Generation of the Combined Prothrombin Activation Peptide (F1·2) during the Clotting of Blood and Plasma

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ABSTRACT We have investigated the pathway of prothrombin activation in blood and plasma. By means of a rapid purification procedure involving chromatography on DEAE-cellulose and hydroxyapatite, we demonstrated that the major prothrombin fragment in serum is that representing the amino-terminal half of prothrombin (i.e. F1·2). The F1·2 isolated was characterized by its size, amino acid and antigenic compositions, amino-terminal residue, and the peptides (designated F1 and F2, respectively) it yielded upon hydrolysis by thrombin. Measurements by the isotope dilution technique showed that F1·2 could account for the fate of at least 90% of the prothrombin originally present in plasma. By contrast, the serum concentration of the fragment representing the amino-terminal third of prothrombin (viz. F1) was <10% that of F1·2. These results demonstrated that the major route of prothrombin conversion in blood or plasma involves the removal of the combined activation fragment (F1·2) as a single peptide.

INTRODUCTION

Four different pathways have been proposed for the conversion of prothrombin to thrombin (Fig. 1); the ultimate products of each appear to be identical. The first to be proposed (Fig. 1, pathway 1) consisted of the rapid, and apparently sequential, loss of the peptides[1] F1 and F2 from the amino-terminus of prothrombin, followed by the scission of the remaining protein (viz., prethrombin 2) between the A and B chains so as to yield the disulfide-bridged thrombin molecule (1, 2). More careful studies indicated that the initial event is the removal, by Factor Xa, of the activation fragment F1·2 (which consists of the F1 and F2 moieties linked as a single peptide) in one step (3–5). The prethrombin 2 formed in this step is then cleaved by Factor Xa to produce thrombin, which in turn catalyzes the cleavage of F1·2 into F1 and F2 (Fig. 1, pathway 2).

Treatment of human (6) or bovine (7, 8) prothrombin with the procoagulant from the venom of the saw scaled viper *Echis carinatus* was shown to yield the amino-terminal peptide F1, and a two-chain, disulfide-bridged molecule with the molecular weight of prethrombin 1. The latter is composed of the B chain bridged to a peptide consisting of the F2 and A moieties. The activation scheme proposed on the basis of these results is summarized as pathway 3 (Fig. 1).

Recent reports contain evidence that cleavage of prothrombin between the A and B moieties is the first step in activation, either by Factor Xa (9) or by *E. carinatus* procoagulant (10, 11). According to this scheme (Fig. 1, pathway 4), the initial product, which is composed of a long peptide (consisting of the F1, F2, and A moieties) disulfide-bridged to the B chain, has proteolytic activity and therefore catalyzes the removal of F1. The two chain product of this reaction also has proteolytic activity, as does thrombin itself, hence F2 can be cleaved off by any of these enzymes to yield thrombin as a final product. However, neither Esmon et al. (5), who studied activation with Factor Xa, nor Franza et al. (6), who used the venom procoagu-

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[1] F1 is the peptide that arises from the amino-terminal region of prothrombin; F2 is the penultimate peptide; prethrombin 1 is prothrombin from which F1 has been removed; prethrombin 2 is prothrombin minus the F1 and F2 regions. When F1 and F2 remain covalently linked as a single peptide, they are referred to as F1·2 (see Fig. 1).

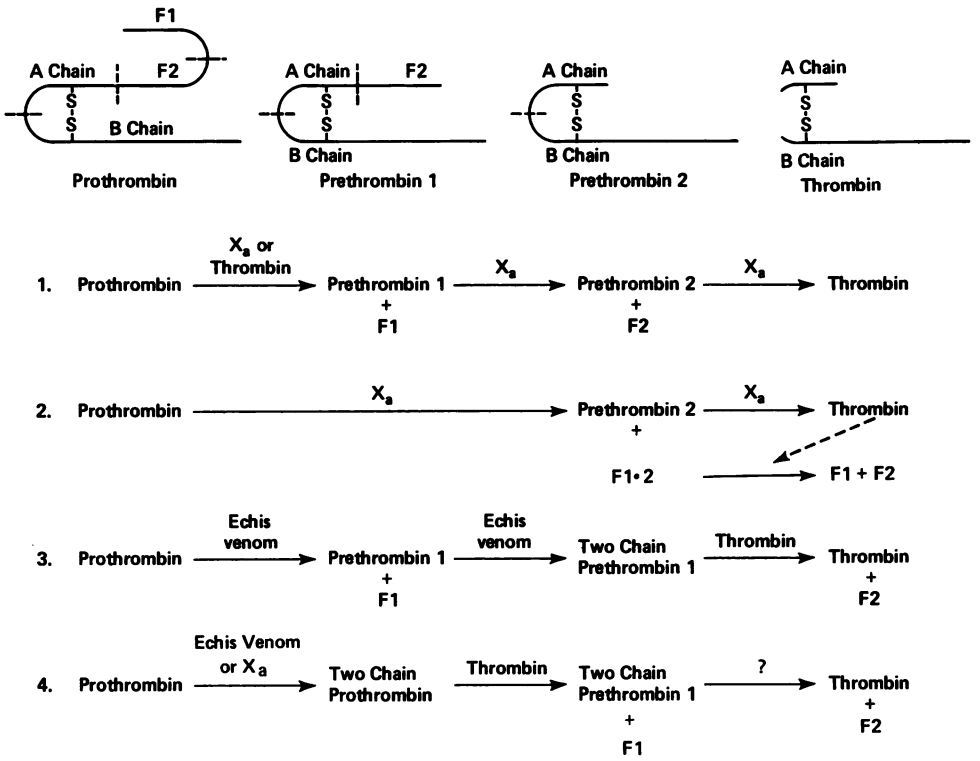

FIGURE 1 Diagrammatic representation (not to scale) of the structures of prothrombin and prethrombins, showing the chains and fragments derived therefrom (above). Proposed pathways of prothrombin conversion to thrombin (below). Species not illustrated include two-chain prethrombin 1, and two-chain prothrombin, which would be produced by cleaving the bond between the A and B moieties in prethrombin 1 and prothrombin, respectively, and peptide F1·2, which would be released by cleaving the bond between the F2 and A moieties of prothrombin.

lant, could find consistent evidence for this mechanism.[2]

The studies leading to the proposal of all the pathways shown in Fig. 1 were carried out in purified systems; few studies of the prothrombin conversion sequence have been done in plasma. The system used by Shapiro and Cooper (12), which consisted of labeled prothrombin added to whole plasma, gave findings consistent with the removal of F1·2 as a single entity, i.e., pathway 2. Immunologic studies also indicated the possibility of a single activation fragment during prothrombin conversion in plasma (13, 14). On the other hand, Fass and Mann (15), who employed a medium containing dilute serum and additional phospholipid to activate fluorescein-labeled prothrombin, concluded that there was sequential loss of F1 and F2, i.e. pathway 1. The present investigation was carried out to determine which of these pathways, if any take(s) place when the prothrombin in human blood or plasma is activated by Factor Xa.

METHODS

Human plasma, anticoagulated with either citrate phosphate dextrose or acid citrate-dextrose, was stored at 4°C until used. Its prothrombin level was about 75% that in fresh plasma.

Russell's Viper venom was obtained from the Miami Serpentarium (Miami, Fla.); DEAE-cellulose (DE 1 Floc and DE 23), from Whatman, Inc. (Clifton, N. J.); hydroxyapatite (Hypatite C), from Clarkson Chemical Co. (Williamsport, Pa.); and Sephadex G-200, from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.). Constant boiling HCl and sodium heparin were obtained from Fisher Chemical Co. (Silver Spring, Md.); soybean trypsin inhibitor, from Worthington Biochemical Corp. (Freehold, N. J.); and tritiated borohydride, from New England Nuclear (Boston, Mass.). The human thrombin (Lot H-1) used for digestion experiments was that employed previously (6).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described by Weber and Osborn (16). An acrylamide concentration of 10% was used through-

[2] Morita et al. reported (11) detecting the two chain intermediate with the molecular weight of prothrombin when *E. carinatus* procoagulant was used in the presence of hirudin, antithrombin III, or *p*-nitrophenyl-*p'*-guanidinobenzoate, but not when it was used in the presence of benzamidine and diisopropylphosphorofluoridate. The latter inhibitor was employed in the experiments of both Esmon et al. (5) and Franza et al. (6).

out. Gels were stained with Coomassie Brilliant Blue and subsequently scanned with a Photovolt densitometer (Photovolt Corp., New York). For determination of the distribution of radioactivity, the gels were sliced with a piano wire-type cutter into 1.2-mm sections which were then dissolved in 30% H_2O_2 at 50°C. Samples were counted in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, Calif.) after suspension in 10 ml of scintillation fluid (Aquasol, New England Nuclear).

Amino acid analyses were carried out with a Beckman 120 amino acid analyzer. Before analysis, samples were hydrolyzed for 24 h in 6 N HCl; no correction was made for differential amino acid destruction during the hydrolysis. Amino-terminal analyses were performed by the dansyl method of Gray and Hartley (17), as modified by Mosesson et al. (18).

Absorption coefficients were determined by measuring the protein concentrations of dialyzed solutions in a Brice-Phoenix differential refractometer at 546 nm; a specific refractive increment of 0.186 ml/g was assumed. Portions of these solutions were then diluted, and the absorbances of the resulting solutions were measured at 280 nm in a Beckman 25 spectrophotometer.

Tritium labeling was done as described by Van Lenten and Ashwell (19). The tritiated samples were stored at −70°C in acetate-buffered 0.15 M NaCl (pH 5.6) until used.

Thrombin was assayed as previously described (1) and standardized against U. S. Standard Thrombin (lot B-3). Prothrombin was measured in a two-stage test (20) with Difco (Difco Laboratories, Detroit, Mich.) two-stage reagent; 1 U of prothrombin was defined as that amount yielding 1 U of thrombin. Factor X was measured by the method of Denson (21) with bovine Factor X-deficient plasma (Diagnostic Reagents, Ltd. Thames, Oxon, England).

RESULTS

Preparation of F1·2. Pools of human plasma (4 liters each) were allowed to warm to ambient temperature (22°C) overnight. These were converted to serum by the addition of Russell's Viper venom and 1 M $CaCl_2$ (final concentrations 1 mg/liter and 10 mM, respectively). Clotting took place within 2.5 min after the addition of $CaCl_2$. The clot was wound, as it formed, on a polyethylene stirring rod. After 90 min, the residual prothrombin level was found to vary between 0 and 20% of that in the starting material; the residual Factor X level, between 10 and 30%. Heparin was then added (final concentration 7.5 mg/liter), the serum was diluted with 0.5 vol of distilled water, damp DE 1 Floc (50 g/liter) was admixed, and the suspension was stirred for an additional 30 min. The adsorbent was packed into a glass column (8-cm diameter) and washed with a volume of 0.026 M TrisCl-0.20 M NaCl, pH 7.4, equal

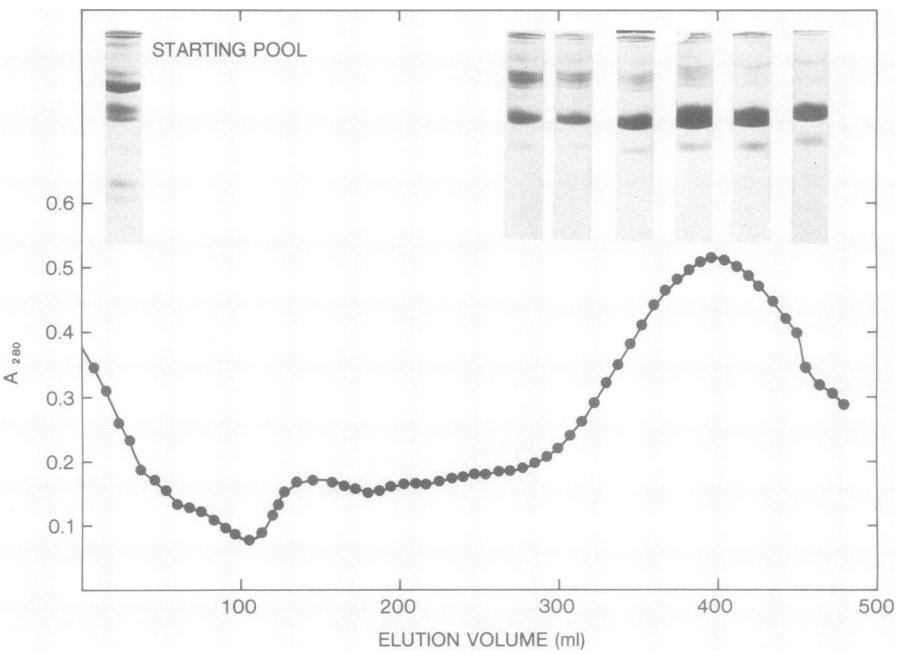

FIGURE 2 Hydroxyapatite chromatography of serum protein eluted from DEAE-cellulose under the conditions described in the text. The hydroxyapatite had been equilibrated with 0.01 M potassium phosphate buffer. After application of the sample, the column was flushed with 250 ml of 0.2 M potassium phosphate buffer, whereupon a linear gradient (total volume 500 ml) from this buffer to 0.7 M potassium phosphate buffer was begun. The pH was 6.8 throughout. Only the elution pattern obtained during application of the gradient is shown. Gels at the top of the figure indicate the electrophoretic patterns of the sample before chromatography and of fractions eluted from the column in the indicated position. When a similar preparation from plasma was chromatographed in this system, the material eluted at the position of the major peak shown here consisted almost entirely of prothrombin.

to that of the starting plasma. The column was subsequently eluted with 1 liter 0.066 M TrisCl-0.50 M NaCl, pH 7.4, and the eluted protein peak was siphoned onto a column (2.2 × 15 cm) of hydroxyapatite equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The column was flushed with 250 ml of 0.2 M potassium phosphate buffer, pH 6.8, and then was eluted with a linear gradient (total volume 500 ml) from this buffer to 0.7 M potassium phosphate buffer, pH 6.8. All chromatography was carried out at 4°C; all pH measurements, at 22°C. The entire procedure required 8 h or less. After chromatography, appropriate fractions were pooled, dialyzed against distilled water, freeze-dried, and stored at −20°C.

Identification of F1·2. When material prepared from serum in this manner was chromatographed, gradient elution yielded a curve with a number of small shoulders and one large peak (Fig. 2). Gel electrophoresis of the fractions in sodium dodecyl sulfate showed the presence of several minor components of high molecular weight and a minor band which migrated at a rate similar to that of F1. Densitometry of the stained gels showed that the major component, which migrated at a rate close to that of thrombin, comprised more than 90% of the sample. Incubation of this component (i.e. fractions from the major chromatographic peak—Fig. 2) with thrombin produced two components which were electrophoretically indistinguishable from F1 and F2, respectively, showing that this component was F1·2 (see below). On the other hand, if plasma rather than serum was fractionated by this procedure, the material eluted from hydroxyapatite at this position, i.e. at an elution volume of ~400 ml, was a virtually homogeneous preparation of prothrombin (≥95% by gel electrophoresis).

Amino-terminal analysis of the F1·2 preparation showed alanine to be the major terminus, though a small amount of threonine was also present. When dansylation of the material was followed by gel electrophoresis, elution of the major band, and subsequent amino-terminal analysis, only dansyl alanine was found. Amino acid analysis was carried out after further purification of F1·2 by exclusion chromatography on Sephadex G-200 (equilibrated and developed with 0.2 M NH_4HCO_3) to remove the high molecular weight contaminants (cf. Fig. 2). There was close agreement between the composition of the isolated material and the sum of the amino acid contents of F1 and F2 derived from human prothrombin[3] (Table I); moreover, the composition was similar to that of bovine F1·2 (22).

Additional proof of the identity of F1·2 was obtained by immunodiffusion of this fragment with rabbit antisera to human prothrombin[4] and F1. Anti-F1 produced a reaction of mutual identity among F1, F1·2, and prothrombin. By contrast, antiserum directed against intact prothrombin gave a precipitin arc which showed spurring across the F1·2 precipitin line, which, in turn, spurred across that of F1 (Fig. 3).

TABLE I

Amino Acid Composition of Human F1·2, Human F1 + F2, and Bovine F1·2

Residue	Human F1·2*	Human F1 + F2‡	Bovine F1·2§
Asp	27.1*	28	32.4
Thr	20.4	19	15.0
Ser	15.3	15	22.8
Glu	40.0	40	37.8
Pro	13.7	16	19.6
Gly	18.4	22	24.1
Ala	21.2	22	20.0
Cys/2	18.4	15	13.3
Val	14.5	16	14.5
Met	1.5	1	0.8
Ile	5.3	5	4.9
Leu	17.5	17	19.2
Tyr	13.1	10	7.3
Phe	7.5	7	7.0
His	5.2	7	2.2
Lys	8.2	7	7.1
Arg	16.2	15	22.5
Trp	ND	7	—
Hexose	ND	6	—
Glucosamine	ND	9	7.5
Sialic acid	ND	5	—
Molecular weight by composition	34,424‖	35,105	

* Values given are moles per mole; those for human F1·2 were calculated on the basis of 40 mol of glutamic acid/mol. ND, not determined.

‡ See text, footnote 3.

§ Reference 22.

‖ Tryptophan, hexose, glucosamine, and sialic acid values from F1 + F2 used to calculate molecular weight of F1·2.

Solubility properties of F1·2. The isolated F1·2 was soluble in distilled water in the pH range of 4.0–8.0 and in 6% perchloric acid. It was not precipitated by 15 min in a boiling water bath, was adsorbed by $BaSO_4$, and was precipitated by 7% TCA.

Sensitivity of F1·2 to thrombin. The rate of digestion of the isolated F1·2 by thrombin was determined by gel electrophoresis (Fig. 4). The half-time for the reaction at a thrombin concentration of 0.5 U/ml was approximately 35 min.

Quantitation of F1·2 in serum. The overall re-

[3] Aronson, D. L., A. P. Ball, A. M. Young, and J. W. Fenton, II. Manuscript in preparation.

[4] Kindly supplied by Dr. S. S. Shapiro, Thomas Jefferson University, Philadelphia, Pa.

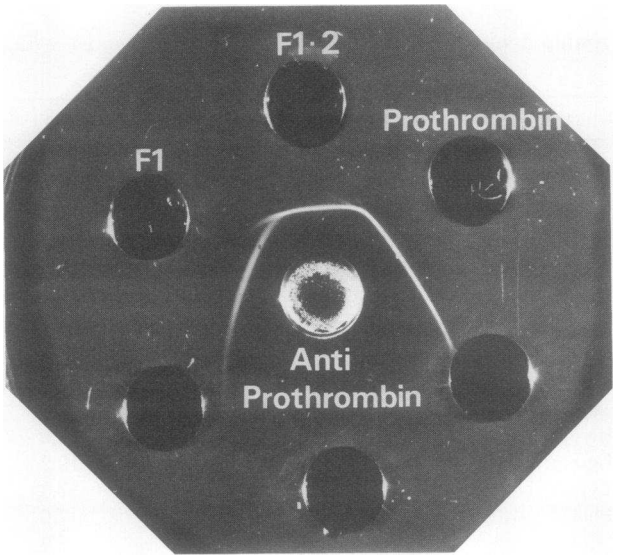

FIGURE 3 Ouchterlony immunodiffusion of rabbit anti-human prothrombin against prothrombin, F1·2, and F1.

covery of F1·2 from the purification procedure described above was 5–10 mg/liter serum. This amounted to ~15% of the value expected on the assumption that (*a*) all prothrombin was converted via the F1·2 pathway and (*b*) there was no degradation of F1·2 during the clotting or the isolation. This assumption was tested by determining the quantity of F1·2 present in serum, which amounted to an assessment of the quantitative importance of the F1·2 pathway in whole plasma. Two different methods of analysis were used; both employed F1·2 labeled with tritium. It was initially shown that when an F1·2 preparation, labeled as indicated in Methods, was subjected to gel electrophoresis in sodium dodecyl sulfate, 90% of the label migrated in the F1·2 band; 10% of the activity migrated as F1 (Fig. 5). After digestion with thrombin, all radioactivity appeared in the F1 region; none was detectable in the region of F2. Recovery of F1·2 in the isolation procedure was then estimated directly in the following manner. Tritiated F1·2 (0.38 mg) was added to 150 ml of serum produced by treating plasma with Russell's Viper venom. Damp DEAE-cellulose (5 g) was then admixed; the label was quantitatively adsorbed, and none was eluted by washing the adsorbent with 0.026 M TrisCl-0.20 M NaCl. Subsequent elution with 0.066 M TrisCl-0.50 M NaCl removed 30% of the label from the adsorbent. Recovery in the hydroxyapatite portion of the procedure was assessed by adding 1.14 mg of tritiated F1·2 to the eluate (from DEAE-cellulose) obtained during the processing of 4 liters of serum. The latter quantity was used in order to maintain the same geometry and protein:hydroxyapatite ratio as those used during the standard isolation. After gradient elution chromatography, the fractions were pooled in the usual manner and radioactivity was measured. The pooled F1·2 contained 50% of the added label. Thus the estimated overall recovery for the purification procedure was found to be 15% (i.e. 50 of 30%), in good agreement with that computed on the basis of the assumption stated above.

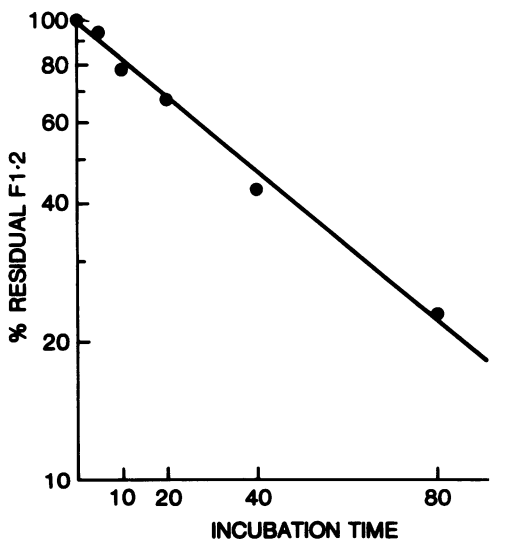

FIGURE 4 Digestion of F1·2 to F1 and F2 by thrombin. The F1·2 (1.6 mg/ml) was dissolved in 0.02 M TrisCl-0.15 M NaCl, pH 7.4, and incubated at 22°C with thrombin (0.5 U/ml). At intervals, samples were removed and added to equal volumes of 1% sodium dodecyl sulfate-0.01 M sodium phosphate. The relative amounts of F1·2 were determined by densitometry of gels stained with Coomassie Brilliant Blue. The abscissa shows the incubation time in minutes.

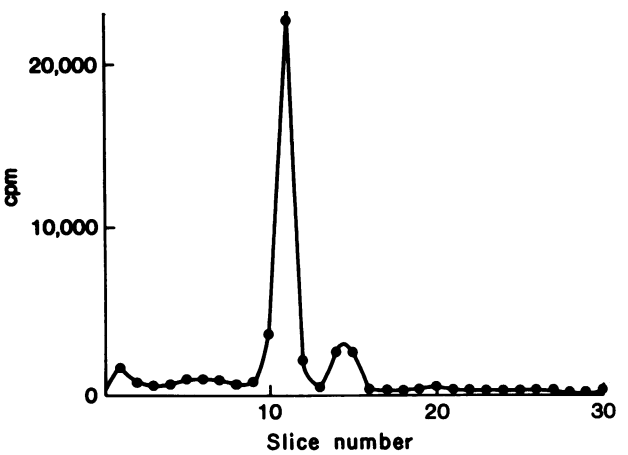

FIGURE 5 Gel electrophoresis of a tritium-labeled F1·2 preparation. After electrophoresis, the gels were sliced into 1.2-mm segments. These were dissolved in H_2O_2 and the radioactivity was measured in a scintillation counter. As depicted, the anode is at the right; slice 1 thus corresponds to material located between the origin and a plane 1.2 mm from the origin.

A more precise estimate of the concentration of F1·2 in serum was obtained by the isotope dilution method. Tritium-labeled F1·2 (2.38 mg; specific activity 3.64 × 10^6 cpm/mg) was added to 3.75 liters of serum prepared as described above. F1·2 was then isolated by the usual procedure; the preparation comprised 90% F1·2 as measured by densitometry after gel electrophoresis. The concentration of the purified material was determined spectrophotometrically($A_{1\,cm}^{1\%}$ at 280 nm = 12.9), and its specific activity was computed. The latter was found to be 5.17 × 10^4 cpm/mg. Thus, if the tritium-labeled F1·2 behaved in the same manner as serum F1·2 during fractionation, the serum contained 70 times the added amount of F1·2, i.e., 165 mg/3.75 liters or ~50 mg/liter (Table II, part C). This is the same concentration as that predicted from the level of prothrombin in plasma (Table II, part A) and from mass recovery after correcting for losses during the purification procedure (Table II, part B).

F1·2 from fresh blood. The purification procedure used in the foregoing experiments was carried out on large volumes of blood bank plasma in order to obtain sufficient material for characterization. However, it could be argued that the age, anticoagulant content, and perturbation of the plasma with Russell's Viper venom resulted in an aberrant pathway of prothrombin activation. Accordingly, an isolation was done from serum derived from fresh whole blood.

TABLE II

Estimates of F1·2 Concentration in Serum

	Fresh plasma	Blood bank plasma
	mg/liter	*mg/liter*
A. From prothrombin level		
Prothrombin concentration	100	75
Theoretical F1·2 concentration*	50	37.5
B. From recovery data		
Mass recovered ~ 7.5 mg/liter		
Recovery of tritiated F1·2 ~ 15%		
Computed F1·2 concentration = 7.5/0.15 ~ 50 mg/liter		
C. By isotope dilution		
Tritiated F1·2 added = 0.635 mg/liter		
Activity of F1·2 added = 3.64 × 10^6 cpm/mg		
Activity of F1·2 isolated = 5.17 × 10^4 cpm/mg		
Purity of F1·2 isolated = 90%		
Computed F1·2 concentration		
$= \frac{3.64 \times 10^6}{5.17 \times 10^4} \times 0.635 - 0.635 = 44.1$ mg/liter		
Corrected‡ F1·2 concentration = 44.1 × 0.9 = 39.7 mg/liter		

* Computed on the assumptions that all prothrombin is activated via the F1·2 pathway and that F1·2 represents half of the prothrombin molecule.

‡ Corrected for the 10% impurity.

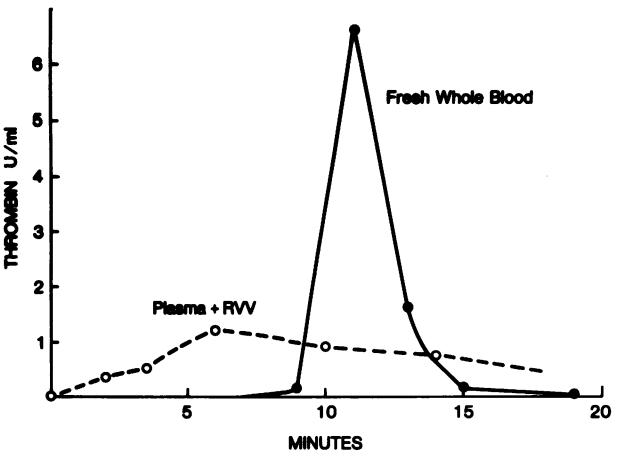

FIGURE 6 Comparison of thrombin generation in fresh whole blood allowed to clot spontaneously and in plasma clotted with Russell's Viper venom (RVV) and $CaCl_2$. The blood was drawn and immediately placed in a glass flask at 35°C. The plasma was incubated at 22°C with Russell's Viper venom and $CaCl_2$ (final concentrations 1 mg/liter and 10 mM, respectively). Thrombin concentration (ordinate) is expressed as Units per milliliter of blood or plasma, respectively.

The steps in the procedure were based on those used for large-scale purification. A 20-ml sample of blood was drawn and immediately placed in a 50-ml glass Erlenmeyer flask prewarmed to 35°C. Visible clot formation took place after 8 min. Measurements made at frequent intervals showed that the thrombin level became detectable at just this point and subsequently rose sharply to a maximum of 6–7 U/ml blood after 11 min (Fig. 6). By contrast, thrombin generation in plasma treated with Russell's Viper venom and $CaCl_2$ at the concentrations used in the present experiments began within 2 min but exhibited only a gradual increase. After 11 min, the level of thrombin activity in the blood system fell precipitously; by 15 min it had nearly returned to the base line (Fig. 6). Similarly, after 15 min this system contained no measurable prothrombin. At this time 150 μg of heparin was added, the cells and clot were removed by centrifugation, and 10 ml of the serum was diluted with 5 ml of water. This solution was immediately applied to a column of DE 23 (2.2 × 7 cm) equilibrated with water. After flushing with 0.026 M TrisCl-0.20 M NaCl, pH 7.4, the column was washed with 0.066 M TrisCl-0.50 M NaCl, pH 7.4, and the protein eluted by the latter was placed on a column of hydroxyapatite (2.2 × 4.5 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. After removal of contaminating proteins with 0.2 M potassium phosphate buffer, pH 6.8, the column was washed with 0.7 M potassium phosphate buffer, also pH 6.8, and the fractions obtained during this final purification step were pooled (cf. Fig. 2). (These procedures were completed within

2 h of drawing the blood.) Two 5-ml portions of this pool were treated with diisopropylphosphorofluoridate (final concentration 1 mM) and concentrated 10-fold with a Minicon B15 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). One of these was then incubated with thrombin (final concentration 1.0 U/ml) for 2 h at 22°C. Gel electrophoresis of the material isolated from fresh serum revealed a major band migrating as F1·2 but no appreciable F1 or F2 (Fig. 7, gel A). Upon treatment with thrombin this band almost completely disappeared, and a new band migrating as F1 was seen (Fig. 7, gel B). When the protein isolated from serum (i.e. not treated with thrombin) was tested by immunodiffusion against rabbit antihuman F1, it gave a reaction of identity with F1. However, when it was allowed to diffuse against rabbit antihuman prothrombin, it exhibited only partial identity with prothrombin (cf. Fig. 3).

DISCUSSION

The objective of the present study was to determine the pathway of prothrombin activation in whole blood and plasma. The approach used, i.e. isolation and quantitation of the fragments generated during clotting, was based on the following assumptions. If the conversion of plasma prothrombin to thrombin proceeds by the sequential removal of F1 and F2 (Fig. 1, pathway 1 or 4), serum should contain substantial amounts of F1 and little F1·2. Moreover, in this case the removal of F1 could represent a controlling step in coagulation, depending upon the relative rates of (*a*) removal of F1, (*b*) conversion of prethrombin 1 to F2 and prethrombin 2 (Fig. 1, pathway 1), and (*c*) cleavage of prethrombin 1 to two-chain prethrombin 1 (Fig. 1, pathway 3). If, on the other hand, the major prothrombin fragment in serum were F1·2, it would indicate that the first step in prothrombin activation is the removal of this fragment as a single peptide (Fig. 1, pathway 2). In this case, the relative importance of prothrombin cleavage to F1 plus prethrombin 1 could be assessed by comparing the amounts of F1 and F1·2 present in serum.[5] A low ratio of F1 to F1·2 concentration would indicate that the F1 removal step has little quantitative significance, and hence that it cannot seriously be considered as a control mechanism in clotting.

Russell's Viper venom was chosen to induce the clotting of blood bank plasma so as to initiate activation at a late stage in the coagulation scheme (i.e. a Factor Xa-dependent step—see Fig. 1) and to ensure adequate prothrombin consumption. Under the conditions chosen, the majority of prothrombin and Factor X

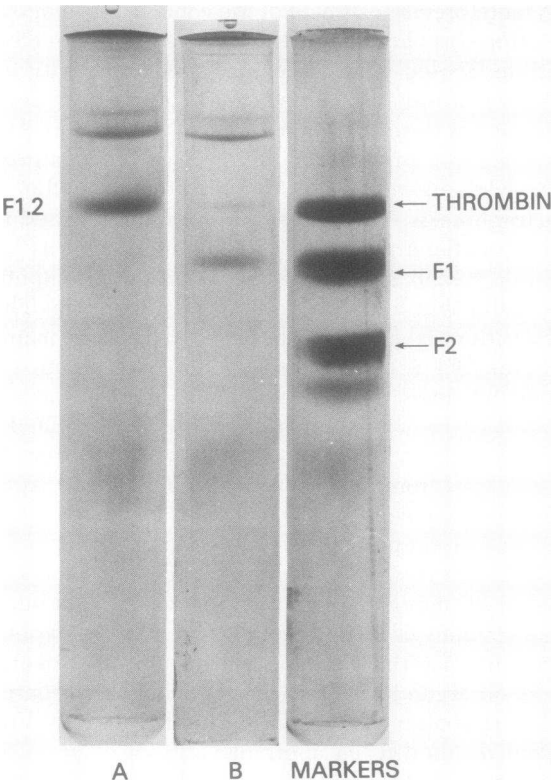

FIGURE 7 Gel electrophoresis of F1·2 isolated after the clotting of whole blood. As depicted, the anode is at the bottom. Gel A is the protein isolated from serum after clotting (see text) and gel B is the preparation shown in gel A after treatment with thrombin (1 U/ml for 2 h). The F2 band is not visible in the photograph of gel B, but a faint band migrating as F2 could be seen on the original gel. The small amount of F1·2 available from whole blood precluded the use of a higher load.

was consumed within 15 min after the addition of Ca^{2+}. Residual prothrombin activity after 90 min was 0–20% of that in the starting plasma. (The blood bank plasma used contained 120 U of prothrombin/ml as opposed to 160 U/ml fresh plasma.)

The preparations obtained by the method described above routinely contained more than 90% F1·2. The major contaminants were F1 and several high molecular weight components. It was impossible to ascertain whether the F1 was formed in the serum or during fractionation. However, if heparin was not added to the serum before fractionation, the material eluted from hydroxyapatite rapidly degraded to F1 and F2.

Identification of the isolated substance as the combined activation peptide, F1·2, was based on its molecular size (Fig. 7), amino acid (Table I) and antigenic (Fig. 3) compositions, amino-terminal residue, and ability to yield F1 and F2 upon hydrolysis by thrombin. The mol wt of human F1·2 calculated from composition data was ~34,500 (Table I). It is there-

[5] Because thrombin, once formed, can catalyze the cleavage of F1·2 to F1 plus F2, the level of F1 in serum would indicate the maximum possible importance of F1 removal.

fore reasonable that this fragment migrated at approximately the same rate as human thrombin (36,500 mol wt) in the electrophoretic system (Fig. 7), as is the assumption that it represented half of the prothrombin molecule (Table II). The amino-terminus of F1·2 was found to be alanine, which is the amino-terminus of prothrombin. Furthermore, the amino acid composition was shown to approximate the sum of the amino acid contents of F1 and F2 isolated after the activation of purified prothrombin (Table I). Although the amino acid composition is similar to that reported for bovine F1·2 (22), there are differences in the serine, threonine, arginine, histidine, proline, and tyrosine contents. However, the sum of the hydroxyamino acids and that of the basic amino acids is similar for bovine and human F1·2. The observation that antiserum to F1 indicated antigenic identity of F1, F1·2, and prothrombin, whereas these antigens exhibited spurring when reacted with anti-human prothrombin (Fig. 3), is consistent with the fact that all three contain the F1 determinants, but F1·2 lacks the prethrombin 2 moiety and F1 lacks the F2 region as well (Fig. 1). Finally, a salient feature of the isolated F1·2 was its rapid hydrolysis by thrombin (Fig. 4 and [4]) to fragments which were electrophoretically indistinguishable from F1 and F2.

The methods for quantitating F1·2 in serum were both based on the assumption that this fragment, when labeled with tritium, would behave identically to the native peptide during fractionation. Although the yield from DEAE-cellulose (30%) was low, it was consistent with that of prothrombin treated in the same manner (23–37%). Likewise, the recovery from hydroxyapatite (50%) was similar to that for human prothrombin. Both the mass recovery and the isotope dilution experiments indicated that the F1·2 pathway must be the major route followed during the activation of prothrombin in whole blood or plasma and, in fact, that it can account for the fate of at least 90% of the prothrombin originally present (Table II).

Even though the isolation from serum obtained by the spontaneous clotting of whole blood showed that the F1·2 pathway is followed in the latter medium, the pattern of thrombin generation observed was quantitatively different from that in plasma clotted with Russell's Viper venom and $CaCl_2$ (Fig. 6). However, despite this difference in the generation profiles, there should have been sufficient thrombin generated in either system to catalyze the cleavage of a substantial portion of the F1·2 (cf. Fig. 4). Several factors may contribute to the stability of this peptide in blood or plasma. First, the fact that the cleavage of F1·2 in the purified system (Fig. 4) was first order at a substrate concentration of ~50 μM suggests that the K_m is at least 0.5 mM. The maximum concentration of F1·2 in serum, ~2 μM is far below this and would permit only a low rate of hydrolysis. Second, the availability of competing substrates may have diminished thrombin activity toward F1·2 still further. Finally, the susceptibility of F1·2 to hydrolysis could be different in the purified and the natural milieu.

The removal of the amino-terminal fragment, F1, from prothrombin has been proposed as a control mechanism in coagulation. Though Stenn and Blout (3) have found evidence that this may be a minor pathway catalyzed by Factor Xa, most data obtained by studying purified systems indicate that the cleavage of F1 from prothrombin is predominantly due to thrombin (3, 4, 22, 23). However, removal of F1 from prothrombin by thrombin is slow compared with the hydrolysis of F1·2 by this enzyme in purified systems (cf. [6] and Fig. 4). Thus the presence of F1·2 in serum is, per se, a strong argument against the F1 pathway having a significant role in the control of coagulation in neat plasma. This conclusion was confirmed by the demonstration (Table II) that the F1·2 pathway is the major route followed during the clotting of plasma. The presence of F1·2 in serum derived from fresh whole blood suggests that it is true for all circumstances in which thrombin is rapidly inactivated. Only in systems lacking sufficient thrombin inhibition, in particular in concentrated solutions of purified clotting factors (22, 24), can the removal of F1 from prothrombin by thrombin become significant.

The foregoing results support the kinetic analysis of Esmon et al. (5), even in whole blood. The noncovalent linkage of the F1·2 and prethrombin 2 moieties of prothrombin (cf. Fig. 1) is necessary for rapid conversion to thrombin. Inasmuch as prethrombin 2 is measured as prothrombin by the two-stage assay, the inability of this procedure to detect prothrombin in serum implies that prothrombin is totally converted to thrombin and does not remain as prethrombin 2. This is consistent with the finding that prethrombin 2, which was activated slowly by Taipan snake venom in the presence of Ca^{2+} and phospholipid, activated very rapidly in the presence of F1·2 or serum (25). In light of these results, any modulation of prothrombin activation in the presence of adequate levels of Factor Xa would seem to occur through (*a*) inhibition of Factor Xa and (*b*) activation and inactivation of Factor V, rather than by removal of the F1 fragment.

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