Promotion of Thrombin-Catalyzed Activation of Factor XIII by Fibrinogen†

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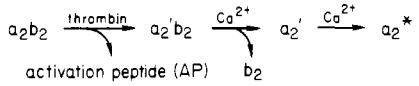
ABSTRACT: High-performance liquid chromatography was used to analyze the kinetics of the thrombin-catalyzed release of the activation peptide from the factor XIII zymogen (fibrin-stabilizing factor). The specificity constant (k_{cat}/K_m) for this reaction, measured at factor XIII concentrations much below K_m, was (0.13–0.16) × 10^6 M^{-1} s^{-1} at pH 7.4, μ = 0.15, and 37 °C. Separate estimates, obtained from the dependence of the initial rates of release of the activation peptide on the concentration of factor XIII, gave values of 10 (±3) s^{-1} for k_{cat} and 84 (±30) μM for K_m, in terms of ab protomers of the zymogen. The thrombin-mediated release of the activation peptide was dramatically enhanced in the presence of fibrinogen. Furthermore, the time course of release, in relation to that of fibrinopeptide A, suggested that some des-A-fibrinogen species (e.g., $\alpha_2B\beta_2\gamma_2$) may be the true activator for promoting the cleavage of the Arg-36 peptide bonds in the a subunits of factor XIII. This observation suggests that generation of factor $XIII_a$ and its substrate (fibrin) is coordinated so that thrombin-mediated zymogen activation proceeds efficiently only after the process of clotting has been initiated by the removal of fibrinopeptide A from fibrinogen.

Factor XIII (fibrin-stabilizing factor), the plasma precursor of the transamidating enzyme (factor $XIII_a$), is generated in the last phase of the coagulation cascade and serves the function of strengthening the structure of the blood clot by catalyzing the formation of γ-glutamyl-ε-lysine peptide bridges between fibrin molecules [for a recent review, see Lorand et al., (1980)]. Activation of the zymogen takes place in the following stages, each of which can be studied in an isolated, purified system:



In the first, limited proteolytic step (Lorand & Konishi, 1964), thrombin causes the release of a 36 amino acid containing N-terminal peptide (AP)[1] from the a subunit (Takagi & Doolittle, 1974). The hydrolytically modified zymogen, still associated in heterologous protomeric units, has no enzymatic activity. However, the ensemble dissociates under the specific influence of Ca^{2+} ions (Lorand et al., 1974; Curtis et al., 1974) which also induce the conformational change ($a_2' \rightarrow a_2^*$) necessary for the unmasking of the active-center cysteine and the generation of active enzyme (a_2^*).

It was recently shown (Credo et al., 1978, 1981) that fibrin(ogen) exerted a significant effect on the dissociation of the $a_2'b_2$ ensemble and on enzyme generation by lowering the otherwise high Ca^{2+} requirement to the concentration of this cation found in plasma (1.5 mM). As such, fibrin(ogen) fulfills an important regulatory role which enables generation of the cross-linking enzyme within the time frame of normal blood clotting. The ability of fibrin(ogen) to promote the generation of factor $XIII_a$ suggested the possibility that fibrin, or some intermediate in the conversion of fibrinogen to fibrin, might also activate the thrombin catalyzed hydrolysis of Arg-36 in the a subunits of the zymogen (i.e., the first step in the conversion sequence). Such regulation could serve to ensure that the activation of factor XIII would not proceed without its substrate, fibrin. In the present paper, we describe a procedure for measuring the kinetics of the release of the activation peptide from human factor XIII and show that the rate of thrombin-mediated release of this peptide is, indeed, dramatically enhanced in the presence of fibrin(ogen).

Materials and Methods

Fibrinogen was purified from human plasma by a previously described method (Higgins & Shafer, 1981) except that, after passage through the lysine–Sepharose column, the material was dialyzed into 0.3 M NaCl and stored at −10 °C.

Human thrombin (4.06 NIH units/μg) was kindly provided by Dr. John W. Fenton II of the New York State Department of Health, Albany, NY. The sample, which was greater than 93% pure by active-site titration (Chase & Shaw, 1970), was stored in 0.3 M NaCl at −70 °C, and α-thrombin accounted for >99% of the enzyme in the preparation. Values of k_{cat} and k_{cat}/K_m reported in this work are based on the concentration of thrombin from its absorbance at 280 nm by using an $E^{1\%}_{280nm}$ value of 18.3 in 0.1 M NaOH and an M_r of 36 500 (Fenton et al., 1977). No corrections were made for the deviation (<7%) of activity from 100%.

Factor XIII was isolated from outdated human plasma by a published procedure (Lorand et al., 1981). The factor XIII was stored in 50 mM Tris-HCl, pH 7.5, with 1.0 mM EDTA at 4 °C, and it was dialyzed at 4 °C into the reaction buffer before use. The concentration of ab protomeric units of factor XIII was determined from its absorbance at 280 nm by using an $E^{1\%}_{280nm}$ value of 13.8 and an equivalent weight of 160 000 (Schwartz et al., 1973) for the ab protomeric unit. Using iodo[^{14}C]acetamide for titrating the active-site potential of the preparation (Curtis et al., 1974), we measured a functional purity of 92%, and no corrections were made for this slight (8%) deviation from 100%.

Reaction Kinetics. The rates of release of peptides (FPA, FPB, AP, and AP′) were measured at 37 °C in reaction

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[1] Abbreviations: AP and AP′, major and minor activation peptides, respectively, released upon treatment of factor XIII with thrombin; φ, fibrinogen; FPA, fibrinopeptide A; FPB, fibrinopeptide B; HPLC, high-pressure liquid chromatography, PEG, poly(ethylene glycol); M_r, molecular weight; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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mixtures of 5–17 mL containing 137 mM NaCl, 2.5 mM KCl, 9.47 mM sodium phosphate (pH 7.4), and 0.1% PEG. In a typical study, fibrinogen and factor XIII were stirred at 37 °C in a polypropylene tube; after removal of a sample for a zero-time point, an aliquot of a diluted stock solution of human thrombin was added. Care was taken to dilute the stock thrombin solution with calibrated Eppendorf pipets into polypropylene tubes containing the reaction mixture with 0.1% PEG 6000. The latter reagent was used to prevent adsorption of thrombin on surfaces (Wasiewski et al., 1976).

Within 2 min after the addition of thrombin, aliquots (0.8–1.2 mL) from the reaction mixture were pipetted into microfuge tubes which had been equilibrated at 37 °C in a water bath. Final amounts of releasable peptides at the completion of the reaction were determined by using long incubation times (more than eight half-lives). Excess thrombin (10-fold greater than that initially present) was sometimes added to samples to shorten the time required for measuring limiting amounts of releasable peptides. To terminate the reaction, 0.1 mL of 3 M $HClO_4$ was added at various times, and volumes were adjusted with water to 1.3 mL. The precipitated protein was centrifuged, and the supernatant solution was transferred to an automatic sample injector (WISP, from Waters Associates) for injection (1 mL) into the HPLC system. Peptides were separated and quantified by reverse-phase HPLC.

HPLC. A Varian 5000 liquid chromatograph was used, and all solvents were HPLC grade. The peptides were detected at 205 nm by using a Model 450 variable-wavelength detector from Waters Associates. For separation, a reverse-phase C_{18} column (0.46 × 25 cm, Partisil-10-ODS-3 from Whatman) was employed, and elution was carried out at room temperature with 0.083 M sodium phosphate buffer (pH 3.1) and acetonitrile by using the following program: 0–10 min, 85% buffer A and 15% buffer B; 10–60 min, a linear gradient from 85% buffer A and 15% buffer B to 10% buffer A and 90% buffer B. Buffer A was 10% acetonitrile–90% phosphate buffer (v/v), and buffer B was 40% acetonitrile–60% phosphate buffer (v/v).

Calculations. The fibrinopeptides (FPA and FPB) and the activation peptides (AP and AP′) were quantified from their peak areas, by utilizing the molar absorptivities of the peptides. A value of 1.12×10^5 M^{-1} cm^{-1} was determined for AP and AP′ by using the procedure previously described for measuring the absorptivities of fibrinopeptides (Higgins & Shafer, 1981), except that the amino acid analysis was carried out by the procedure of Koop et al. (1982).

To minimize errors due to possible degradation of AP over prolonged incubation periods without fibrinogen, the first order release of AP was analyzed at degrees of conversion of less than 60%, and the limiting, final value for AP concentration (denoted as $[AP]_F$) was calculated from the initial concentration of the factor XIII protomers (i.e., the ab subunit ensemble).

Results and Discussion

Treatment of factor XIII by thrombin resulted in the release of two species of peptides, AP and AP′ (Figure 1). As indicated by the data in Table I, the amino acid compositions of both AP and AP′ were consistent with the reported structure for the activation peptide (Takagi & Doolittle, 1974). The similar amino acid composition of the two peptides leaves open the possibility that they may differ with respect to their Gln or Asn content, and the finding of two activation peptides could be due to the presence of a factor XIII variant in the pooled plasma from which this zymogen was purified.[2]

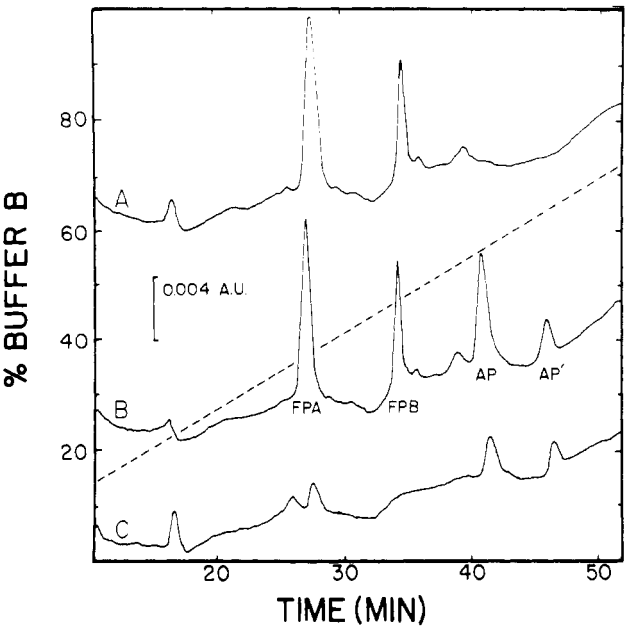

FIGURE 1: HPLC elution profiles for fibrinopeptides and for factor XIII activation peptides. Aliquots (0.8 mL) of reaction mixtures containing 0.27 nM thrombin at pH 7.4 (37 °C, $\mu = 0.15$) and 0.1% PEG were quenched at the times specified with 0.1 mL of 3 M $HClO_4$. Precipitated protein was removed by centrifugation. The supernatant solution was adjusted to 1.3 mL with water and 1 mL of the resulting solution subjected to HPLC. (A) 0.2 μM fibrinogen, after 15 min of reaction; (B) 0.2 μM fibrinogen and 0.2 μM factor XIII protomer after 18 min of reaction; (C) 0.4 μM factor XIII protomer after 66 min of reaction. The initial part of the chromatograms (not shown) contained a peak at the void volume. The broken line indicates the percentage of buffer B in the gradient (see Materials and Methods).

Table I: Amino Acid Composition of Activation Peptides Derived from the Reaction of Human Factor XIII with Human Thrombin[a]

amino acid	AP	AP′	lit. data[b]
aspartic acid	4.4	4.7	5
glutamic acid	3.9	4.2	4
arginine	3.6	3.5	4
serine	3.0	2.6	3
glycine	3.3	2.8	3
threonine	2.8	2.7	3
proline	4.0	4.0	4
alanine	4.0	3.7	4
valine	3.0	3.1	3
leucine	1.8	2.7	2
phenylalanine	1.0	1.0	1

[a] The amino acid composition was obtained by dividing the molar amount of each amino acid by the molar amount of peptide analyzed. The latter quantity was obtained by dividing the total amount of amino acids analyzed by 36, the number of amino acid residues in the activation peptide. [b] From the amino acid sequence for the activation peptide reported by Takagi & Doolittle (1974).

Because AP accounted for 80% for the total activation peptides, we focused only on the time course of the release of AP, and in the calculations, $[AP]_F$ was set equal to 0.8 times the initial concentration of added factor XIII protomers (i.e., $0.8[ab]_0$). In the presence of fibrinogen, the combined amount

[2] Incubation (at room temperature) of the acidified reaction mixture for several hours prior to HPLC resulted in the appearance of a shoulder on the AP and AP′ peaks. Conversion of AP to AP′ (or vice versa) was not observed, however. The appearance of a shoulder in the AP and AP′ peaks could reflect a N–O acyl shift in the amino-terminal *N*-acetylserine residue of the activation peptide. Further work is required to establish the structural basis for the difference in AP and AP′ and for the alteration of these peptides in acid.

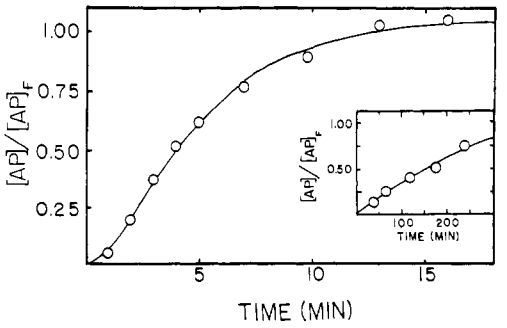

FIGURE 2: Thrombin-mediated release of activation peptide from 0.194 μM factor XIII protomer in the presence of 0.45 μM fibrinogen at pH 7.4 (37 °C, μ = 0.15), 0.1% PEG, and 0.565 nM thrombin. The solid line in the inset, pertaining to the release of AP in the absence of added fibrinogen, was obtained from a least-squares fit of the data to eq 4 and yielded a value for k_{cat}/K_m of 0.13 × 10^6 M^{-1} s^{-1}.

of AP + AP′ released from factor XIII upon digestion with thrombin was within 5% of that expected from the amount of zymogen present. In the absence of fibrinogen, however, a quantitative yield of AP and AP′ could not be obtained because partial degradation of the activation peptides occurred during the long time necessary for their release.

The thrombin-mediated release of the activation peptide directly from factor XIII could be interpreted in terms of the Michaelis–Menten equation:

$$d[AP]/dt = 0.8[ab]_0 k_{cat} e/(0.8[ab]_0 + K_m) \quad (1)$$

When $[ab]_0 \ll K_m$, eq 1 simplifies to

$$d[AP]/dt = 0.8[ab]_0 k_{cat} e/K_m \quad (2)$$

which upon integration gives rise to

$$\ln (1 - [AP]/[AP]_F) = -k_{cat} et/K_m \quad (3)$$

or

$$[AP]/[AP]_F = 1 - \exp(-k_{cat} et/K_m) \quad (4)$$

where $[AP]_F$ is the final concentration of the activation peptide, or $0.8[ab]_0$, and e is the concentration of thrombin.

The time dependence of the release of AP at concentrations of factor XIII protomer below 1 μM could be represented by eq 3 or 4 (e.g., see inset to Figure 2) with a value of 0.13–0.16 μM^{-1} s^{-1} for k_{cat}/K_m.[3] In an attempt to determine K_m, the initial rates of release of AP were determined at several concentrations of factor XIII, but even at the highest concentration of protomers ($[ab]_0$ = 70 μM), the thrombin was not sufficiently saturated to give a reliable value for K_m. Nevertheless, using eq 1, we could calculate estimates of K_m = 84 (±30) μM and k_{cat} = 10 (±3) s^{-1} from the initial rates obtained with 5.59 and 56.5 μM AP-yielding protomers.

The addition of fibrinogen, at concentrations well below that of this protein in normal plasma (~8 μM), caused a dramatic increase in the thrombin-catalyzed release of AP from the factor XIII zymogen, which is illustrated by the example given in Figure 2. As seen from the inset, in the absence of fibrinogen, a 50% release of AP was elicited in 150 min. However, in the presence of 0.45 μM fibrinogen (main graph), 50% release of AP was obtained in less than 4 min. Furthermore, the effect of fibrinogen on the reaction appeared to be dose dependent (Figure 3). Additionally, fibrinogen caused the time dependence for the release of AP to change from the simple first-order reaction found in the absence of fibrinogen

[3] The initial substrate concentrations used in these experiments (<1 μM) were much less than the value of K_m ~84 μM. Thus, use of eq 3 was justified.

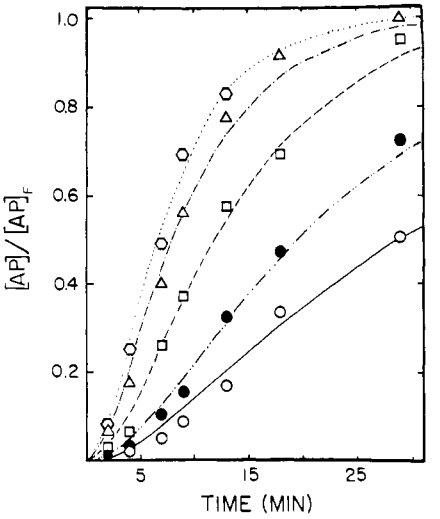

FIGURE 3: Dependence of the release of activation peptide from 0.2 μM factor XIII protomer on the concentration of fibrinogen, in the presence of 0.29 nM thrombin at pH 7.4, (37 °C, μ = 0.15) and 0.1% PEG. Fibrinogen concentrations were 0.05 (○), 0.10 (●), 0.20 (□), 0.40 (△), and 0.90 μM (open hexagon).

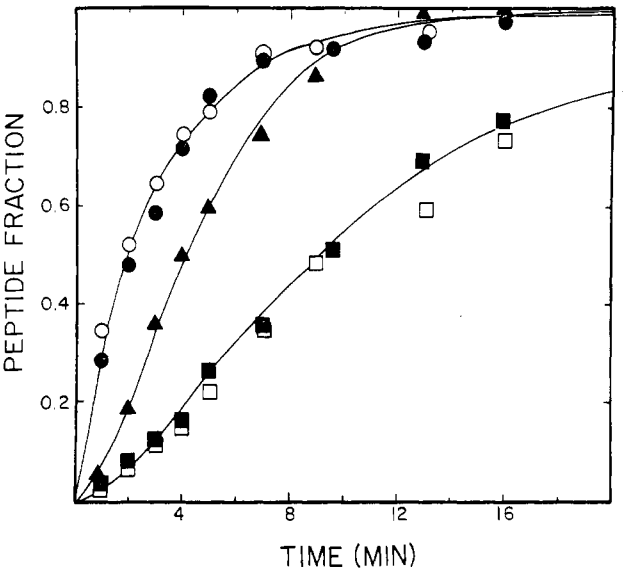

FIGURE 4: Sequence of release of fibrinopeptides and activation peptide from factor XIII at pH 7.4 (37 °C, μ = 0.15) in 0.1% PEG by using 0.565 nM thrombin, 0.445 μM fibrinogen, and 0.258 μM factor XIII protomer. FPA, circles; FPB, squares; AP, triangles. The open circles and squares are data points obtained for the release of FPA and FPB in the absence of factor XIII. The ordinate (peptide fraction) is the quotient between the amount of peptide released at the indicated time and that released at completion of the reaction.

to a more complex process, which exhibited a distinct lag phase. This lag could not be ascribed to competition between factor XIII and fibrinogen for thrombin, since only a small fraction of the thrombin was saturated at the fibrinogen concentrations used.[4] Thus, the lag in the appearance of AP indicated that release of AP in the presence of fibrinogen was preceded by another reaction. Figure 4 shows the time course for release of FPA, FPB, and AP when 0.2 μM factor XIII

[4] The degree of saturation of thrombin by fibrinogen can be estimated from the fibrinogen concentrations, $[\phi]$, and the relationship:

$$\frac{2[\phi]}{2[\phi] + K_m} = \frac{2[\phi]}{2[\phi] + 7.2}$$

where K_m (7.2 μM) is the Michaelis constant for the interaction of thrombin with the Aα-chains in fibrinogen (Higgins et al., 1983).

protomer was activated by thrombin in the presence of 0.45 μM fibrinogen. It is clear that under the conditions of this experiment AP was released after FPA but before FPB.[5] This finding is in contrast to a preliminary report by Greenberg et al. (1982) that in plasma the release of FPA occurs simultaneously or slightly precedes the hydrolytic conversion of the a subunits of factor XIII to a'. It is possible, however, that different experimental conditions might account for the quantitative difference between our results and those of Greenberg et al. (1982).

It has long been known that during clotting in plasma, factor XIII activity is adsorbed to fibrin (Lorand & Dickenman, 1955), and it has also been shown by immunochemical analysis that only the noncatalytic b subunits of the zymogen appear in serum (Ikematsu et al., 1981). Moreover, fibrinogen preparations were found to be contaminated by factor XIII to various degrees (Lorand, 1961a,b), which suggested strong interactions between the two plasma proteins. Direct measurements by Greenberg & Shuman (1982) of the binding of factor XIII to fibrinogen, coupled to a solid support, yielded a dissociation constants as low as 10 nM. Since the plasma concentration of fibrinogen ($\sim$8 μM) is much larger than that of factor XIII [20 nM; see Skrzynia et al. (1982)], the fraction of factor XIII complexed with fibrinogen [ϕ···ab] should be given by

$$\frac{[\phi\cdots ab]}{[\phi\cdots ab] + [ab]} = \frac{[\phi]}{[\phi] + K_D} = \frac{8 \times 10^{-6}}{8 \times 10^{-6} + 10^{-8}} = 0.999$$

Thus, unless other plasma components compete with fibrinogen for factor XIII, essentially all of the factor XIII in plasma should be complexed with fibrinogen. Greenberg & Shuman (1982) pointed out that the tight binding of factor XIII to fibrinogen would ensure that the zymogen will be localized at the site of clotting. Our present work discloses the possibility of a more subtle form of control whereby interaction of factor XIII together with some intermediate of the fibrinogen–fibrin conversion causes the a subunits of the factor XIII zymogen to become more susceptible to cleavage by thrombin at Arg-36. Further work is required to define more exactly the nature of the activating species and to quantify the extent to which fibrogen promotes release of AP in plasma. On the basis of the data given in Figure 4, either fibrinopeptide A or des-A-fibrinogen (i.e., $\alpha_2B\beta_2\gamma_2$) could qualify as an activator. Addition of 0.48 μM FPA (isolated by HPLC after treatment of fibrinogen with thrombin), however, had no detectable effect on the thrombin- (7.6 nM) catalyzed release of AP from 0.15 μM factor XIII. Thus, des-A-fibrinogen appears to be the more likely candidate for serving as the activator in the thrombin-catalyzed release of AP. Whatever the exact nature of the activator, the observations presented in this paper, together with the findings that fibrin(ogen) also facilitates the conversion of $\alpha_2'b_2$ to $a_2^* + b_2$ (Credo et al., 1978, 1981), provide a molecular basis for a coordinated generation of factor $XIII_a$ and its substrate, fibrin, in juxtaposition of each other.

[5] The plots of Figure 4 also indicate that when fibrinogen was in excess of factor XIII, the presence of factor XIII had little effect on fibrinopeptide release (compare open and closed symbols for release of FPA and FPB in Figure 4).

Registry No. Factor XIII, 9013-56-3; thrombin, 9002-04-4.

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