overall levels is suggestive that not all FPB is being cleaved from the B5-chain.

FPB Degradation to Des-Arg FPB—Previous studies have observed the formation of des-Arg FPB (31, 32) and noted that the addition of EACA to the Fg solution prior to thrombin addition prevents the formation of des-Arg FPB. To test whether the enzymatic degradation of FPB occurs during the process of blood coagulation or after sample quenching, the following experiments were performed. 1) EACA (50 mmol/liter) was added to the collecting tubes prior to the addition of blood; 2) EACA (50 mmol/liter) was added to the mixture of coagulation inhibitors used to quench the reaction. 3) An experiment was performed where no EACA was added. The overlaid HPLC chromatograms illustrated in Fig. 5 represent the 0- (Ia/b), 5- (IIa/b), 10- (IIia/b), and 20-min (IVa/b) time points plotted as absorbance (214 nm) versus time (seconds). Panel A shows the experiment with EACA (50 mmol/liter) added prior to the distribution of blood. Panel B shows the experiment with no addition of EACA. FPA is seen first over FPB at 5 min in both panels A and B. FPB generation was unchanged throughout the course of these experiments. Therefore, EACA had no effect on FPA separation or stability. FPB remains uncleaved in panel A where EACA was added prior to the addition of blood. Without EACA, des-Arg FPB was formed with time (B, IIIb and IIIc). When EACA was added to the quench buffer,
des-Arg FPB was also observed (data not shown). Thus an enzyme is acting upon FPB during the course of blood coagulation and des-Arg FPB formation was occurring after the samples had been quenched. Whatever carboxypeptidase is cleaving the carboxyl-terminal arginine from FPB is selective. EACA is only inhibiting this carboxypeptidase from attacking FPB since FPA remained unchanged with/without EACA addition within the time frame of this experiment. In panel B (without EACA addition) there also appears to be several unresolved peaks present to the left of des-Arg FPB, that are not present in panel A. These could be other degradation products that are not yet identified, but are also inhibited by EACA.

Several carboxypeptidases can be responsible for causing this type of cleavage of FPB. These carboxypeptidases include: carboxypeptidase-B (46), carboxypeptidase-N (47), and carboxypeptidase-U (48). All are basic carboxypeptidases capable of cleaving carboxyl-terminal lysines or arginines. Previous studies showed that a potent carboxypeptidase-B inhibitor was unable to inhibit cleavage of FPB, unlike EACA (32). Carboxypeptidase-U or TAFI has previously been suggested to play a role in the premature lysis of clots from hemophilic plasma (49). In order to determine if TAFI was capable of cleaving FPB to des-Arg FPB we incubated TAFI (70 nmol/liter) with Fg (2.5 mg/ml), thrombin (10 nmol/liter), and thrombomodulin (4 nmol/liter) for 35 min at 25 °C. Results showed the same pattern of FP generation that was seen in whole blood (Figs. 3–5) (data not shown). The three forms of FPA were seen as well as FPB and des-Arg FPB. No apparent degradation of FPA was detectable during the allotted time interval of the experiment. Next we wanted to test activated TAFI with the substrates FPA and FPB directly to determine if TAFI was capable of cleaving the carboxyl-terminal arginine of FPA. Therefore, we incubated the stock FPs (10 μmol/liter final) with activated TAFI (70 nmol/liter final) 1 h at 22 °C. From these experiments we were able to identify FPA cleavage products as well as FPB cleavage products from HPLC analyses (data not shown), indicating that
FPA is also a substrate candidate for TAFI, but does not appear during the time course observed for our reactions. In contrast, FPB cleavage to des-Arg FPB occurs almost to completion during this time period.

Analyses of Fg/Fn and FXIII Activation in Whole Blood—Analyses of Fg depletion from solution is seen in the Western blots in Fig. 6. The quenched time points from 1 to 20 min are illustrated above the Western blot. Clotting occurred at 4 ± 0.2 min (arrow, CT). Fg/Fn is seen in solution (Fg) up to 4 min. At clot time Fg/Fn is almost totally (≥95%) out of solution. These results are similar to what was seen previously in our laboratory on whole blood analysis (24). At 4 min, just prior to clot time there is also evidence of a high molecular weight cross-linked product of Fg/Fn (X-Link). Prior to clot time, thrombin is generated at low (≤10 nmol/liter) levels. These levels of thrombin are enough to activate FXIII allowing transglutaminase activity to be present by 4 min. Since FPA release is already detected at 3 min, Fn is capable of forming complementary overlapping protofibrils at this time. Another possibility for the soluble cross-linked product is that Fg monomers are cross-linked by FXIIIa. Although from previous studies (50), it is known that Fg undergoes cross-linking more slowly than Fn, the preferred substrate for FXIIIa.

Analyses of FPA versus FXIII activation in quenched time points from a whole blood series is depicted in Fig. 7. The Western blot developed using polyclonal α-Fg is illustrated in the inset with time points from 0 to 5.7 min labeled above. Time points extending to 20 min were conducted but by 5.7 min conversion to the activated form of FXIII had reached a maximum. The top band seen in the immunoblot is the A-chain of FXIII (FΧIIIa) and the bottom band is seen as the activated form of FXIII (FΧIIIαa). A relative fraction of activated FXIII is calculated from this immunoblot. Clot time occurred at 3 ± 0.04 min.
min. The FPA data determined by HPLC methods and plotted as FPA (μM) is presented up to 5.7 min, which is also at a maximum at this time. This graph shows a direct correlation \( r^2 = 0.9795 \) between FXIII activation and FPA release. The burst between 4 and 11 μM FPA illustrates that FXIII is activated rapidly and coincidently with FPA cleavage.

**Analysis of the Clot for Cross-linking and FPB**—The insoluble material (clots) from the whole blood were analyzed for cross-linking on reduced SDS-PAGE gels by direct stainig methods (“Experimental Procedures”) and Western blotting with monoclonal α-FPB. The washed clots were solubilized at approximately 2.5 mg/ml. The results are seen in Fig. 8 depicting the reduced disulfide bonds, with Fg standard (Fg Std) on the left of the gel with the α-, β-, and γ-chain labeled on the left. A Fn standard (Fn Std), consisting of the β-chain and γ-γ dimer is on the right side of the gel. Time points from 4 to 20 min are labeled above the gel. Clot time (arrow, CT) is seen at 4 ± 0.2 min. Because of variability in sample manipulation, comparisons are best made between the amount of γ-γ monomer versus γ-γ dimer to β-chain content at each time point. At the 4-min time point γ-γ monomer is equal to γ-γ dimer in the clot. By 5 min the γ-γ monomer is totally converted to γ-γ dimer. Previous studies have inferred that γ-γ dimer formation occurs only after Fn II formation, following the release of FPB (51). However, FPB release is just starting to occur at the time that γ-γ dimer formation is complete in this study. Therefore, it appears that the formation of cross-links is contemporaneous with clotting and requires only FPA release and initial protofibril overlapping. FPB release does not appear to be a prerequisite for γ-γ dimer formation.

The β-chain FPB content was also evaluated in these clots using an α-FPB monoclonal antibody (supplied by B. Kudryk) (Fig. 8B). The immunoblot is lined up directly below the SDS-PAGE of the cross-linking. Fg and Fn standard are seen to the left and right of the immunoblot, respectively followed by 4–20-kDa bands (Fig. 8). The immunoblot inset shows time points from 1 to 5.7 min, the A-chain of FXIII and its activated form are seen on the right of the time points. From the 4-min time point γ-γ monomer and γ-γ dimer are present. By 5 min γ-γ monomer is no longer present. A Western blot of the quenched time point clots (4–20 min) are shown in part A with monoclonal α-FPB. The β-chain of the Fg standard is shown to the left of the time points, and the β-chain of the Fn standard is shown to the right. FPB can be seen to be attached to the β-chain.

**Summary of Fg Cleavage, FP Formation, and Cross-linking**—The results from a whole blood experiment of Fg depletion, FP formation, and γ-γ cross-linking (Figs. 4, 6, and 8) are correlated in Fig. 9. Clot time (arrow, CT) is seen at 4 ± 0.2 min. FPA formation is represented as FnI (A), FPB formation (combined FPB and des-Arg FPB) as Fn II (B), and des-Arg FPB (C) are plotted as fibrinopeptides (μM) versus time (min). Fg (●) levels in the soluble phase and γ-γ dimer (○) formation in the insoluble phase are plotted as a relative fraction versus time (min) on the secondary y axis. It is clear to see that at clot time, Fn I has already formed (≈14 μM/liter) and is cross-linked (≈90%). This correlates with the Fg levels that have been depleted from solution (≈60%) and is present in the clot. At clot time, Fn II formation occurs after Fg is (≈95%) in the clot and to a lesser extent then Fn I. This is followed by a second process that degrades FPB to des-Arg FPB.

**DISCUSSION**

This study represents an account of Fg/Fn processing during the biologically relevant TF induced clotting of non-anticoagulated, warm, whole blood. Previous studies which have been conducted to study Fg/Fn processing have either utilized purified Fg with/without cells (i.e. human umbilical vein endothelial cells or platelets) (2, 15, 26, 52, 53) or plasma or blood with chelators (i.e. EDTA, sodium citrate) present (30, 33, 35, 37, 38). Studies of blood processing in this manner provides useful insights but leaves open the question of what is actually occurring in native blood. Chelators influence cellular metabolism and numerous plasma protein functions ranging from vitamin K-dependent zymogen and FXIII activation to the cross-linking of Fg/Fn. Additional processes may also come into play with native blood that may not be observed in purified systems. Until recently, studying the biochemistry of the TF induced coagulation of non-anticoagulated whole blood had not been

![Fig. 7. Comparison of FPA release with FXIII activation.](image)

![Fig. 8. Analysis of whole blood clots.](image)
initial clot that is a composite of Fn I and Fg with γ-γ cross-links. At this point significant FPB has not been released from the formed clot. Thrombin continues to remove the remaining FPA s and some of the FPBs to produce the final clot, which is composed of Fn I, Fn II with quantitative cross-linked γ-chains. A carboxypeptidase-B-like enzyme specifically degrades FPB to des-Arg FPB.

Some of the results seen in this study of whole blood Fn formation are predicted from what has previously been reported in less complicated systems. FPA release occurs first followed by release of FPB (Figs. 4 and 5B), as has been shown to occur in numerous studies (1–4, 55). Mechanisms proposed to explain the more rapid cleavage at Aα-16 include kinetic models in which $k_{B-16} \gg k_{B-14}$ (27, 56). In this theory, thrombin is equivalently accessible to both Aα- and Bβ-chains and FPA is released faster because the thrombin cleavage rate constant is greater. A contrasting sequential model hypothesizes that FPA release must occur first in order to expose the Bβ peptide site to thrombin (57). Regardless of the model used, released FPA precedes FPB. In purified Fg and thrombin reaction systems, quantitative release of FPA and FPB occur. However, in the whole blood system most Bβ remains intact. Only about ~30% of FPB is released over a 20-min time interval. The unexpected early termination of FPB release is interesting since FPB release is terminated when thrombin levels are high (~3 μmol/liter) (25). Thrombin, which is also being consumed by AT-III, is still being produced in massive quantities. Therefore, it is difficult to explain why the release of FPB suddenly stops. The termination of Bβ-chain cleavage and resulting non-quantitative cleavage is also curious since it has been thought that this cleavage is necessary for normal protofibril and fiber assembly. With FPB release preferentially affecting lateral aggregation (58).

In whole blood FXIII activation is detected prior to clot formation and coincident with the interval when the lowest levels of thrombin are detected. FXIII activation correlates with FPA release (Fig. 7) making transeptamine activity available to cross-link the overlapped fibrils as soon as they are formed. The cross-linking of Fn by FXIII has been thought to be an important step which occurs subsequent to FPB and lateral aggregation, in reinforcing the structure of a thrombus (26). In contrast γ-γ cross-linking is nearly complete before FPB is released from the clot (Fig. 9) and is not a subsequent step in forming the thrombus. The presence of clot cross-links prior to the release of FPB suggests that lateral aggregation upon release of FPB is not required for transglutaminase activity to begin. The cross-linking of the clots prior to the release of FPB might conceivably suggest the creation of an environment which traps FPB in the formed Fn II polymers. This possibility is ruled out by immunoblotting which detects most FPB still attached as the Bβ-chain in the clots even at the 20-min time point (Fig. 5B). Therefore, the exposed binding site of FPB (G-H-R) suggested to allow for lateral association (59) may occur only to a small degree. Clot organization in terms of FPA (Fn I) and FPB (Fn II) have been studied previously (15). Fibrin deposition has been directly correlated to FPA not FPB release at the onset of gelation (57). These results are comparable to the whole blood studies seen here, where Fn I formation (FPA release) appears to be crucial to Fn deposition versus Fn II (FPB release).

FPB cleaved from Fn I in the clot is cleaved by a carboxypeptidase-B-like enzyme that produces des-Arg FPB (Figs. 3 and 4). Des-Arg FPB has been previously observed but was thought to arise from the action of an irrelevant carboxypeptidase contaminant of assay samples (31, 32). Des-Arg FPB has also been identified in plasma (60). One study analyzed conversion of
the B

contributes to this process by its ability to activate FPA (13). Thrombin simultaneously acts upon Fg formation of the initial soluble Fn is seen approximately the same rate. The subsequent

domains from another Fg molecule. FXIII sites in the E domain with adjacent D complementary overlap of the exposed released from the molecules allowing for the agent responsible for the formation of des-Arg FPB is TAFI. The main difference between FPA and FPB is that the majority of FPA is removed from the clot, followed by a slow degradation process. Whereas, FPB is released in small amounts and is degraded immediately.

This study leads to an insight into what is seen in whole blood when initiated with a small amount of TF. The techniques developed here are sufficiently robust for quantitation of FPs and soluble and insoluble Fn products in media as complicated as blood. From these results and using these types of analyses we are able to compare results that are seen in the synthetic/reconstituted models developed in our laboratory (66, 67). These studies are conducted in the hopes to eventually explaining blood coagulation using non-invasive techniques in normal and diseased states.

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FPB to des-Arg FPB in anticoagulated blood by adding carboxypeptidase-B and measuring the levels produced (38). The natural enzyme that causes this cleavage in our study has not been identified, apart from its inhibition by EACA (Fig. 5).

Several carboxypeptidases with similar cleavage specificity to carboxypeptidase-B are capable of removing the carboxyl-terminal arginine. Carboxypeptidase-N (47) has been proposed to serve as a regulator for several blood peptides including peptides (FDP-6A and -6D) released from Fg/Fn in the initial stage of plasmin degradation (61) and EACA has been shown to be an inhibitor of carboxypeptidase-N (62). The TAFI or procarboxypeptidase-U is activated by thrombin-thrombomodulin and is thought to contribute to clot stability by interference in the plasmin dissolution of a clot by removing terminal lysines required for plasmin binding. It has also been identified as being cross-linked to fibrin during the latter part of the coagulation cascade (63). Previously published results show that EACA is a competitive inhibitor of TAFI (64) and an inhibitor of fibrinolysis (65).

In our studies with purified Fg, thrombin, thrombomodulin, TAFI provides an activity with the appropriate specifications. From our whole blood studies with EACA addition (Fig. 5) and our in vitro studies with TAFI acting on Fg, and purified FPA and FPB, it is tempting to suggest that the agent responsible for the formation of des-Arg FPB is TAFI. The pattern of selective cleavage of FPB was observed in Fg experiments, although TAFI was also capable of cleaving isolated FPA. However, preliminary experiments using monoclonal α-TAFI (at 1.5 and 3 μmol/liter final), which inhibits the formation of activated TAFI (41), was unable to prevent the cleavage of FPB. Potato carboxypeptidase inhibitor (1 μmol/liter) which has been shown to inhibit TAFI (64) was also unable to inhibit the cleavage of FPB. Overall, the significance of the cleavage specificity toward FPB and the identification of the carboxypeptidase-B-like enzyme can be a link between the balance of forming a thrombus and fibrinolysis and remains to be identified.

Levels of FPA in whole blood/serum are decreased over time. The final levels of FPA detected at 20 min are ~30% less then those seen at 4.5 min. This observation was also seen in a whole blood study (25) in which FPA was measured by immunoassay. Together these observations suggest that there are also FPA degradation processes occurring. FPA also contains a carboxyl-terminal arginine that could be susceptible to cleavage by a carboxypeptidase-B-like enzyme. The main difference between FPA and FPB is that the majority of FPA is removed from the clot, followed by a slow degradation process. Whereas, FPB is released in small amounts and is degraded immediately.

FIG. 10. Summary of whole blood clotting. At the onset of clot formation, thrombin simultaneously acts upon Fg (D-E-D) and FXIII. FPA (~30–40%) is released from the molecules allowing for complementary overlap of the exposed sites in the E domain with adjacent D domains from another Fg molecule. FXIII is being activated (XIIIa) at approximately the same rate. The subsequent formation of the initial soluble Fp is seen to be cross-linked (D-D). Thrombin continues to activate XIII and cleave the remaining FPA molecules, yielding an initial clot that is a composite of Fg, Fn, and γ-γ-cross-links, with FPB still attached to the B-chain. The initial clot is continuously acted upon by thrombin to release the remaining FPAs and some of the FPBs to yield a final clot. The released FPB is selectively acted upon by a carboxypeptidase-B like enzyme cleaving the carboxyl-terminal arginine to produce des-Arg FPB. Together these results suggest that Fn I, Fn II, and cross-linking are not seen as separate processes but appear to occur simultaneously.
