Effect of Fibrinogen and Ca^{2+} on the Thrombin-Catalyzed Proteolytic Event That Triggers Activation of Factor XIII

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Formation of stable blood clots requires formation of glutamyl-ε-lysyl peptide cross-links between certain glutamyl and lysyl residues in fibrin. Formation of these cross-links is catalyzed by the transamidase factor $XIII_a$. Production of this enzyme is initiated by thrombin-catalyzed limited proteolysis of the inactive zymogen factor XIII during the final stage of the blood clotting cascade.[1] This initial proteolytic event in the reaction pathway for conversion of factor XIII to factor $XIII_a$ involves cleavage at Arg-a36 and release of a 36 amino acid residue activation peptide from the a chains of the a_2b_2 molecules comprising plasma factor XIII.[2]

Using an HPLC procedure developed to monitor the release of peptides upon treatment of human factor XIII with human α-thrombin, two peptides, AP and AP′, with amino acid compositions consistent with that reported[2] for activation peptide (TABLE 1), were produced upon treatment of factor XIII with thrombin.[3] The appearance in lower amounts (< 20%) of the second activation peptide (AP′) may reflect the presence of a factor XIII variant in the pooled plasma from which the protein was purified, or it could reflect partial hydrolysis at a Gln or Asn residue during purification. Treatment of factor XIII, or isolated AP and AP′, with alkaline phosphatase had no effect on the relative amounts or elution positions of AP and AP′, suggesting that activation peptide does not exist in different states of phosphorylation as does fibrinopeptide A (FPA). Incubation of acidified mixtures of AP and AP′ for several hours prior to HPLC resulted in the appearance of a shoulder on both the AP and AP′ peaks, but no interconversion of the AP and AP′. The appearance of the shoulder may well reflect an N-O acyl shift in the *N*-acetylseryl residue at the amino terminus of the activation peptide. Additional studies are required, however, to determine the structural basis for the different elution positions of AP and AP′ and for the acid catalyzed alterations of these peptides. All kinetic parameters reported in this work are for the release of the major activation peptide, AP.

Studies of the dependence of the time dependence of the release of AP on the concentration of factor XIII indicated a value of $0.14 \times 10^6 M^{-1}s^{-1}$ for the specificity constant k_{cat}/K_m for thrombin-catalyzed release of AP. The value of K_m, which was too high to determine accurately, was estimated to be 84 ($\pm$ 30) μM. Comparison of the K_m value for this process with estimates[4] of 20 nM for the factor XIII concentration in blood plasma, suggested thrombin-catalyzed activation of factor XIII might be too inefficient to be physiologically significant unless the process was enhanced by other plasma constituents. FIGURE 1 shows that the presence of 0.45 μM fibrinogen produces a dramatic enhancement in the thrombin-catalyzed release of activation peptide. The promoting effect of fibrinogen increased with its concentration, and under the experimental conditions a near maximal effect was obtained at 0.9 μM fibrinogen, well below the plasma fibrinogen concentration ($\sim$ 8 μM). When the concentration of fibrinogen was not in large excess of the factor XIII, there was a clearly discernible lag in the release of activation peptide. Under these conditions FPA appeared prior

TABLE 1. Amino Acid Composition of Activation Peptides Derived from the Reaction of Human Factor XIII with Human Thrombin

Amino acid	AP	AP′	Literature[a]
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] From the amino acid sequence for the activation peptide reported in reference 2.

to activation peptide, which in turn appeared prior to fibrinopeptide B, FPB (FIGURE 2). This observation suggested that fibrin I or FPA, rather than fibrinogen, might be the immediate promoter of thrombin-catalyzed activation of factor XIII. Whereas FPA had no effect, fibrin I and II promoted thrombin-catalyzed release of AP from factor XIII. More importantly, as illustrated in FIGURE 3, the lag in the release of AP seen with fibrinogen was eliminated when fibrin I or II was the promoter. A kinetic analysis of the time dependence of the release of AP with fibrinogen as the promoter indicated that both fibrin I and II were equally effective in promoting thrombin-catalyzed activation of factor XIII.

FIGURE 4 illustrates that the inhibitor of fibrin polymerization, glycyl-L-prolyl-L-arginyl-L-proline (GPRP),[5,6] antagonized the promoting effect of fibrinogen. Because GPRP does not inhibit thrombin-catalyzed release of FPA from fibrinogen,[7] the simplest explanation of the antagonism by GPRP, is that fibrin monomer is not a promoter of thrombin-catalyzed release of AP from factor XIII, and that the ability

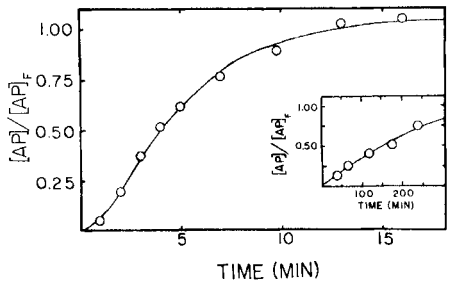

FIGURE 1. Thrombin-mediated release of activation peptide from 0.194 μM factor XIII protomer in the presence and absence (inset) of 0.45 μM fibrinogen at pH 7.4 (37°C, μ = 0.15), 0.1% PEG, and 0.565 nM thrombin. (Janus *et al.*[3] With permission from *Biochemistry.*).

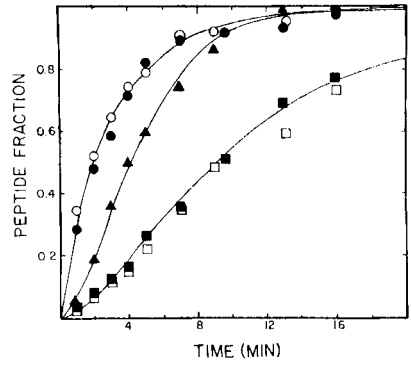

FIGURE 2. Sequence of release of fibrinopeptides and activation peptide from factor XIII at pH 7.4 (37°C, μ = 0.15) in 0.1% PEG by using 0.565 nM thrombin, 0.445 μM fibrinogen, and 0.258 μM factor XIII protomer. FPA, circles; FPB, squares; AP, triangles. The open circles and squares are data points obtained 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. (Janus *et al.*[3] With permission from *Biochemistry.*)

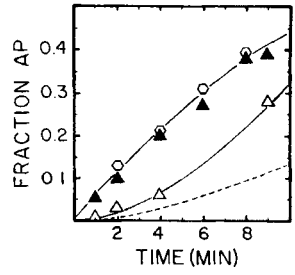

FIGURE 3. Time dependence of the release of AP, containing factor XIII and either fibrinogen (△) or polymeric fibrin I (▲) or fibrin II (○). Reaction mixtures contained 0.29 nM thrombin, 0.1 μM factor XIII, and 0.1 μM fibrinogen or 0.1 μM (with respect to fibrin monomer) polymeric fibrin. Reactions were carried out in 9.5 mM phosphate buffer, pH 7.4, 0.14 M NaCl, and 0.1% PEG, 37°C. (Lewis *et al.*[8] With permission from *Biochemistry.*)

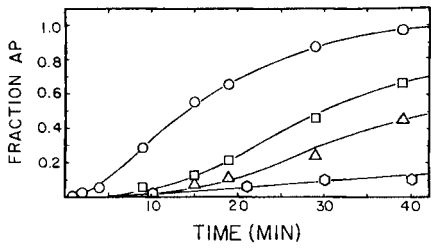

FIGURE 4. Thrombin-catalyzed release of AP from 0.10 μM factor XIII, with 0.10 μM fibrinogen added, in the absence (○) and presence of 0.10 (□), 0.21 (△), and 0.490 mM (◇) GPRP. Reactions were carried out as described in FIGURE 3. (Lewis *et al.*[8] With permission from *Biochemistry.*)

of GPRP to act as an antagonist is due to its suppression of the polymerization of fibrin.

These studies of the fibrin-promoted thrombin-catalyzed release of AP from factor XIII were done in the absence of calcium. In the absence of added calcium, enzymically active factor $XIII_a$ is not generated, because Ca^{2+} is required for steps subsequent to initial proteolysis in the reaction pathway for the conversion of factor XIII to factor $XIII_a$. Thus, in the absence of added Ca^{2+}, the fibrin in the reaction mixture remains non-cross-linked during measurements of the time dependence of AP release. Ca^{2+}-promoted cross-linking of fibrin, however, could well alter its competence as a promoter. Therefore, we were prompted to determine the effect of Ca^{+} on thrombin-catalyzed release of AP. The plots in FIGURE 5 show that the presence of 1.4 mM Ca^{2+} dramatically reduced the effectiveness of fibrinogen. FIGURE 6 shows that the effect of Ca^{2+} can be partially reversed by the presence of plasma levels of fibrinogen. Interestingly, 1.4 mM Ca^{2+} had no effect on the unpromoted rate of release of AP

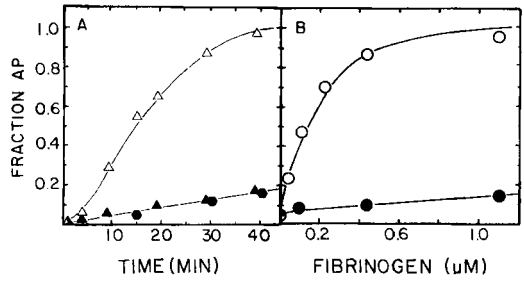

FIGURE 5. Panel A: Effect of Ca^{2+} on the thrombin-catalyzed release of activation peptide (AP) from 0.10 μM factor XIII in the presence of 0.10 μM fibrinogen, with (▲) and without (△) 1.4 mM $CaCl_2$. Also shown is the thrombin-mediated release of AP from 0.10 μM factor XIII without fibrinogen and in the presence of 1.4 mM $CaCl_2$ (●). Reactions were carried out as described in FIGURE 3. Panel B: Dependence of thrombin-catalyzed release of AP from 0.10 μM factor XIII on the fibrinogen concentration in the absence (○) and presence (●) of added 1 mM $CaCl_2$. The ordinate is the fraction of AP released in 15 minutes. The reaction conditions are as described in FIGURE 3, except the buffer contains 0.01 M Tris instead of 9.5 mM phosphate. Control experiments showed that the fibrinogen-promoted release of AP from factor XIII was the same in both buffers when Ca^{2+} was absent and that Ca^{2+} at 1 mM antagonized the effect of 0.1 μM fibrinogen to the same extent in 0.01 M Tris and 9.5 mM phosphate buffer. (Lewis *et al.*[8] With permission from *Biochemistry.*)

from factor XIII observed in the absence of fibrinogen. The data in FIGURE 6 indicate that only a fraction of the AP is released at an enhanced rate in the presence of Ca^{2+}. Thus, in the presence of 6 μM fibrinogen and 1 mM Ca^{2+}, the rate of AP release fell to the unpromoted rate after 20% of the AP had been released, whereas in the absence of added Ca^{2+}, the presence of 6 μM fibrinogen resulted in an enhanced rate of AP release until well over 90% of the AP was released. This observation indicated that the time-dependent loss in promoting activity might be due to cross-linking of fibrin by the activated factor XIII. Comparison of the time dependence of AP release and cross-linking of fibrin revealed that in the presence of Ca^{2+} the rate of AP release decreased to that seen in the absence of fibrin when 40% of the γ chains of fibrin had become cross-linked. This result suggests that if non-cross-linked fibrin is a promoter and cross-linked fibrin is not, the promoting activity of fibrin must involve the interaction of more than one repeating unit of fibrin with each molecule of factor XIII.

Consistent with the notion that formation of cross-links inactivated fibrin as a promoter of thrombin-catalyzed release of AP from factor XIII, the thiol reagent methyl methanethiosulfonate (MMTS) inactivated the activated factor XIII and substantially blocked the effect of Ca^{2+} (FIGURE 7). The failure of MMTS to block completely the inhibitory effect of Ca^{2+} suggests that Ca^{2+} may have secondary inhibitory effects on the fibrin-promoted reaction.

Preliminary studies of the dependence of thrombin-catalyzed AP release on the concentration of factor XIII and fibrin indicate that fibrin reduces the K_m for the thrombin-catalyzed reaction, consistent with the possibility that fibrin forms a complex with factor XIII, which exhibits a high affinity for thrombin. Interestingly, γ-thrombin, a form of thrombin that interacts poorly with fibrinogen, did not exhibit fibrin-promoted release of AP, although in the absence of fibrin, the rate of γ-thrombin-catalyzed release of AP from factor XIII was only fivefold lower than that of normal α-thrombin. This observation suggests the possibility that in fibrin-promoted α-thrombin-catalyzed release of AP from factor XIII, the fibrin interacts with both the factor XIII and thrombin and thereby presents the factor XIII scissile bond to the active site of thrombin.

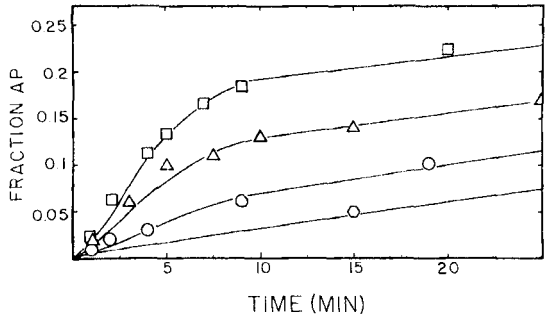

FIGURE 6. Thrombin-catalyzed release of AP from 0.10 μM factor XIII with 0.10 (○), 1.1 (△), and 6 μM (□) fibrinogen in the presence of 1.0 mM $CaCl_2$. The lowest line and (○) represent the release of AP in the absence of fibrinogen (see FIGURE 5A). Reaction conditions are as described in FIGURE 3, except that the curve for 1.1 μM fibrinogen was determined in 0.01 M Tris buffer. (Lewis *et al.*[8] With permission from *Biochemistry*.)

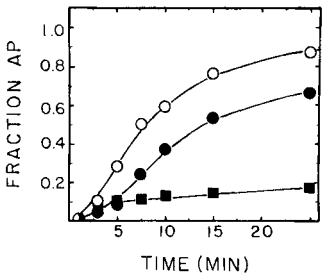

FIGURE 7. Ability of MMTS to block the inhibitory effects of Ca^{2+} on thrombin-catalyzed release of AP from factor XIII. Reaction mixtures contained 0.29 nM thrombin, 1.1 μM fibrinogen, 0.1 μM factor XIII, and (■) no MMTS and 1 mM Ca^{2+}, (●) 1.2 mM MMTS and 1 mM Ca^{2+}, or (○) 1.2 mM MMTS and no added Ca^{2+}. Reaction conditions are as described in FIGURE 3 except the buffer species was 0.01 M Tris. (Lewis *et al.*[8] With permission from *Biochemistry*.)

The unique ability of polymeric non-cross-linked fibrin to promote thrombin-catalyzed release of AP could ensure that production of factor $XIII_a$ is initiated when its substrate is present and that production of additional factor $XIII_a$ is terminated upon cross-linking of fibrin γ chains. This mode of regulation may serve to ensure against wasteful and possibly deleterious activation of factor XIII. It is our hypothesis that the final degree of cross-linking of a fibrin clot should increase with the amount of fibrin-associated factor $XIII_a$, because the ability of a molecule of factor $XIII_a$ to catalyze formation of cross-links should be lost as the factor $XIII_a$ becomes enmeshed in the network of cross-links it produces. If this contention is correct, a high density of factor $XIII_a$ might be expected to result in a more heavily cross-linked clot, which would be more difficult for plasmin to dissolve, and more likely to lead to thrombosis. Thus, the regulation of factor $XIII_a$ described in this paper may be an important determinant for the formation of stable fibrin blood clots, which can later be efficiently dissolved by plasmin. Investigations are in progress to test this hypothesis.

REFERENCES

1. LORAND, L. & K. KONISHI. 1984. Arch. Biochem. Biophys. **105:** 58-67.
2. TAKAGI, T. & R. F. DOOLITTLE. 1974. Biochemistry **13:** 750-756.
3. JANUS, T. J., S. D. LEWIS, L. LORAND & J. A. SHAFER. 1983. Biochemistry **22:** 6269-6272.
4. SKRZYNIA, C., H. M. REISNER & J. MCDONAGH. 1982. Blood **60:** 1089-1095.
5. LAUDANO, A. P. & R. F. DOOLITTLE. 1978. Proc. Natl. Acad. Sci. USA **75:** 3085-3089.
6. LAUDANO, A. P. & R. F. DOOLITTLE. 1980. Biochemistry **19:** 1013-1019.
7. HIGGINS, D. L., S. D. LEWIS & J. A. SHAFER. 1983. J. Biol. Chem. **258:** 9276-9282.
8. LEWIS, S. D., T. J. JANUS, L. LORAND & J. A. SHAFER. 1985. Biochemistry **24:** 6772-6777.