α-Thrombin-Catalyzed Activation of Human Platelet Factor XIII: Relationship between Proteolysis and Factor XIIIa Activity†

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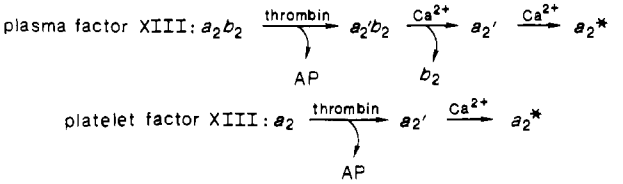
ABSTRACT: The kinetics of activation of platelet factor XIII, an *a*-subunit dimer, were characterized by determining rate constants for activation peptide (AP) release, generation of activity, and exposure of the active-site thiol group. The specificity constant (k_{cat}/K_m) for α-thrombin-catalyzed AP release, 1.2×10^5 M^{-1} s^{-1}, was found to be similar to that for AP release from the tetramer plasma factor XIII (a_2b_2) [Janus, T. J., Lewis, S. D., Lorand, L., & Shafer, J. A. (1983) *Biochemistry 22*, 6269–6272], implying that the *b* subunits of plasma factor XIII do not hinder α-thrombin-catalyzed cleavage of AP from the *a* subunit. Platelet factor XIIIa activity was generated at a rate approximately twice the rate of AP release. This difference in rates was shown to be consistent with a reaction pathway for activation of platelet factor XIII wherein full factor XIIIa activity is generated when one AP is removed from the dimeric zymogen so that removal of the second AP has no detectable effect on catalytic activity. In accord with this conclusion, the rate constant for exposure of the active-site thiol group, as measured by the incorporation of [1-^{14}C]-iodoacetamide, was about twice that observed for the removal of AP. The observed stoichiometry of labeling with iodoacetamide of only one active-site thiol group per dimer for active factor XIIIa, together with the values of the rate constants for AP release, generation of activity, and exposure of the active-site thiol group, suggests that either (i) release of one molecule of AP per platelet factor XIII dimer triggers subunit interactions that cause both active-site thiol groups to become exposed with one thiol group becoming unreactive upon alkylation of the other or (ii) release of one molecule of AP per platelet factor XIII dimer triggers subunit interactions that expose one active-site thiol group and prevent simultaneous exposure of both active-site thiol groups even after the second molecule of AP is released.

Factor XIIIa, the last enzyme in the blood coagulation cascade, is a transamidase that catalyzes formation of γ-glutamyl-ε-lysyl peptide cross-links between polypeptide chains in adjacent fibrin monomers and between fibrin and other plasma proteins [for reviews, see Lorand et al. (1980) and Shafer and Higgins (1988)]. Cross-links between fibrin molecules increase the mechanical strength of fibrin clots (Roberts et al., 1974; Gerth et al., 1974), whereas the cross-links between fibrin and adhesive glycoproteins such as thrombospondin and fibronectin may serve to anchor fibrin clots to the site of injury (Shafer & Higgins, 1988). Additionally, the cross-linking of fibrin to α_2-antiplasmin which occurs during blood clotting increases the resistance of fibrin clots to the fibrinolytic activity of plasmin and appears to be an important determinant of the lifetime of fibrin clots (Sakata & Aoki, 1980, 1982; Jansen et al., 1987).

Factor XIII, the zymogen form of factor XIIIa, is found in the body in two forms. In plasma, factor XIII exists as a tetramer comprised of two *a* subunits and two *b* subunits. A homodimeric form containing only the *a* subunits of plasma factor XIII is located within platelets and placental tissue (Bohn, 1972; Schwartz et al., 1973, 1974). The two forms of factor XIII (a_2b_2 and a_2) are both activated by α-thrombin

Scheme I



as shown in Scheme I. α-Thrombin acts upon both plasma and platelet factor XIII to release a 37-aminoacyl N-terminal peptide, activation peptide (AP),[1] from the amino terminus of the *a* subunits (Takagi & Doolittle, 1974; Ichinose et al., 1986). In the presence of calcium ion, the *b* subunits dissociate from α-thrombin-treated plasma factor XIII (Lorand et al., 1974; Cooke & Holbrook, 1974; Chung et al., 1974), leaving a cleaved dimer (a_2) identical with the product of thrombin action on platelet factor XIII. In the continued presence of calcium ion, the active-site thiol group is unmasked (Curtis et al., 1973, 1974; Cooke & Holbrook, 1974; Chung et al., 1974), resulting in the conversion of a_2' to a_2*, the catalytically competent form of factor XIIIa.

Activation of plasma factor XIII is a highly regulated process, with fibrin serving as a cofactor both in the Ca^{2+}-

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[1] Abbreviations: AP, activation peptide; BPB, bromophenol blue; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PEG, poly(ethylene glycol); PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; r-factor XIII, yeast recombinant human platelet factor XIII; TANEP, 0.1 M Tris–acetate, 0.15 M NaCl, 1 mM EDTA, and 0.1% PEG, pH 7.5; Tris, tris(hydroxymethyl)aminomethane.

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dependent conformational change necessary for exposure of the active-site thiol (Credo et al., 1978, 1981) and in the initial proteolytic event (Janus et al., 1983; Lewis et al., 1985). In two previous studies comparing activation of plasma and platelet factor XIII (Chung et al., 1974; Greenberg et al., 1987), it was proposed that the *b* subunits inhibit access of α-thrombin to the scissile bond at Arg-*a*37. In one of these studies (Greenberg et al., 1987), it was also proposed that fibrin interacts with plasma factor XIII to remove the inhibition of α-thrombin-catalyzed cleavage by the *b* subunits. The α-thrombin-catalyzed initial proteolytic event, cleavage of AP, was not directly measured in these studies. Instead, the studies relied upon the measurement of factor XIIIa activity to provide information about the initial proteolytic cleavage event. The possible complications associated with such a determination prompted us, in the present study, to characterize directly the kinetics for the α-thrombin-catalyzed event in the activation of platelet factor XIII. Comparison of these kinetics to those previously determined for plasma factor XIII (Janus et al., 1983) indicates that the *b* chains of plasma factor XIII *do not* alter the access of α-thrombin to the scissile bond at Arg-*a*37. Additional findings presented in this paper show that release of only one AP per dimer fully activates platelet factor XIII.

Materials and Methods

Platelet-Derived Factor XIII. Platelet factor XIII was purified from 3-week-old human platelets by using a published procedure (Schwartz et al., 1974) with small variations. Instead of sonication, a Sorvall Omni-mixer (setting 5, 4 × 10-s bursts) was used to lyse the platelets. Gel filtration chromatography was conducted using Sephacryl S-300 in 50 mM HEPES–NaOH, 0.15 M NaCl, and 1 mM EDTA at pH 7.5. Fractions of highest specific activity were concentrated by ultrafiltration under N_2 gas. When necessary, a second chromatography using DE-52 or a second 40% ammonium sulfate precipitation was added to purify further the platelet factor XIII. Platelet factor XIII purified by this method was estimated to be >90% pure by SDS–PAGE and Coomassie staining. The purified platelet factor XIII was stored at 4 °C in 50 mM HEPES–NaOH, 0.15 M NaCl, and 1 mM EDTA, pH 7.5, and retained 87% of its activity under these conditions after 9 weeks.

Yeast Recombinant Human Platelet Factor XIII (r-Factor XIII). Factor XIII was subcloned as a *Pst*I fragment from a full-length cDNA isolated by Ichinose et al. (1986). The cDNA was expressed by joining to the yeast *ADH2*-4c promoter (Russell et al., 1983) and the *TPI1* terminator (Alter & Kawasaki, 1982). This expression cassette was subcloned into a yeast 2μ-based expression vector containing the *Schizosaccharomyces pombe* triosephosphate isomerase (TPI) gene, and the construction was transformed into the *tpi* yeast strain ZM118, genotype (*a*/α *pep4*::*URA3*/*pep4*::*URA3*; *leu2-3,112*/*leu2-3,112* Δ*tpi*::*URA3*/Δ*tpi*::*URA3 bar1-1*/*bar1-1* [*cir*°]). A 12-L fermentor was inoculated with yeast and fed glucose continuously until the absorbance reached 30–45 (at 600 nm). The glucose feed was then stopped, and the cells were fed continuously with 2% ethanol for the duration of the fermentation, usually 24 h.

Unless otherwise stated, all buffered solutions contained 5 mM EDTA and 5 mM 2-mercaptoethanol. Whole cells were harvested, solvent-exchanged, and concentrated by using a spiral cartridge system (Amicon, Danvers, MA) to yield a final concentration of 50% (packed wet weight to volume) in deionized H_2O. The resulting cell suspension was then diluted with concentrated lysis buffer to give a final cell concentration of 40% in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15 mM EDTA, 5 mM 2-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride. Cell lysis was performed in a glass bead mill (DynaMill, Glen Mills, MA) using 100-μm acid-washed glass beads. Cell debris was removed from the lysate by centrifugation at 4000*g* for 45 min. The lysate was further clarified by addition of streptomycin sulfate to a final concentration of 2% (w/v) followed by centrifugation at 9000*g* for 60 min. The resulting supernatant was fractionated by addition of PEG-8000 to 12% (w/v) and the factor XIII containing precipitate harvested by centrifugation. This crude factor XIII precipitate was then redissolved in 50 mM Tris-HCl, pH 7.8, and applied to a 6 × 27 cm (diethylaminoethyl)cellulose fast-flow Sepharose (Pharmacia LKB, Piscataway, NJ) column, washed with 2 column volumes of the same buffer, and then eluted with a linear gradient consisting of 1 L each of the following: buffer A—50 mM imidazole, pH 6.3; buffer B—50 mM imidazole, pH 6.3, and 150 mM NaCl. The factor XIII containing peak fractions were then pooled, and the factor XIII was precipitated by dialysis against 50 mM piperazine, pH 6.0. The resulting crystalline precipitate was then redissolved in 50 mM glycine, pH 7.6, and chromatographed on a 4.7 × 80 cm S-200 Sephacryl (Pharmacia LKB, Piscataway, NJ) column. The peak fractions containing factor XIII were then pooled, filter-sterilized, and either used directly or stored as piperazine precipitate at 4 °C.

The resulting product appeared to be >99% pure as judged by silver-stained SDS–PAGE, by gel filtration chromatography, and by the virtual absence of any detectable yeast protein in antiyeast Western blots. Yeast recombinant human factor XIII (r-factor XIII) appears to be indistinguishable from placental or platelet-derived human factor XIII by all of the criteria thus far studied. Like the placenta and platelet-derived factor XIII, native r-factor XIII (i) is a homodimer with an M_r of 165 000 as determined by equilibrium sedimentation (David C. Teller, unpublished observations), (ii) is blocked to N-terminal Edman degradation, and (iii) yields upon treatment with α-thrombin a 4-kDa N-blocked activation polypeptide and a 79-kDa active protein (factor XIIIa). Additionally, the N-terminal sequence of the first 20 aminoacyl residues (as determined by Edman degradation) of α-thrombin-generated r-factor XIIIa is identical with that of platelet and placenta-derived factor XIII, and all three proteins catalyze the rapid formation of fibrin cross-links, yielding a similar pattern of γ dimers and α multimers when analyzed by reducing SDS–PAGE.

Platelet Factor XIIIa Assays. Concentrations of platelet factor XIII were determined from the absorbance of stock solutions using a value of 13.8 for $E^{1\%}_{280}$ and a molecular weight of 150 000 (Schwartz et al., 1973; Chung et al., 1974). Assays of platelet factor XIII, both for quantifying platelet factor XIII in fractions during purification and for measuring the activity of purified enzyme, were performed by using a modification of a previously described procedure (Curtis & Lorand, 1976). A solution prepared by mixing 20 μL of 4 mM dansylcadaverine in 50 mM Tris-HCl, pH 7.5, 50 μL of 0.4 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, 200 μL of the platelet factor XIII containing sample, and 1200 μL of 50 mM Bicine–NaOH, pH 9.0, was equilibrated at 37 °C in a cuvette. The solution was mixed with 50 μL of 500 units/mL α-thrombin in 25 mM Tris-HCl/25% glycerol, pH 7.5, and incubated for 10 min to activate the factor XIII. A 50-μL aliquot of 0.2 M DTT in 50% glycerol was then added, and after 1 min, 200 μL of 2% *N*,*N*′-dimethylcasein in 50 mM Bicine–NaOH, pH 9.0, 37 °C, was mixed into the solution. The increase in fluorescence ($\lambda_{excitation}$ = 360 nm, $\lambda_{emission}$ = 500 nm) was continuously

monitored at 37 °C. Activities were measured in fluorescence units per time, with a fluorescence unit defined as the maximum increase in dansylcadaverine fluorescence due to factor XIIIa activity under the assay conditions [1 fluorescence unit (FU) = fluorescence of a completely reacted sample − fluorescence of a blank with 200 μL of buffer substituted for 200 μL of the factor XIII containing solution]. Slopes of linear plots of FU (FU < 0.1) vs time were divided by the amount (milligrams) of factor XIII in the assay cuvette to yield the specific activity for factor XIIIa (7FU min^{-1} mg^{-1} for pure factor XIIIa).

Human α-thrombin was a generous gift of Dr. John W. Fenton II of the New York State Department of Health, Albany, NY. The thrombin was >98% α-thrombin and had an active-site content of 96%.

Kinetics of AP release were measured by HPLC as previously described (Janus et al., 1983). The reaction buffer contained 0.1 M Tris–acetate, 0.15 M NaCl, 1 mM EDTA, and 0.1% PEG all at pH 7.5 (TANEP). In some determinations (as specified), the reaction buffer also contained 14 mM $CaCl_2$.

Kinetics for the generation of platelet factor XIIIa activity were measured by using an adaptation of the dansylcadaverine incorporation assay (Curtis & Lorand, 1976; Curtis et al., 1974). For each time point, reaction mixtures were prepared consisting of 0.10 μM platelet factor XIII, 14 mM $CaCl_2$, 20 nM α-thrombin, and 100–115 μL of TANEP to bring the volume to 125 μL. (The $CaCl_2$ was added immediately prior to the addition of α-thrombin to minimize α-thrombin-independent calcium activation of factor XIII.) At the indicated activation times, 10 μL of 30 μM PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, Calbiochem) was added to quench the thrombin activity. In control experiments, the resulting concentration (2.2 μM) of PPACK was shown to inhibit the α-thrombin activity within a few seconds without affecting the factor XIIIa activity, which was immediately measured by transferring a portion of the quenched reaction mixture into a waiting assay solution equilibrated in a fluorescence cuvette at 37 °C. Fluorescence activity was measured as described above. The fraction of maximal activity was determined by dividing the factor XIIIa activity observed by the maximal activity resulting from rapid activation with 220 nM α-thrombin.

Kinetics of active-site thiol exposure of platelet factor XIII were measured as previously described (Curtis et al., 1974). An incubate consisting of platelet factor XIII, α-thrombin, and 14 mM $CaCl_2$ in TANEP was prepared and treated as described above for measurements of the kinetics of generation of factor XIIIa activity. At specified time intervals, aliquots were pipetted into vials containing, as specified in the legends to Figures 3 and 4, amounts of PPACK to inactivate α-thrombin and amounts of [1-^{14}C]iodoacetamide (New England Nuclear, 24.1 mCi/mmol) to alkylate exposed active-site thiol of factor XIIIa. Control experiments showed that the presence of PPACK at these specified concentrations did not affect the ability of iodoacetamide to alkylate factor XIIIa. After reaction with [1-^{14}C]iodoacetamide at 37 °C for 12 or 30 min, aliquots were applied to Whatman 3MM filter paper (1 cm^2), washed and dried as previously described (Curtis et al., 1973), and counted in 2.5 mL of scintillation cocktail (Bio-Safe II, Research Products International Corp.) in a Beckman LS 3801 liquid scintillation system.

Assay of α-Thrombin-Mediated Cleavage of Factor XIIIa. Cleavage of factor XIIIa by α-thrombin was assessed in a manner similar to that described previously (Takahashi et al., 1986). Platelet factor XIII (1.0 μM) in TANEP was activated by 20 nM α-thrombin at 37 °C in the absence of and in the presence of 1.5 or 14 mM $CaCl_2$ for 0 min, 4 min (1.5 mM $CaCl_2$ only), 30 min, and 2 h. Aliquots (20 μL) were quenched at the indicated times by addition of 2 μL of 30 μM PPACK, after which 5 μL of a solution comprised of 0.15 M Tris-HCl, pH 6.8, 40% glycerol, 5% SDS, 0.0075% BPB, and 125 mM DTT was added. The quenched samples were heated at 95 °C for 10 min and immediately transferred to a dry ice–ethanol bath where they remained until gel electrophoresis. Samples (9.5 μL) were applied to an 11.3% polyacrylamide gel prepared with an acrylamide:bis(acrylamide) ratio of 30:0.8 (Laemmli, 1970). The lanes were examined for the presence of the intact factor XIII *a* subunit (M_r 80000), the *a'* subunit (M_r 76000) that results upon α-thrombin-catalyzed release of AP, and the *a'* subunit fragment (M_r 56000) that results from a second α-thrombin-catalyzed cleavage of factor XIIIa (Schwartz et al., 1973).

Results and Discussion

Upon treatment with α-thrombin, platelet factor XIII yielded two activation peptides, AP and AP', which were quantified by HPLC. As observed previously for plasma factor XIII, the yield of AP' was <25% of that for AP, and no substantial difference was observed in the rate of release of the two. AP', the minor activation peptide, may be the result of a variant form of factor XIII present in the pooled blood products used to purify factor XIII. Consistent with this view, α-thrombin-catalyzed cleavage of recombinant platelet factor XIII, the expression product of a single gene, liberated a single activation peptide that cochromatographed with the major activation peptide (AP) from platelet-derived factor XIII. Further work is necessary to determine the difference between the structure of AP and AP'.

A specificity constant (k_{cat}/K_m) of 1.2×10^5 M^{-1} s^{-1} (at pH 7.5, Γ/2 = 0.15, 37 °C, in the absence of added Ca^{2+}) was determined for α-thrombin-catalyzed release of AP from platelet factor XIII (Figure 1A). This value is similar to the specificity constants observed under similar conditions for AP release from plasma factor XIII, $(1.3–1.6) \times 10^5$ M^{-1} s^{-1} (Janus et al., 1983). Moreover, in side by side experiments (data not shown), the time dependencies of the fraction of AP release from platelet and plasma factor XIII were indistinguishable at equivalent concentrations of α-thrombin. Addition of Ca^{2+} had little effect on the specificity constant for the release of AP from platelet factor XIII. AP release in the presence of 14 mM $CaCl_2$ occurred at a rate only slightly less ($k_{cat}/K_m = 0.94 \times 10^5$ M^{-1} s^{-1}) than the rate of AP release in the absence of added $CaCl_2$ (Figure 1). Additionally, recombinant human platelet factor XIII functioned as a substrate for thrombin in a manner similar to factor XIII that was purified from human platelets ($k_{cat}/K_m = 1.1 \times 10^5$ M^{-1} s^{-1} for AP release, Figure 1B). Recombinant and platelet-derived factor XIII also were essentially indistinguishable in experiments where the time dependence of the appearance of factor XIIIa activity was measured (see below). Because of its ready availability, the recombinant protein was used in most of the experiments described in this study.

The similarity of the specificity constants for α-thrombin-catalyzed AP release from platelet and plasma factor XIII is an indication that the *b* subunits of plasma factor XIII do not inhibit α-thrombin-mediated cleavage of AP from the *a* subunits. Although our results indicate that α-thrombin-catalyzed cleavage of AP from platelet and plasma factor XIII proceed at similar rates, slow dissociation of the *b* subunits from plasma factor XIII may limit the rate of generation of active enzyme

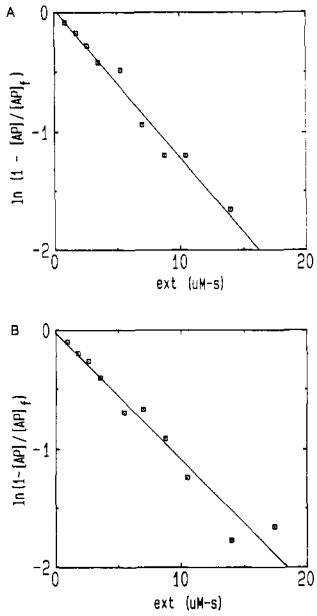


FIGURE 1: (A) Release of AP from human platelet factor XIII. (B) Release of AP from recombinant human platelet factor XIII. Platelet factor XIII (0.10 μM) was activated with 2.9 nM α-thrombin in TANEP buffer. In the above determinations, a least-squares fit of the data (represented by the lines) yielded $k_{cat}/K_m = 1.2 \times 10^5$ M^{-1} s^{-1} for human platelet-derived factor XIII and $k_{cat}/K_m = 1.1 \times 10^5$ M^{-1} s^{-1} for r-factor XIII. In the plots, e, t, [AP], and $[AP]_f$ represent the α-thrombin concentration, time, the concentration of AP at the plotted time, and the final concentration of AP, respectively.

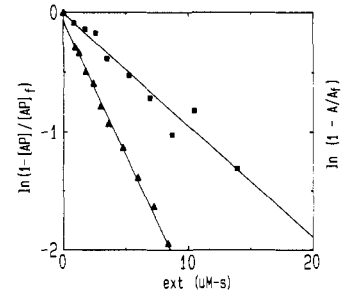


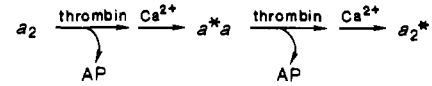
FIGURE 2: α-Thrombin-catalyzed AP release from factor XIII (■) and generation of factor XIIIa activity (▲) in the presence of 14 mM $CaCl_2$. Each time point for the time dependence for generation of factor XIIIa was obtained from separate 125-μL incubates containing 0.10 μM platelet factor XIII, 14 mM $CaCl_2$, 20 nM α-thrombin, and TANEP buffer. At the indicated time, 10 μL of 30 μM PPACK was added (final concentration 2.2 μM). A 100-μL sample of the incubate was then pipetted into a 1.67-mL assay mixture containing 50.2 μL of 0.2 M DTT in 50% glycerol, 55.0 μL of 0.4 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, 20.0 μL of 4 mM dansylcadaverine in 50 mM Tris-HCl, pH 7.5, 1200 μL of 50 mM Bicine–NaOH, pH 9.0, 144.8 μL of TANEP, and 200 μL of 2% N,N'-dimethylcasein in 50 mM Tris-HCl, pH 7.5, and the factor XIIIa activity was measured in the fluorometer. AP release was measured as in Figure 1, except that 14 mM $CaCl_2$ was present in the incubate. The best-fit lines yield values of 2.2×10^5 M^{-1} s^{-1} (eq 9) and 0.94×10^5 M^{-1} s^{-1} (eq 1) for the rate constant for the appearance of activity ($k_{app\,act}$) and the rate constant for AP release (k_{cat}/K_m), respectively.

from the $a_2'b_2$ intermediate and cause factor XIII activity to be generated more slowly from plasma factor XIII than from platelet factor XIII.

It has been proposed elsewhere that the b chains inhibit a-chain cleavage at Arg-a37 of plasma factor XIII in the absence of fibrin (Chung et al., 1974; Greenberg et al., 1987). This proposal was based on observations of the generation of factor XIIIa activity upon treatment of plasma and platelet factor XIII. Although it remains to be established why earlier analyses of the rates of generation of factor XIIIa activity suggested that the b chains inhibit access of α-thrombin to Arg-a37, inactivation of factor XIIIa subsequent to its generation (see below) and slow dissociation of the b subunits from $a_2'b_2$ might have confounded relationships between the appearance of enzymic activity and AP release. In these earlier studies where attempts were made to relate generation of activity to thrombin-catalyzed cleavage of AP, the latter problem was addressed by allowing time for the thrombin-cleaved intermediate $a_2'b_2$ to convert to a_2* prior to determination of enzymic activity. Differences in the stability of $a_2'b_2$ and a_2* under the experimental conditions used in the earlier studies, however, might have produced unrecognized complications in the relationship between the approach of enzymic activity to its final value and the rates of thrombin-catalyzed release of AP from plasma and platelet factor XIII.

To obtain more information regarding the relationship between the initial α-thrombin-catalyzed proteolytic event and the appearance of factor XIIIa activity, the specificity constant for α-thrombin-catalyzed generation of factor XIIIa activity from platelet factor XIII was measured in the presence of 14 mM $CaCl_2$. This level of $CaCl_2$ was determined in separate control experiments to stabilize platelet factor XIIIa activity for the times used to measure the specificity constant for the appearance of catalytic activity. As seen in Figure 2, the specificity constant ($k_{app\,act} = 2.2 \times 10^5$ M^{-1} s^{-1}) for the α-thrombin-catalyzed appearance of platelet factor XIIIa activity

Scheme II



is approximately twice that measured for AP release ($k_{cat}/K_m = 0.94 \times 10^5$ M^{-1} s^{-1}) from platelet factor XIII under the same conditions in the presence of 14 mM $CaCl_2$. Thrombin-independent calcium-promoted activation of factor XIII was found to be <8% of the total over the time span in which α-thrombin-catalyzed appearance of factor XIIIa activity was measured, ensuring that this phenomenon (Credo et al., 1978) made only a small contribution to the measured rate of generation of catalytic activity.

It might be argued that the disparity between the rates of proteolysis and the generation of activity is inconsistent with the accepted view that AP release is on the reaction pathway for generation of factor XIIIa activity. It is possible, however, to account for the 2-fold difference in rates without excluding AP release from the reaction pathway. Consider Scheme II where a*a and a_2* are active platelet factor XIII dimers from which one AP and two APs, respectively, have been cleaved. If f is the fraction of total AP released, then

$$f = 1 - e^{-kt} \tag{1}$$

where $k = (k_{cat}/K_m)$[α-thrombin] and K_m is much larger than the initial concentration of factor XIII. At a given extent of AP release (f), the fractions of molecules with zero (f_{a_2}), one (f_{a^*a}), and two ($f_{a_2^*}$) AP cleavages are given by the binomial distribution. Thus

$$f_{a_2} = (1 - f)^2 \tag{2}$$

$$f_{a^*a} = 2f - 2f^2 \tag{3}$$

$$f_{a_2^*} = f^2 \tag{4}$$

where the right side of eq 2–4 respectively represents the probability that a molecule will have undergone either zero, one, or two AP cleavages. If a*a and a_2* possess equal specific enzymatic activities (c), the observed activity (A) at any time

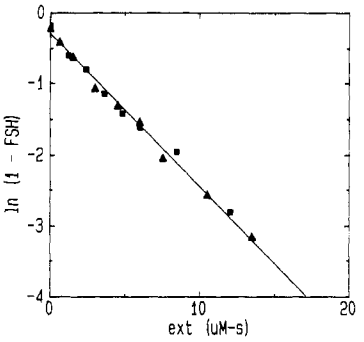


FIGURE 3: Kinetics for exposure of the active-site thiol group of platelet factor XIII. Incubates containing 1.26 μM platelet factor XIII and 14 mM $CaCl_2$ in TANEP were activated with 5 nM (▲) or 20 nM (■) α-thrombin. At each time point, aliquots were pipetted into vials containing amounts of 30 μM PPACK and 420 μM [1-^{14}C]iodoacetamide to yield final concentrations of 2.5 and 39.8 μM, respectively. Alkylation was performed for 30 min (20 nM α-thrombin experiment) or 12 min (5 nM α-thrombin experiment). Aliquots (10 μL) were pipetted onto filter papers and treated as described under Materials and Methods. The fraction incorporation represents averages of two individual determinations in the 20 nM α-thrombin experiment and three determinations in the 5 nM α-thrombin experiment. Incorporation at 0 min was determined by alkylation for 12 min immediately after addition of 14 mM $CaCl_2$ to 1.26 μM platelet factor XIII containing no thrombin. FSH is the fraction of exposable thiols that have been exposed at time t, and 1 - FSH is the fraction of exposable thiols that have not yet been exposed at time t. FSH was calculated from the ratio of carbamylmethyl incorporation at time t to the limiting carbamylmethyl incorporation observed after complete AP release.

t after addition of α-thrombin to a solution containing factor XIII at an initial concentration $[XIII]_0$ is

$$A = (f_{a^*a} + f_{a_2^*})[XIII]_0 c \quad (5)$$

Since the final activity (A_f) is given by the relationship

$$A_f = [XIII]_0 c \quad (6)$$

it follows from eq 1–5 that

$$A/A_f = 1 - e^{-2kt} \quad (7)$$

and

$$-\ln (1 - A/A_f) = 2kt = 2(k_{cat}/K_m)[\alpha\text{-thrombin}]t \quad (8)$$

Since the specificity constant describing the appearance of activity ($k_{app\,act}$) was determined by fitting the time dependence of the generation of factor XIIIa activity to the equation

$$-\ln (1 - A/A_f) = k_{app\,act}[\alpha\text{-thrombin}]t \quad (9)$$

$$k_{app\,act} = 2(k_{cat}/K_m) \quad (10)$$

Thus, the disparity between the rate constants describing release of AP from platelet factor XIII and generation of factor XIIIa activity can be reconciled, if the a^*a heterodimer from which only one AP has been cleaved is as active as the a_2^* dimer that has lost both activation peptides.

The relationship between the rate of exposure of the active-site thiol group in the a subunit (Cys-314) to the rate of release of AP and generation of factor XIIIa activity was characterized by studying the reaction of [1-^{14}C]iodoacetamide with α-thrombin-treated factor XIII. Previous studies have demonstrated that iodoacetamide alkylates the active-site thiol group in plasma and platelet factor XIIIa (Curtis et al., 1973; Chung et al., 1974). Measurements in the present study of the dependence of the extent of alkylation of factor XIII on the incubation time with α-thrombin yielded a second-order rate constant of 2.2×10^5 M^{-1} s^{-1} for α-thrombin-catalyzed exposure of the active-site thiol group of platelet factor XIII. The correspondence of the rate of exposure of Cys-314 and

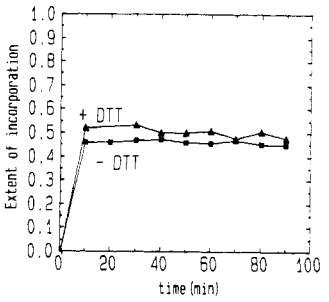


FIGURE 4: Time dependence of the reaction of activated platelet factor XIIIa with [1-^{14}C]iodoacetamide. A 50-μL incubate containing platelet factor XIII zymogen was activated by treatment with 20 nM α-thrombin for 30 min at 37 °C in TANEP containing 14 mM $CaCl_2$. The α-thrombin was then quenched by addition of 5 μL of 30 μM PPACK (final concentration 2.7 μM). Alkylation was initiated by addition of 10 μL of 840 μM [1-^{14}C]iodoacetamide (final concentration 130 μM) at 37 °C both without (■) and with (▲) 6.4 mM DTT present. Aliquots (5 μL) were pipetted onto filter papers at the indicated times for determination of radioactivity incorporated into factor XIIIa as described under Materials and Methods.

the appearance of activity (Figure 3) suggests that, like catalytic competence, exposure of the active-site thiol group is not altered by release of the second molecule of AP from the dimeric zymogen. Still, three situations could account for the equal enzymic activities of a^*a and a_2^*. (i) Two operationally independent active-site thiols are produced upon release of one AP from the dimeric zymogen. (ii) Two thiols linked by negatively cooperative interactions become exposed upon release of one AP so that reaction of one thiol with a substrate or an alkylating reagent causes the second thiol to become inaccessible.[2] (iii) Release of the first molecule of AP triggers a conformational rearrangement that exposes one active site, but negatively cooperative subunit interactions prevent exposure of a second active site when the second molecule of AP is released. The observation of a limiting incorporation of only one carbamylmethyl group per dimer (Figure 4), which confirms previous reports of half-of-the-sites reactivity of factor XIIIa,[3] excludes the first alternative and implicates the existence of negatively cooperative interactions between the active sites as stipulated by alternatives ii and iii. These alternatives suggest that negatively cooperative subunit interactions, triggered by either AP release or alkylation of one of the active-site thiol groups, prevent alkylation of both active-site thiol groups by [1-^{14}C]iodoacetamide. The 2-fold difference between the rate constants for AP release and the generation of activity is not easily reconciled without postulating cooperative subunit interactions. Since equal rate constants for AP release and generation of activity should have been observed if the functional factor XIII in a 50% impure preparation were comprised of independent, noninteracting subunits, it is difficult to attribute the stoichiometry of the alkylation reaction to an undetected 50% impurity in factor XIII. In summary, our observations suggest that either (i) release of one molecule of AP per platelet factor XIII dimer triggers subunit interactions that cause both active-site thiol groups to become exposed with one thiol group becoming unreactive upon alkylation of the other or (ii) release of one molecule of

[2] This possibility is similar to that proposed by Levitski et al. (1971) to account for half-of-the-sites reactivity with cytidine triphosphate synthetase.

[3] Chung et al. (1974) reported half-of-the-sites reactivity with platelet factor XIII using [1-^{14}C]iodoacetamide as an alkylating agent. Seelig and Folk (1980) reported half-of-the-sites reactivity under certain conditions with plasma factor XIII, using [^{14}C]- and [^{3}H]iodoacetic acid as alkylating agents.

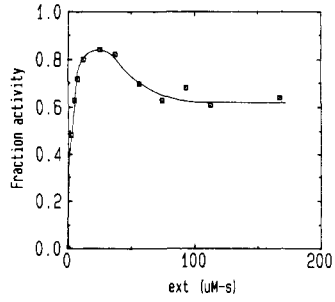


FIGURE 5: Generation and decay of platelet factor XIIIa activity in the presence of 1.5 mM $CaCl_2$. Platelet factor XIII (0.10 μM) was activated with 20 nM α-thrombin in the presence of 1.5 mM $CaCl_2$ in TANEP. At the indicated times, activity was measured as described in the legend to Figure 2. The velocity corresponding to 100% activity was determined after rapid activation with 220 nM α-thrombin for 5 min. Measurements of the initial rates for the appearance of activity yielded a value of 2.6×10^5 M^{-1} s^{-1} for $k_{app\,act}$.

AP per platelet factor XIII dimer triggers subunit interactions that expose one thiol and prevent simultaneous exposure of both even after the second molecule of AP is released.

As mentioned earlier, 14 mM $CaCl_2$ was required in the kinetic studies of the generation of factor XIIIa to stabilize factor XIIIa activity. At a lower, physiological $CaCl_2$ concentration of 1.5 mM, measurements of the initial rate of appearance of factor XIIIa activity yielded a rate constant (2.6×10^5 M^{-1} s^{-1}) similar to that obtained with 14 mM $CaCl_2$ (data not shown), but at 1.5 mM $CaCl_2$, the factor XIIIa activity decayed to a level of approximately 60% activity (Figure 5), where 100% activity was obtained by rapidly activating factor XIII with a higher concentration of α-thrombin. An experiment was also performed in which platelet factor XIII, activated with α-thrombin in the absence of $CaCl_2$, lost all activity after 2 h of continued incubation. It was of interest to determine whether loss of activity in the absence of stabilizing concentrations of $CaCl_2$ could be correlated with the secondary thrombin cleavage that has been reported to split the a' subunit into 56- and 24-kDa fragments (Schwartz et al. 1973; Takahashi et al., 1986). A gel electrophoresis study of the appearance of various thrombin cleavage products revealed that such a correlation could not be made. Even after incubation with 20 nM α-thrombin for 2 h in the absence of $CaCl_2$, conditions resulting in complete loss of platelet factor XIIIa activity, most of the factor XIII was present as the 76-kDa a' subunit, with barely detectable amounts of the 56-kDa fragment apparent. Hence, it is likely that the inactivation of factor XIIIa seen at low $CaCl_2$ levels (1.5 mM) under the conditions used in this study is caused by a process other than the secondary thrombin cleavage that produces the 56-kDa fragment.[4]

Although the function of platelet factor XIII in blood clotting is unclear (Lopaciuk et al., 1976; Francis & Marder, 1987), it is nonetheless valuable to study its activation because the properties of the a subunits apart from the b subunits can be investigated and because, after the b subunits dissociate from plasma factor XIII, the active forms of plasma and platelet factor XIII are identical. We have shown that α-thrombin releases AP from platelet factor XIII and plasma factor XIII at similar rates in the absence of fibrin, indicating that the b subunits do not sterically hinder the access of thrombin to the scissile bond at Arg-a37. Our work has also demonstrated that cleavage of one AP is enough to generate full activity from platelet factor XIII and that the second AP cleavage does not appear to increase the reactivity of factor XIIIa toward iodoacetamide or the competence of factor XIIIa to catalyze incorporation of dansylcadaverine into casein. This conclusion, however, does not necessarily imply that the first thiol exposed in a platelet factor XIII dimer remains permanently active. Negatively cooperative interactions between two subunits in a dimeric enzyme (such as factor XIIIa) might cause the active-site residues to oscillate alternately between accessible and inaccessible states, as proposed by Seydoux et al. (1974) in their model for negatively cooperative interactions. Further work is necessary to establish the existence and possible physiological relevance of negatively cooperative interactions in factor XIIIa catalysis.

[4] Further studies are necessary to characterize the inactivation process. It should be noted, however, that the relative contribution of the secondary proteolytic cleavage should be a function of the thrombin concentration and other experimental conditions. For example, a thrombin concentration much higher than that used in this study might cause the thrombin-promoted secondary cleavage to become the predominant mode of inactivation of factor XIIIa in the absence of Ca^{2+}.

REFERENCES

Alter, T., & Kawasaki, G. (1982) *J. Mol. Appl. Genet. 1*, 419–434.

Bohn, H. (1972) *Ann. N.Y. Acad. Sci. 202*, 256–272.

Chung, S. I., Lewis, M. S., & Folk, J. E. (1974) *J. Biol. Chem. 249*, 940–950.

Cooke, R. D., & Holbrook, J. J. (1974) *Biochem. J. 141*, 79–84.

Credo, R. B., Curtis, C. G., & Lorand, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4234–4237.

Credo, R. B., Curtis, C. G., & Lorand, L. (1981) *Biochemistry 20*, 3770–3778.

Curtis, C. G., & Lorand, L. (1976) *Methods Enzymol. 45*, 177–191.

Curtis, C. G., Stenberg, P., Chou, C.-H. J., Gray, A., Brown, K. L., & Lorand, L. (1973) *Biochem. Biophys. Res. Commun. 52*, 51–56.

Curtis, C. G., Brown, K. L., Credo, R. B., Domanik, R. A., Gray, A., Stenberg, P., & Lorand, L. (1974) *Biochemistry 13*, 3774–3780.

Francis, C. W., & Marder, V. J. (1987) *J. Clin. Invest. 80*, 1459–1465.

Gerth, C., Roberts, W. W., & Ferry, J. D. (1974) *Biophys. Chem. 2*, 208–217.

Greenberg, C. S., Achyuthan, K. E., & Fenton, J. W., II (1987) *Blood 69*, 867–871.

Ichinose, A., Hendrickson, L. E., Fujikawa, K., & Davie, E. W. (1986) *Biochemistry 25*, 6900–6906.

Jansen, J. W. C. M., Haverkate, F., Koopman, J., Nieuwenhuis, H. K., Kluft, C., & Boschman, Th. A. C. (1987) *Thromb. Haemostasis 57*, 171–175.

Janus, T. J., Lewis, S. D., Lorand, L., & Shafer, J. A. (1983) *Biochemistry 22*, 6269–6272.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Levitski, A., Stallcup, W. B., & Koshland, D. E., Jr. (1971) *Biochemistry 10*, 3371–3378.

Lewis, S. D., Janus, T. J., Lorand, L., & Shafer, J. A. (1985) *Biochemistry 24*, 6772–6777.

Lopaciuk, S., Lovette, K. M., McDonagh, J., Chuang, H. Y. K., & McDonagh, R. P. (1976) *Thromb. Res. 8*, 453–465.

Lorand, L., Gray, A. J., Brown, K., Credo, R. B., Curtis, C. G., Domanik, R. A., & Stenberg, P. (1974) *Biochem. Biophys. Res. Commun. 56*, 914–922.

Lorand, L., Losowsky, M. S., & Miloszewski, K. J. M. (1980) *Prog. Hemostasis Thromb. 5*, 245–290.

Roberts, W. W., Kramer, O., Rosser, R. W., Nestler, F. H. M., & Ferry, J. D. (1974) *Biophys. Chem. 1*, 152–160.

Russell, D. W., Smith, M., Cox, D., Williamson, V. M., & Young, E. W. (1983) *Nature 304*, 652-654.
Sakata, Y., & Aoki, N. (1980) *J. Clin. Invest. 65*, 290-297.
Sakata, Y., & Aoki, N. (1982) *J. Clin. Invest. 69*, 536-542.
Schwartz, M. L., Pizzo, S. V., Hill, R. L., & McKee, P. A. (1971) *J. Biol. Chem. 246*, 5851-5854.
Schwartz, M. L., Pizzo, S. V., Hill, R. L., & McKee, P. A. (1973) *J. Biol. Chem. 248*, 1395-1407.
Seelig, G. F., & Folk, J. E. (1980) *J. Biol. Chem. 255*, 9589-9593.
Seydoux, F., Malhotra, O. P., & Bernhard, S. A. (1974) *CRC Crit. Rev. Biochem. 2*, 227-257.
Shafer, J. A., & Higgins, D. L. (1988) *CRC Crit. Rev. Clin. Lab. Sci. 26*, 1-41.
Takagi, T., & Doolittle, R. F. (1974) *Biochemistry 13*, 750-756.
Takahashi, N., Takahashi, Y., & Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8019-8023.
Tamaki, T., & Aoki, N. (1981) *Biochim. Biophys. Acta 661*, 280-286.

Molecular Cloning of cDNA for Proteasomes (Multicatalytic Proteinase Complexes) from Rat Liver: Primary Structure of the Largest Component (C2)†

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ABSTRACT: Proteasomes (multicatalytic proteinase complexes) from rat liver are composed of at least 13 nonidentical components [Tanaka, K., Yoshimura, T., Ichihara, A., Ikai, A., Nishigai, M., Morimoto, M., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K., & Takagi, T. (1988) *J. Mol. Biol. 203*, 985-996]. The nucleotide sequence of one major component (C2) of the proteasomes has been determined from a recombinant cDNA clone isolated by screening a rat liver cDNA library with a mixture of synthetic deoxyribonucleotides as a probe. The sequence was composed of 1174 nucleotides including a coding region for the entire protein and noncoding regions of both the 5′- and 3′-sides. The polypeptide deduced from the open reading frame consisted of 263 amino acid residues, and its molecular weight was calculated to be 29 516. The partial amino acid sequences of several fragments (approximately 45% of the total residues), which were obtained by cleavage of C2 with lysyl endopeptidase and cyanogen bromide, were determined by automated Edman degradation and found to be in complete accordance with those deduced from the cDNA sequence. The amino acid composition of C2, determined by chemical analysis, was also consistent with that deduced from the cDNA sequence, indicating that the cloned cDNA actually encoded component C2. Computer analysis revealed little structural similarity of C2 to other proteins reported so far. Northern blot hybridization analyses showed that the mRNA encoding this novel protein C2 was expressesd in all the rat tissues examined and in a variety of eukaryotic organisms such as amphibia, birds, and mammals with slight species-specific differences in size. This finding suggests that the gene encoding proteasomes has been conserved in eukaryotes during evolution.

During investigations on the intracellular proteolytic system, very large proteases have been found in mammalian cells [for recent review, see Rivett (1989)]. This enzyme was recently named "multicatalytic proteinase", because of its unique catalytic properties, that is, cleavage of peptide bonds on the carboxyl side of basic, hydrophobic, or acidic amino acid residues (Wilk & Orlowski, 1983; Dahlmann et al., 1985; Tanaka et al., 1986a). These multiple proteolytic activities seem to be manifested at independent catalytic sites within the single large enzyme complex. We demonstrated that these multicatalytic proteinases are widely distributed in a variety of eukaryotic cells ranging from human to yeast cells (Tanaka et al., 1988a). Recently, these proteinases have been reported to be responsible for ATP-stimulated breakdown of intracellular proteins in mammalian cells (McGuire et al., 1988; Tanaka & Ichihara, 1988; Driscoll & Goldberg, 1989; Matthews et al., 1989). In addition, we showed that these enzymes are present in the nucleus as well as in the cytoplasm of various cells (Arrigo et al., 1988; Tanaka et al., 1989).

On the other hand, ring-shaped 19S-22S particles have been found in a wide variety of eukaryotic organisms and shown to have common structural characteristics, such as an unusually large size, a unique symmetrical shape, and a multisubunit structure (Martins de Sa et al., 1986, Arrigo et al.,

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