Role of Calcium Ion in the Generation of Factor XIII Activity†

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ABSTRACT: The involvement of calcium ion in the activation of both plasma factor XIII (a_2b_2) and platelet factor XIII (a_2) was investigated. The second-order dependence of the rate constant for exposure of the active-site thiol group of α-thrombin-cleaved plasma factor XIII ($a_2'b_2$) on the concentration of calcium ion suggested that the binding of two calcium ions is required for transformation of the $a_2'b_2$ tetramer to enzymatically active factor XIIIa. Fibrinogen, previously reported to lower the calcium ion concentration required for efficient activation of $a_2'b_2$ [Credo, R. B., Curtis, C. G., & Lorand, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4234–4237], was found in the present study to increase the rate of exposure of the active-site thiol group. Whereas calcium ion is required for exposure of the active-site thiol group in cleaved plasma factor XIII ($a_2'b_2$), exposure of an active-site thiol group in cleaved platelet factor XIII (a_2') occurs in the absence of calcium ion. The rate constant (2.2×10^5 M^{-1} s^{-1}) for α-thrombin-catalyed exposure of the active-site thiol group of platelet factor XIII zymogen (a_2) in the presence of calcium ion was greater than the rate constant (0.7×10^5 M^{-1} s^{-1}) determined in the absence of calcium ion. This difference in rate constants was shown to be consistent with the observation that in the presence of calcium ion, cleavage of one subunit of the a_2 dimer to form an $a'a$ heterodimer results in equal accessibility of the active-site thiol groups on both the cleaved and uncleaved subunits of the heterodimer to alkylation by [1-^{14}C]iodoacetamide. However, in the absence of calcium ion, only the thiol group on the cleaved subunit in the $a'a$ heterodimer is exposed, with the uncleaved subunit remaining unreactive toward [1-^{14}C]iodoacetamide. Thus, during the activation of factor XIII, calcium ion behaves as an allosteric effector that induces an intersubunit interaction to expose the active-site thiol group on the uncleaved a subunit of a once-cleaved, $a'a$ heterodimer.

Factor XIIIa (fibrin-stabilizing factor) is a transglutaminase that functions in the terminal stage of the blood coagulation cascade, wherein it catalyzes formation of isopeptide cross-links between the fibrin repeating units that comprise the fibrin matrix of blood clots. Factor XIIIa also cross-links certain plasma proteins, such as fibronectin and α_2-antiplasmin, to the fibrin clot (Folk, 1980; Lorand et al., 1980; McDonagh, 1987; Sakata & Aoki, 1980, 1982; Jansen et al., 1987; Mosher, 1975; Mosher & Schad, 1979). The interfibrin cross-links endow fibrin clots with increased mechanical strength, whereas the cross-links between fibrin and fibronectin and α_2-antiplasmin, respectively, may serve to tether the fibrin clot to the exposed subendothelium at the site of injury (Mosher, 1975; Mosher & Schad, 1979) and delay the onset of plasmin-catalyzed dissolution of the fibrin clot until the occurrence of substantial permanent tissue repair (Sakata & Aoki, 1980, 1982; Jansen et al., 1987). Individuals unable to form active factor XIIIa suffer from a delayed bleeding syndrome (Duckert, 1972; Bohn, 1978; Lorand et al., 1980) after trauma and also are at increased risk for cerebral hemorrhage (Duckert, 1972; Lorand et al., 1980).

Factor XIIIa can be generated from two zymogen forms of the enzyme, namely, plasma factor XIII (an a_2b_2 tetramer that circulates freely in plasma) and platelet factor XIII (an a_2 dimer that is found primarily in platelets and the placenta) (Bohn, 1972; Schwartz et al., 1971, 1974; Lorand et al., 1974). Activation of plasma factor XIII is initiated by α-thrombin-catalyzed proteolysis at Arg-37 in the a subunits to yield the cleaved a' subunit and the 37-aminoacyl activation peptide (AP)[1] (Takagi & Doolittle, 1974; Ichinose et al., 1986). Exposure of the cleaved zymogen ($a_2'b_2$) to calcium ion (>10 mM) results in (i) substantial dissociation of the b subunits from $a_2'b_2$, (ii) exposure of an active-site thiol group in the a subunit (Cys-a314), and (iii) generation of factor XIIIa enzymic activity (Curtis et al., 1973, 1974; Cooke & Holbrook, 1974; Chung et al., 1974). Exposure of the active-site thiol group and generation of factor XIIIa activity can be separately assessed via titration of the active-site thiol group with alkylating agents (Curtis et al., 1973; Chung et al., 1974), and measurements of the rate of factor XIIIa catalyzed incorporation of dansylcadaverine, a fluorescent lysine analogue, into *N*,*N*′-dimethylcasein (Curtis & Lorand, 1976). Dissociation of the b subunits appears to be regulated by fibrinogen. In the presence of plasma levels of fibrinogen, the calcium ion requirement for dissociation of the b subunits and exposure of Cys-a314 is lowered to the calcium ion concentration found in plasma (1–1.5 mM) (Credo et al., 1978, 1981). Interestingly, α-thrombin-catalyzed release of AP from plasma factor XIII is promoted by its natural substrate, fibrin (Lewis et al., 1985; Greenberg et al., 1987). This promoting effect may prevent wasteful and perhaps deleterious generation of factor XIIIa in the absence of fibrin.

In the present paper, we report studies indicating that exposure of the active-site thiol group after thrombin-catalyzed

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[1] Abbreviations: AP, activation peptide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); 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; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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release of AP from plasma factor XII (a_2b_2) is dependent upon the binding of two calcium ions to the $a_2'b_2$ tetramer, whereas α-thrombin-catalyzed release of AP from platelet factor XIII (a_2) results in the exposure of the active-site thiol group in the absence of calcium ion, even though addition of calcium ion is required for expression of enzymatic activity. Moreover, we show that in the absence of calcium ion, release of one molecule of AP from the a_2 dimer results in selective exposure of the active-site thiol group in the cleaved subunit and that calcium ion causes the active-site thiol group in the uncleaved subunit to become as accessible as the thiol group in the cleaved subunit.

Materials and Methods

Materials. Human plasma factor XIII and plasma factor XIII *b* subunits were prepared according to procedures previously described (Lorand et al., 1981). Recombinant human platelet factor XIII was a gift from Zymogenetics, Inc. The properties of recombinant human platelet factor XIII and human platelet-derived factor XIII have been previously reported to be similar (Hornyak et al., 1989). Fibrinogen was prepared by precipitating human plasma with β-alanine as previously described (Lewis & Shafer, 1984).

Generation of $a_2'b_2$ and Appearance of the Active-Site Thiol Group. A 100-μL aliquot of plasma factor XIII was mixed with a solution containing 840 μM [1-^{14}C]iodoacetamide (NEN, 24.1 mCi/mmol) in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5 at 37 °C. At t = 0, a small volume of α-thrombin diluted in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5, was added such that the final composition of the reaction mixture was 1.21 μM plasma factor XIII, 45 μM [1-^{14}C]iodoacetamide, and 19 nM α-thrombin. Incubation of plasma factor XIII with 19 nM α-thrombin under these conditions results in greater than 99% cleavage of AP after 30-min incubation (Janus et al., 1983). The α-thrombin activity was quenched after 30 min of incubation by the addition of 11 μL of 30 μM PPACK (final molar ratio of PPACK to α-thrombin = 160). A 5-μL sample of the incubate was pipetted onto a 1 cm^2 piece of Whatman 3MM filter paper at this time and placed in a 10% trichloroacetic acid (TCA) bath to determine the fraction of active-site thiol group exposure prior to addition of calcium ion. A small volume of $CaCl_2$ solution (0.04–0.4 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, depending upon the final concentration of $CaCl_2$ desired) was added to the incubate containing α-thrombin-cleaved factor XIII, and 5-μL samples were pipetted onto filter papers at various times thereafter. Immediately after being spotted, the filter papers were placed into a bath of 10% TCA and washed as previously described (Curtis et al., 1973, 1974). The amount of radioactively labeled factor XIIIa precipitated on the filter papers was determined as noted previously (Hornyak et al., 1989).

In the experiment with fibrinogen, a 61.3-μL incubate with a composition of 2.24 μM plasma factor XIII, 82 μM [1-^{14}C]iodoacetamide, and 35 nM α-thrombin was used to cleave the factor XIII. After addition of the PPACK quench, 51.7 μL of 23.2 μM fibrinogen (previously dialyzed against 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5) was added to the cleaved factor XIII to standardize conditions to those of the experiments not containing fibrinogen. Calcium ion was added and the experiment completed as described above. No clotting of fibrinogen was observed during the time course of the experiment, consistent with complete activation of the α-thrombin by PPACK prior to addition of the fibrinogen.

Rate of Exposure of the Active-Site Thiol Group of Platelet Factor XIII. The rate of exposure of the active-site thiol group of platelet factor XIII was measured essentially as described by Hornyak et al. (1989). Incubates (300 μL) of 1.26 μM platelet factor XIII in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5, were prepared. After addition of $CaCl_2$ to the appropriate concentration (1.5 or 14 mM), a 20-μL aliquot was withdrawn and incubated with [1-^{14}C]iodoacetamide to determine the amount of incorporation before addition of α-thrombin. α-Thrombin was then added to the solutions (final concentrations were 1.20 μM platelet factor XIII, 15.3 nM α-thrombin, and 0, 1.4, or 13.3 mM $CaCl_2$ depending upon the experiment), and exposure of the active-site thiol group was measured as previously described. As a control, an experiment was performed with 5.5 mM EDTA in the medium to eliminate the possibility that a small amount of free calcium ion adventitiously present in the buffer containing no added calcium ion was responsible for the observed exposure of the platelet factor XIII active-site thiol group in the absence of added calcium ion.

Electrophoretic Determination of Subunits Containing an Exposed Active-Site Thiol Group and Quantification of Results. Two 200-μL incubates at 37 °C were prepared in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.1% PEG, pH 7.5, with final concentrations of 2.0 μM platelet factor XIII, 20 nM α-thrombin, and either 0 or 14 mM $CaCl_2$. The $CaCl_2$ was added in a small volume from a solution of 0.4 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, immediately before addition of α-thrombin. Aliquots (20 μL) were removed from the incubate at various times after addition of α-thrombin and added to 0.5-mL polyethylene tubes each containing 2.0 μL of 30 μM PPACK (final concentration = 2.4 μM) and 2.3 μL of 840 μM [1-^{14}C]iodoacetamide (final concentration = 80 μM). Incorporation of [1-^{14}C]iodoacetamide was allowed to occur at 37 °C for 10 min before dilution of the [1-^{14}C]iodoacetamide by the addition of 2.1 μL of 0.1 M unlabeled iodoacetamide (final concentration = 8.0 mM). After incubation at 37 °C for 10 min, 1.4 μL of 2 M β-mercaptoethanol was added and the incubation continued for an additional 10 min. The mixture was then quenched with the addition of 6.95 μL of SDS-reducing quench buffer (initial concentration of quench buffer = 0.15 M Tris-HCl, 40% glycerol, 5% SDS, 0.0075% bromophenol blue, and 125 mM DTT, pH 6.8) and heated at 95 °C for 10 min. The samples were stored at −20 °C prior to gel electrophoresis. For quantification of the incorporation of [1-^{14}C]iodoacetamide in the *a* and *a'* subunits of platelet factor XIII, the same procedure was followed except that the incubate consisted of 5.0 μM recombinant human platelet factor XIII, 14 mM $CaCl_2$, and 20 nM α-thrombin in a buffer of 33 mM HEPES–NaOH, pH 7.5, 30 mM Tris–acetate, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and 0.03% PEG 8000. Also, the [1-^{14}C]iodoacetamide/PPACK incubate contained 0.61 mM DTT. For a zero time point, a 20-μL incubate was prepared without α-thrombin but otherwise identical in concentration with the 200-μL incubate described above. After addition of $CaCl_2$ to 14 mM at t = 0 with the mixture equilibrated at 37 °C, the incorporation procedure described above (beginning with the addition of PPACK and [1-^{14}C]-iodoacetamide) was performed.

Separation and Analysis of a and a' Subunits. The *a* and *a'* subunits (M_r = 83K and 79K, respectively) were separated by using SDS–PAGE with a gel of 7.5% polyacrylamide. The subunit bands were visualized by staining with Coomassie blue, and the gel was dried on Whatman 3MM filter paper. Autoradiography was performed by exposing film to the gel at −70 °C to detect radioactivity present in the bands. Following development of the autoradiogram, a soft laser scanning

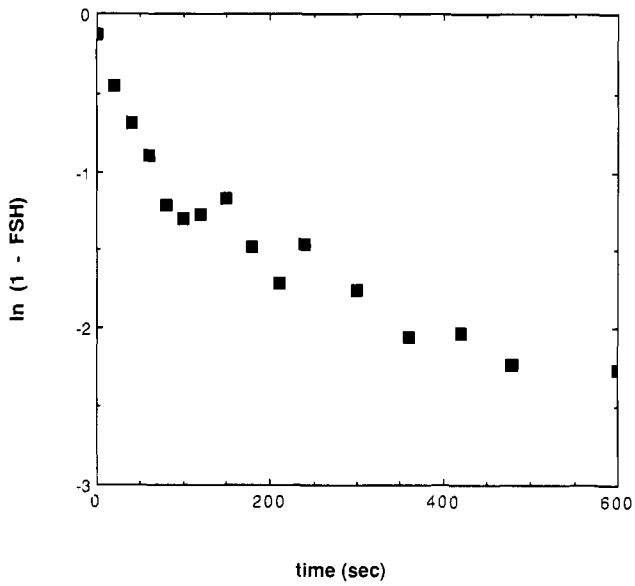

FIGURE 1: Time dependence of exposure of the active-site thiol group of $a_2'b_2$. Cleaved plasma factor XIII ($a_2'b_2$) at a concentration of 1.01 μM was activated by 12 mM $CaCl_2$ in the presence of 38.2 μM [1-^{14}C]iodoacetamide. Samples of the reaction mixtures were precipitated on filter papers to quantify covalent incorporation of iodoacetamide into protein as described under Materials and Methods. FSH is the fraction of thiol group that is exposed and accessible to alkylation by [1-^{14}C]iodoacetamide at the sampling time. The limiting value of [1-^{14}C]iodoacetamide incorporation into factor XIIIa is one [1-^{14}C]carbamylmethyl group per factor XIIIa dimer.

densitometer (Zeineh, Model SL-TRFF, Biomed Instruments, Inc.) in conjunction with an Apple IIe computer was used to quantify incorporation of radiochemical label in subunit bands on the autoradiogram.

RESULTS

Exposure of the Active-Site Thiol Group in Cleaved Plasma Factor XIII ($a_2'b_2$). The time dependence of the exposure of the active-site thiol group subsequent to α-thrombin-catalyzed release of AP from plasma factor XIII (a_2b_2) was monitored at several calcium ion concentrations. Plots of ln (1 - FSH), where FSH is the fraction of thiol group that is exposed and accessible to alkylating reagent (RI) at the sampling time, versus time were linear only to the first 50–80% of the reaction, suggesting that exposure of the active-site thiol group may not be a one-step irreversible first-order process. One such plot is illustrated in Figure 1.

In accord with this observation and previous work (Curtis et al., 1974), we envisioned the following reaction pathway for calcium ion induced exposure of the active-site thiol group:[2]

$$a_2'b_2 + n\mathrm{Ca}^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$\underset{\text{unexposed thiol}}{a_2'b_2{:}(\mathrm{Ca}^{2+})_n} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{exposed thiol}}{a_2'{:}(\mathrm{Ca}^{2+})_n} + (b_2 \text{ or } 2b)$$

[2] There are conflicting conclusions regarding the state of association of the b chains liberated concomitantly with zymogen activation. Chung et al. (1974) suggest (on the basis of gel filtration evidence) that free b chains exist as a b_2 dimer, but a recent hydrodynamic and electron microscopy study (Carrell et al., 1989) instead concludes that the monomeric form is most likely. The validity of the latter conclusion needs to be confirmed, however, since the interpretation of molecular dimensions in this study has been questioned by Bishop et al. (1990), who present arguments indicating that the conclusions of Carrell et al. (1989) regarding the state of association of the a chains may be incorrect due to a flawed interpretation of molecular dimensions.

At the indicated incubation times, the exposed active-site thiol was quantified and labeled by alkylation with [1-^{14}C]iodoacetamide (RI) via the reaction:

$$a_2'{:}(\mathrm{Ca}^{2+})_n + \mathrm{RI} \xrightarrow{\text{very fast}} \mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n + \mathrm{I}^- + \mathrm{H}^+$$

This reaction was assumed to be fast relative to the rate of exposure of the active-site thiol group, since the extent of labeling of the active-site thiol group after various incubation times with calcium ion was unaltered by doubling the concentration of [1-^{14}C]iodoacetamide used to label the exposed thiol group (data not shown).

Early in the reaction [before reverse reactions (k_{-2}) become significant], the velocity of the reaction can be written as

$$v = \mathrm{d}[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]/\mathrm{d}t = k_2[a_2'b_2{:}(\mathrm{Ca}^{2+})_n] \quad (1)$$

If $a_2'b_2{:}(\mathrm{Ca}^{2+})_n$ is at steady-state during the early part of the reaction, then

$$\mathrm{d}[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]/\mathrm{d}t = [k_1k_2/(k_{-1} + k_2)][a_2'b_2][\mathrm{Ca}^{2+}]^n \quad (2)$$

If

$$[a_2'b_2{:}(\mathrm{Ca}^{2+})_n] \ll [a_2'b_2] \text{ and } [\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n] \ll [a_2'b_2] \quad (2a)$$

then

$$\mathrm{d}[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]/\mathrm{d}t = -\mathrm{d}[a_2'b_2]/\mathrm{d}t \quad (3)$$

and

$$\mathrm{d}[a_2'b_2]/[a_2'b_2] = -[k_1k_2/(k_{-1} + k_2)][\mathrm{Ca}^{2+}]^n\,\mathrm{d}t \quad (4)$$

Integrating from time = 0 to t:

$$[a_2'b_2]_t/[a_2'b_2]_0 = \exp\{-[k_1k_2/(k_{-1} + k_2)][\mathrm{Ca}^{2+}]^n t\} \quad (5)$$

From eq 2a and 3, it follows that

$$[a_2'b_2]_t/[a_2'b_2]_0 + [\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_t/[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_f = 1 \quad (6)$$

and thus

$$[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_t/[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_f = 1 - \exp\{-[k_1k_2/(k_{-1} + k_2)][\mathrm{Ca}^{2+}]^n t\} \quad (7)$$

Hence:

$$\ln(1 - \mathrm{FSH}) = -k_{\mathrm{obs}}t \quad (8)$$

where

$$\mathrm{FSH} = [\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_t/[\mathrm{R}a_2'{:}(\mathrm{Ca}^{2+})_n]_f \quad (9)$$

$$k_{\mathrm{obs}} = k[\mathrm{Ca}^{2+}]^n \quad (10)$$

and

$$k = k_1k_2/(k_{-1} + k_2) \quad (11)$$

Then

$$\log k_{\mathrm{obs}} = \log k + n \log [\mathrm{Ca}^{2+}] \quad (12)$$

A plot of log k_{obs} and log [Ca^{2+}] should yield the number of calcium ions involved in b-chain dissociation and exposure of the active-site thiol group of $a_2'b_2$.

Figure 2 shows that a plot of log k_{obs} and log [Ca^{2+}] has a slope = 1.8. The simplest interpretation of this result is that the binding of two calcium ions is necessary for the release of the b subunits from $a_2'b_2$ and subsequent exposure of the active-site thiol group.

It has been reported previously that fibrinogen acts as a cofactor for the Ca^{2+}-dependent dissociation of the b subunits and unmasking of the active-site thiol group by lowering the calcium ion requirement for the reaction to concentrations

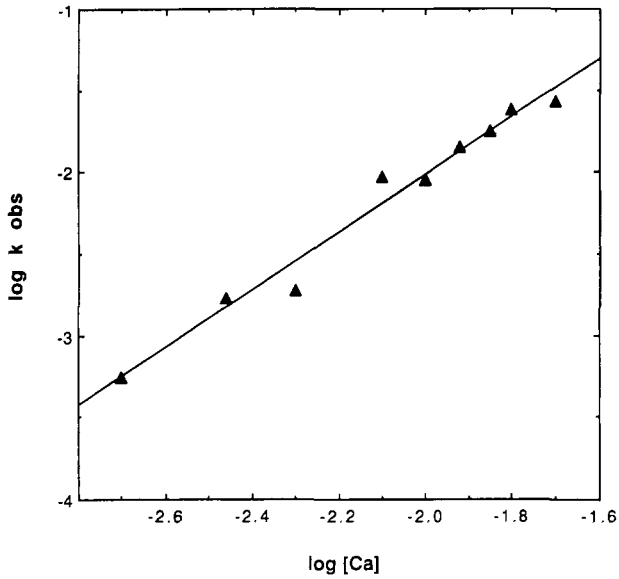

FIGURE 2: Calcium ion dependence of the rate of exposure of the active-site thiol group of $a_2'b_2$. Rates were determined as described under Materials and Methods. A linear least-squares fit of the data, represented by the line, yielded a slope of 1.8.

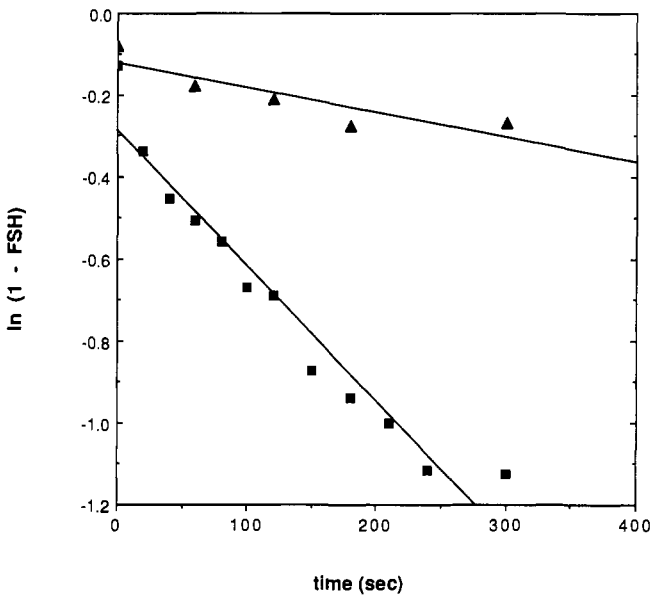

FIGURE 3: Effect of fibrinogen on the rate of exposure of the active-site thiol group of $a_2'b_2$. 1.05 μM $a_2'b_2$ (final concentration) was activated by 2 mM $CaCl_2$ in the absence (▲) and presence (■) of 12 μM fibrinogen. A linear least-squares fit of the data yielded rate constants of 0.06×10^{-3} and 3.3×10^{-3} s^{-1} in the absence and presence of fibrinogen, respectively. FSH represents the fraction of exposable thiol groups exposed at time t, and 1 – FSH represents the fraction of exposable thiol groups not yet exposed at time t.

found in plasma (Credo et al., 1978). Our experiment (Figure 3) shows that fibrinogen exerts its effect by increasing the rate of exposure of the active-site thiol group. A 6-fold enhancement in the rate of exposure of the active-site thiol group was observed when $a_2'b_2$ was incubated with fibrinogen in the presence of 2 mM $CaCl_2$.

Half-of-the-sites incorporation of label in $a_2'b_2$ was observed for alkylation both in the presence and in the absence of fibrinogen. This finding is consistent with the studies of Chung et al. (1974) and Seelig and Folk (1980) demonstrating half-of-the-sites reactivity for a_2b_2 in the absence of fibrinogen.

Exposure of the Active-Site Thiol Group in Cleaved Platelet Factor XIII (a_2'). Although factor XIII purified from

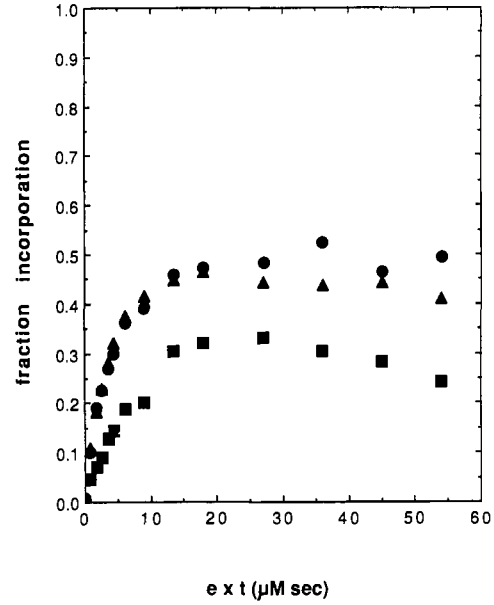

FIGURE 4: Effect of calcium ion on the rate of α-thrombin-catalyzed exposure of the active-site thiol group of platelet factor XIII. 1.20 μM platelet factor XIII was activated as described under Materials and Methods in the absence (■) and in the presence of 1.5 mM (▲) and 14 mM (●) $CaCl_2$. The axis labels e and t represent the α-thrombin concentration and time, respectively, and fraction incorporation refers to the fraction of platelet factor XIII a subunits alkylated with [1-^{14}C]iodoacetamide.

human plasma generally exhibited little incorporation (10–20% of maximal incorporation) of [1-^{14}C]iodoacetamide after cleavage by α-thrombin but prior to addition of calcium ion, we observed incorporation of [1-^{14}C]iodoacetamide into cleaved platelet factor XII zymogen (a_2') even before calcium ion was added to the solution containing a_2'. To eliminate the possibility that this finding was due to an adventitious alteration in the a chains, a_2b_2 was reconstituted by incubating platelet factor XIII with free b chains that were isolated from human blood plasma. The presence of the b chains suppressed the incorporation (after cleavage with α-thrombin) of [1-^{14}C]-iodoacetamide to a level that was 27% of that observed for a_2' from the same source that was not preincubated with b subunits. [The incorporation observed in the presence of b subunits may reflect incomplete reconstitution of a_2b_2 by the added b chains in addition to the incorporation of 0.05–0.1 mol/mol of tetramer exhibited by native ($a_2'b_2$) plasma factor XIII.] As expected, after addition of 10 mM $CaCl_2$ to the reconstituted $a_2'b_2$, exposure of the active-site thiol group occurred at a rate comparable to the rate of exposure of the active-site thiol group of native $a_2'b_2$ in the presence of 10 mM $CaCl_2$. The simplest interpretation of these results is that the difference in the accessibility of the thiol groups in $a_2'b_2$ and a_2' reflects the effect of the b chains and not some unidentified difference in the structure of the a_2' chains of the plasma and platelet factor XIII.

Effect of Calcium Ion on Rate of Exposure of the Active-Site Thiol Group in Platelet Factor XIII. The observation that there was no further time-dependent increase in the exposure of the active-site thiol group subsequent to α-thrombin-catalyzed release of AP from platelet factor XIII (a_2), together with the observation that the rate of thiol group exposure was directly proportional to the α-thrombin concentration (data not shown), suggested that the α-thrombin-catalyzed proteolytic step, rather than a subsequent conformational rearrangement of the cleaved a chains, limits the rate of exposure of the active-site thiol group in platelet factor XIII

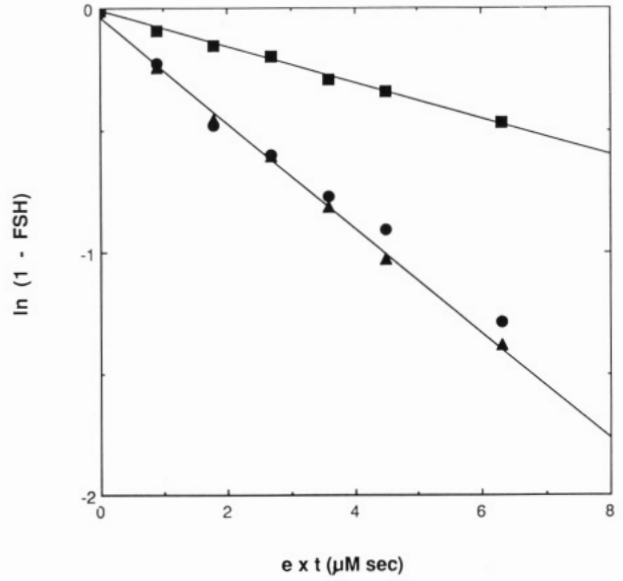

FIGURE 5: Determination of the initial rate constants for α-thrombin-catalyed exposure of the active-site thiol group in platelet factor XIII. Exposure of the active-site thiol group was measured in the presence of 0 (■), 1.5 (▲), and 14 mM (●) $CaCl_2$. A least-squares fit yielded rate constants of 0.7×10^5 M^{-1} s^{-1} for no $CaCl_2$, 2.0×10^5 M^{-1} s^{-1} for 14 mM $CaCl_2$, and 2.2×10^5 M^{-1} s^{-1} for 1.5 mM $CaCl_2$. FSH is the fraction of exposable thiol groups exposed at time t and 1 − FSH is the fraction of exposable thiol groups that have not yet been exposed at time t. The end point used to calculate the rate constants for each of the reactions was the limiting incorporation of one [1-^{14}C]carbamylmethyl group per a_2 dimer.

(a_2). Studies of the time dependence of the thrombin-catalyzed exposure of the active-site thiol group in platelet factor XIII (Figure 4) showed that in the absence of calcium ion (and also in the presence of 5.5 mM EDTA), incorporation of [1-^{14}C]iodoacetamide only reached a level of 60–70% of the maximal incorporation of one molecule of iodoacetamide per dimer, before declining after further incubation. In contrast, addition of calcium ion appeared to maintain the reactivity of the active-site thiol group. Half-of-the-sites incorporation of [1-^{14}C]iodoacetamide was observed when 1.5 and 14 mM $CaCl_2$ was present in incubation mixtures, with preservation of the reactivity of the thiol group upon prolonged incubation at the higher calcium ion concentration. Moreover, there appeared to be a distinct difference in the rate of exposure of the active-site thiol group when calcium ion (both 1.5 and 14 mM) was present compared to when it was absent. Upon further analysis of the data (Figure 5), it was evident that exposure of the active-site thiol group in the absence of calcium ion could be described by a single process before the k_{-2} reaction or some other competing process began to limit accessibility of the thiol group to alkylation. In the absence of calcium ion, exposure of the active-site thiol group occurred with a rate constant of 0.7×10^5 M^{-1} s^{-1}. This value is somewhat less than the rate constant of 1.1×10^5 M^{-1} s^{-1} for the α-thrombin-catalyzed cleavage of activation peptide (AP) from platelet factor XIII previously reported by Hornyak et al. (1989). Interestingly, in the presence of 1.5 and 14 mM $CaCl_2$, the rate constant for exposure of the active-site thiol group increased to $(2.0–2.2) \times 10^5$ M^{-1} s^{-1}, approximately equal to the rate constant reported by Hornyak et al. (1989) for the generation of factor XIIIa activity and exposure of the active-site thiol group.

Effect of Calcium Ion on the Subunit Disposition of the Exposed Active-Site Thiol Group in Platelet Factor XIII. In our previous report, we had shown that the 2-fold difference between the rate of AP release from factor XIII and the rate

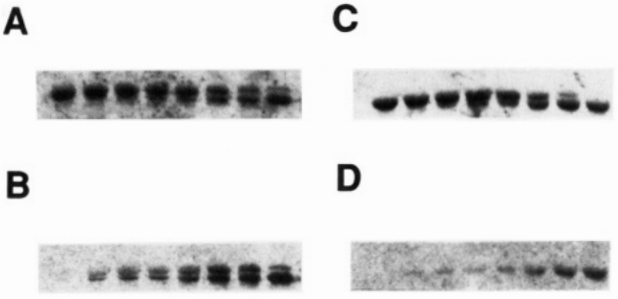

FIGURE 6: Effect of calcium ion on the exposure of the active-site thiol group in the a and a' subunits. Platelet factor XIII was activated by α-thrombin, quenched with PPACK (at 0, 30, 60, 90, 120, 300, 600, and 1800 s), and treated and prepared for electrophoresis as described under Materials and Methods. 0.87 μg of platelet factor XIII was added per lane. Autoradiography to develop the bands was performed at −85 °C for 28 days. (A) Polyacrylamide gel, 14 mM $CaCl_2$. (B) Autoradiogram of polyacrylamide gel, 14 mM $CaCl_2$. (C) Polyacrylamide gel, no $CaCl_2$. (D) Autoradiogram of polyacrylamide gel, no $CaCl_2$.

of exposure of the active-site thiol group and generation of factor XIIIa activity in the presence of calcium ion indicated that cleavage of only one AP from the platelet factor XIII dimer was sufficient to generate full exposure of the active-site thiol group and full factor XIIIa activity. Our finding here, that, in the absence of calcium ion, the rate of exposure of the active-site thiol group was nearly equal to that of AP release, suggested the possibility that in the $a'a$ heterodimer, only one thiol group could react with iodoacetamide in the absence of calcium ion but that both thiol groups became reactive toward iodoacetamide in the presence of calcium ion. By cleaving platelet factor XIII to increasing degrees with α-thrombin, stopping the reaction, and observing the pattern of subsequent [1-^{14}C]iodoacetamide incorporation in a and a' subunits following analysis by gel electrophoresis (Figure 6), we could discern a clear difference in the pattern of exposure of the active-site thiol group of cleaved platelet factor XIII in the presence and absence of calcium ion. In the absence of $CaCl_2$, only the active-site thiol group in the α-thrombin-cleaved subunit (a') was subject to alkylation. In the presence of calcium ion, however, both the a and a' subunits were alkylated. The observation that alkylation of a' occurs in the absence of calcium ion indicates that exposure of the active-site thiol group in a' subsequent to α-thrombin-catalyzed release of AP does not require calcium ion. Since the active-site thiol group in the uncleaved homodimer (a_2) is inaccessible for reaction with iodoacetamide, the observed α-thrombin-promoted reaction in the presence of calcium ion of the active-site thiol group in the intact a subunit must involve alkylation of the active-site thiol group in the a subunit of the $a'a$ heterodimer. This conclusion suggests that the once-cleaved $a'a$ heterodimer (which we have previously shown to possess full catalytic activity in the presence of calcium ion) undergoes a calcium ion induced intersubunit interaction that results in exposure of the active-site thiol group on the uncleaved subunit. This intersubunit interaction may also be associated with generation of enzymic activity, since no factor XIIIa activity is observed in the absence of calcium ion, even though the thiol group on the cleaved a' subunit is exposed.

Accessibility of the Active-Site Thiol Group in the Uncleaved Subunit of the a'a Heterodimer. To determine the accessibility of the a-subunit thiol group relative to that of the a' subunit, we resorted to a quantitative analysis of the autoradiographic data.

Incorporation of [1-^{14}C]iodoacetamide in the a and a' subunits subsequent to incubation of factor XIII with α-

thrombin for time t can be expressed by combining the equation describing α-thrombin-catalyzed AP release from the a subunit and the set of equations describing the proportions of the aa, $a'a$, and $a'a'$ ensemble in solution (Hornyak et al., 1989). If f_{AP} is the fraction of AP released at time t, then

$$f_{AP} = 1 - \exp(-kt) \quad (13)$$

where $k = (k_{cat}/K_M)[\alpha\text{-thrombin}]$ and K_M is much larger than the initial concentration of factor XIII. The fractions of factor XIII dimers with zero (f_{a_2}), one (f_{a^*a}), and two ($f_{a_2^*}$) AP cleavages (Hornyak et al., 1989) are

$$f_{a_2} = (1 - f_{AP})^2 \quad (14)$$

$$f_{a^*a} = 2f_{AP} - 2f_{AP}^2 \quad (15)$$

$$f_{a_2^*} = f_{AP}^2 \quad (16)$$

The amounts of radiolabeled a subunit (a-CM)[3] and a' subunit (a'-CM) can be expressed as:

$$a\text{-CM} = Af_{a^*a}[\text{FXIII}]_0 \quad (17)$$

$$a'\text{-CM} = [(1 - A)f_{a^*a} + f_{a_2^*}][\text{FXIII}]_0 \quad (18)$$

where A represents the fraction of radiolabeled thiol group in the once-cleaved heterodimer ($a'a$) that resides in the intact a subunit. These equations assume half-of-the-sites reactivity and equal incorporation of radiochemical label in the $a'a$ and $a'a'$ forms of cleaved platelet factor XIII as documented previously by Chung et al. (1974) and Hornyak et al. (1989). Substitutions in the above equations yield

$$a\text{-CM} = 2A\,[\exp(-kt) - \exp(-2kt)][\text{FXIII}]_0 \quad (19)$$

$$a'\text{-CM} = \{1 - \exp(-2kt) + 2A\,[\exp(-2kt) - \exp(-kt)]\}[\text{FXIII}]_0 \quad (20)$$

Hence:

$$a'\text{-CM}/(a'\text{-CM} + a\text{-CM}) = \{1 - \exp(-2kt) + 2A\,[\exp(-2kt) - \exp(-kt)]\}/[1 - \exp(-2kt)] \quad (21)$$

Comparison of the observed densitometric data with the theoretical curve generated from eq 21 for the case where the accessible thiol group of the $a'a$ heterodimer is expressed exclusively on the intact subunit ($A = 1$) with the theoretical curve obtained from eq 21 for the case where the active-site thiol group is equally accessible on the cleaved and uncleaved subunits ($A = 0.5$) suggests that, in the presence of calcium ion, the active-site thiol group on the $a'a$ heterodimer is distributed equally between the uncleaved and the cleaved subunits (Figure 7). This result suggests the existence of a calcium ion induced intersubunit interaction which can transmit a conformational change across the $a'a$ subunit interface of the heterodimer that exposes the active-site thiol group in the uncleaved subunit in the presence of calcium ion. In the absence of calcium ion, exposure of the active-site thiol group occurs only on the cleaved subunit,[4] but this effect is not sufficient to generate factor XIIIa activity.

Exposure of an active-site thiol group in the absence of calcium ion with the lack of any apparent enzymatic activity raised the possibility that cleaved factor XIII under these conditions could exhibit either a burst of enzymatic activity, indicative of a single enzymatic turnover event, or formation of a stable acyl-enzyme complex. An experiment investigating

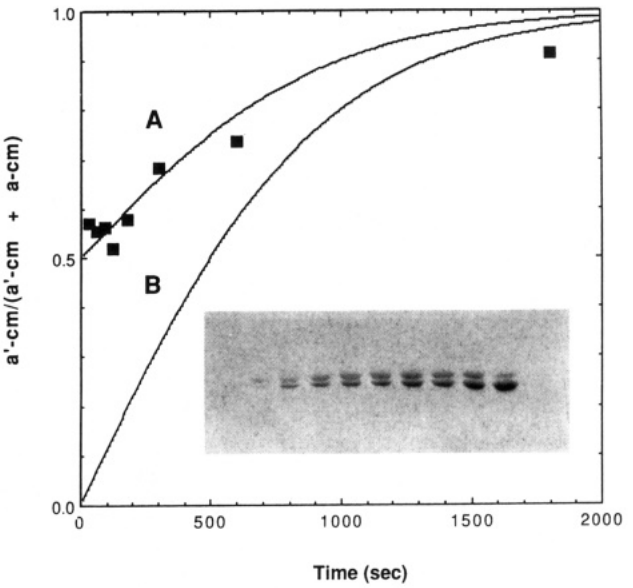

FIGURE 7: Quantitative analysis of exposure of the active-site thiol group. Curve A is the theoretical plot of eq 21 for $A = 0.5$ and curve b for $A = 1$. Data points are generated from densitometry of the autoradiogram (inset), with thrombin incubation times of 0, 30, 60, 90, 120, 180, 300, 600, and 1800 s. a'-CM and a-CM refer to the amounts of 1-^{14}C-carbamylmethylated cleaved and uncleaved subunits at each time of thrombin incubation, respectively. The expression of a'-CM/(a-CM + a'-CM) signifies the fraction of total label in the a' subunit after quenching the thrombin in each factor XIII containing incubate at time t.

these possibilities (data not shown) was devised in which platelet factor XIII was activated by α-thrombin in the presence of fibrinogen at a 1:2 molar ratio of factor XIII to fibrinogen (1.0 μM factor XIII and 2.0 μM fibrinogen) and subjected to reducing and nonreducing SDS–PAGE on a 7.5% polyacrylamide gel. Without $CaCl_2$ present, no cross-linking of fibrin was detected (<5% γ-dimer formation relative to a control containing calcium ion that featured 100% γ-dimer formation), nor was there any indication that a covalent complex of factor XIII and fibrin formed, as would have been evidenced by the appearance of a band at M_r = 120 000–130 000 in the reducing sample (corresponding to a covalent complex of the a subunit and the fibrin γ-chain; 5% complex formation would have been detected) or by disappearance of the M_r = 80 000 band on the nonreducing sample (corresponding to loss of the free a subunit due to formation of a complex of the a subunit and fibrin; 20% loss would have been detected). This negative result suggests that exposure of the active-site thiol group per se is not sufficient for any enzymatic function and that other residues, perhaps on the opposite a subunit, must assist Cys-a314 in initiating catalytic activity.

Discussion

Previous studies of the binding of calcium ion to factor XIII and factor XIIIa (Lewis et al., 1978) demonstrated specific binding of calcium ion to the a subunits, with the presence of 1–1.5 (possibly 2) high-affinity sites ($K = 0.1$ mM). At higher free calcium ion concentrations (2.5 mM), binding of up to eight ions per molecule of plasma factor XIII and factor XIIIa was reported. Our finding that two calcium ions are involved in the conversion of the cleaved zymogen ($a_2'b_2$) to the activated enzyme over the range of calcium ion concentrations studied (2–20 mM) suggests that additional lower affinity sites for calcium ion binding are involved in this conversion, since the sites for calcium ion binding described by Lewis et al. (1978) would have been saturated at the calcium ion concentrations used in this study. If the sites characterized by

[3] CM = carbamylmethylated.

[4] The case when $A = 0$ is represented when cleavage of AP occurs in the absence of calcium. In this case, a'-CM/(a'-CM + a-CM) = 1 at all values of time t.

Lewis et al. (1978) were sufficient to mediate exposure of the active-site thiol group, we would have observed apparent rate constants that were independent of the concentration of calcium ion at calcium ion concentrations above 2.5 mM. Additional low-affinity calcium ion binding sites must be involved not only in the conversion of the cleaved zymogen to the activated enzyme but also in the nonphysiologic activation of factor XIII by 50 mM calcium ion (in the absence of thrombin) reported by Credo et al. (1978) as well as in the enhanced stability of factor XIIIa observed at higher calcium ion concentrations.

The kinetics of *b*-chain dissociation and exposure of the active-site thiol group have been studied by using DTNB as a spectrophotometric probe for exposure of the active-site thiol group during activation of plasma factor XIII and as a ligand to measure inhibition of factor XIIIa activity (Cooke et al., 1974). A fast and a slow phase of the Ca^{2+}-induced reaction was noted in the spectrophotometric studies, with the slow phase attributed to light scattering due to precipitation of *a'* chains. In both the spectrophotometric and inhibition experiments, the rate of the initial, fast reaction was observed to be dependent upon the calcium ion concentration, although square dependencies of the rate upon calcium ion concentration were not apparent. The results of a later study (Freyssinet et al., 1978), employing polarization of fluorescence to measure the Ca^{2+}-induced dissociation of plasma factor XIIIa, agree more closely with our findings. In this study, a biphasic reaction is also observed, with the proportion of the total reaction attributable to the fast initial phase increasing with the calcium ion concentration. At $[Ca^{2+}]$ <20 mM, a nonlinear dependence of the initial rate was observed, although a kinetic analysis to determine the kinetic order of the dissociation reaction with respect to the concentration of calcium ion was not performed. The dependence of the extent of the fast phase of the reaction upon the calcium ion concentration may be due to the fact that at higher calcium ion concentrations a greater fraction of the process is completed before significant competing reactions can begin to slow the rate.

Our observation that the active-site thiol group is exposed in a_2' in the absence of calcium ion differs from the finding of Chung et al. (1974), who reported no incorporation of [1-^{14}C]iodoacetamide in a_2' in the absence of calcium ion.[5] It is important to note, however, that our conclusion that the thiol group is accessible in a_2' in the absence of calcium ion is consistent with the findings of Cooke (1974), whose studies document a clear difference in the reactivity of a_2' and $a_2'b_2$ with DTNB in the absence of calcium ion. Cooke showed that a_2' was completely inactivated by exposure to DTNB, whereas $a_2'b_2$ was not and thus he concluded (as we have) that the active-site thiol group in a_2' in accessible in the absence of calcium ion. The finding that AP release alone is sufficient to cause exposure of the active-site thiol group in the cleaved subunit of platelet factor XIII, but that calcium ion is required for exposure of the active-site thiol group in the uncleaved subunit of an *a'a* heterodimer, demonstrates that AP cleavage and calcium ion binding have distinct functions in the activation pathway for factor XIII. Calcium ion appears to behave as an allosteric effector in the generation of factor XIIIa activity. Since the active-site thiol group is not exposed in $a_2'b_2$, the *b* chains in this complex either sterically mask the active-site thiol group or effect a structural change in the *a* subunits preventing thiol group exposure until the *b* chains are released. The absence of activity in a_2' under conditions when an active-site thiol group is exposed suggests that residues of the *a* chain other than Cys-*a*314 are required for development of activity.

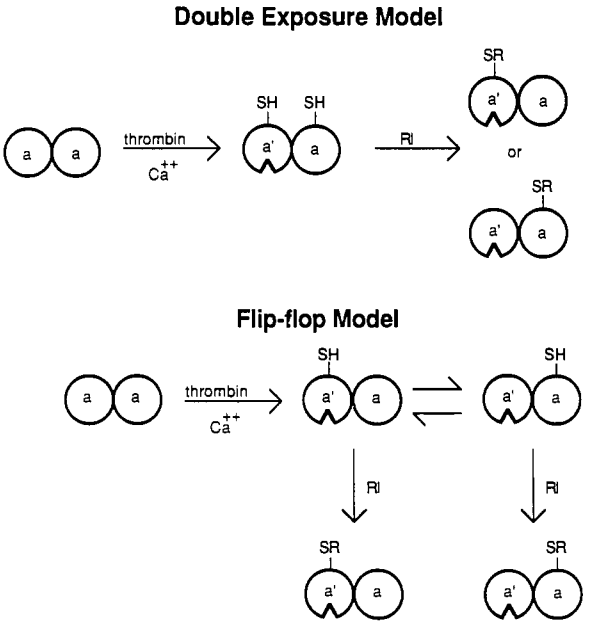

FIGURE 8: (A) Double-exposure model for reactivity of the active-site thiol groups of factor XIIIa. (B) Flip-flop model for reactivity of the active-site thiol groups of factor XIIIa. In the double-exposure model, both thiol groups in the *a'a* heterodimer are exposed. After alkylation, a negative cooperative interaction prevents labeling of a second thiol group. In the flip-flop model, only one active-site thiol group is exposed in the *a'a* heterodimer, but this thiol rapidly alternates between the cleaved and the uncleaved subunit.

Since *a'a* and *a'a'* are fully active in the presence of calcium ion (Hornyak et al., 1989), and since exposure of the active-site thiol group in the uncleaved *a* subunit of *a'a* is only observed in the presence of calcium ion, both thiol groups must be capable of exposure in catalytically competent factor XIIIa, yet [as documented in this and previous studies (Hornyak et al., 1989; Chung et al., 1974)] only half-of-the-sites incorporation of [1-^{14}C]iodoacetamide into factor XIIIa is observed under conditions that yield full expression of factor XIIIa catalytic activity. Two different models (the double-exposure and flip-flop models) for exposure of the active-site thiol group in factor XIIIa can be proposed to resolve this paradox (Figure 8). In the double-exposure model, cleavage of AP in the presence of calcium ion results in an allosteric interaction,

[5] It should be noted, however, that in the 1974 study a_2 was cleaved by 0.25 unit/mL thrombin (approximately 2.5 nM) at 25 °C for 30 min. Assuming a value of $k_{cat}/K_M = 0.55 \times 10^5$ M^{-1} s^{-1} for AP release from platelet factor XIII at 25 °C (Hornyak et al., 1989) leads to the conclusion that less than 22% of the AP would have been released under the conditions of Chung et al. (1974). The assumed value of k_{cat}/K_M for AP release is half the value reported by Hornyak et al. (1989) for this constant at 37 °C and reflects an estimate of the effect upon k_{cat}/K_M of the temperature difference between the two studies. If, as our analysis suggests, the platelet factor XIII assayed for incorporation of [1-^{14}C]-iodoacetamide was not substantially cleaved by thrombin in the studies of Chung et al. (1974), we could account for the failure of Chung et al. to observe incorporation of [1-^{14}C]iodoacetamide into an active-site cysteine under their experimental conditions. [It should be noted that our estimate of the extent of cleavage in the study of Chung et al. is based upon the assumption that human α-thrombin with a specific activity of ~0.1 NIH unit/pmol was used. This is the specific activity for pure human α-thrombin (Lewis & Shafer, 1984). Use of less active α-thrombin by Chung et al. would have resulted in an even lower extent of cleavage.] The observation of Chung et al. (1974) that the factor XIII generated under their conditions is fully active [rather than ~40% active as one might calculate using the equations of Hornyak et al. (1989) that relate the extent of cleavage of a_2 to the extent of activation] might well be a consequence of the fact that Chung et al. assayed for factor XIIIa activity in the presence of 40 mM calcium ion, conditions that result in thrombin-independent activation of factor XIII.

mediated by calcium ion, across the *a* subunits that exposes both active-site thiol groups, each with equal accessibility. When either one is alkylated by [1-^{14}C]iodoacetamide, a negative cooperative interaction occurs across the subunit interface to eliminate the accessibility of the unmodified thiol. In the flip-flop model, binding of calcium ion after AP cleavage induces a rapid alternation in the accessibility of the active-site thiol groups. Alkylation with [1-^{14}C]iodoacetamide then traps a thiol in its exposed position, preventing exposure of the second thiol. Currently, no data are available to distinguish between these models.

Regardless of which model most accurately describes the true behavior of the enzyme, the second active-site thiol group is not exposed when one active-site thiol group is alkylated. Alternating-sites reactivity has been demonstrated for several enzymes including mitochondrial ATPase (Choate et al., 1979) and succinyl-coenzyme A synthetase (Wolodko et al., 1981), although the physiological relevance of this phenomenon remains to be established. It is possible that the behavior of the alkylated factor XIIIa mimics the behavior of the enzyme in the acyl-enzyme complex, after its attack at the Gln-γ398 carbonyl of fibrinogen but before its displacement by Lys-γ406 of the adjacent fibrin unit.[6] It is tempting to speculate that when factor XIIIa is positioned close to the cross-linking site, as it is in the covalent acyl-enzyme complex with Gln-γ398 of fibrinogen, the inaccessibility of the unacylated active-site thiol group on the adjacent subunit might serve to prevent the occurrence of nonspecific cross-linking reactions between the constituents of the fibrin clot. It is also conceivable that negatively cooperative intersubunit interactions in factor XIIIa might facilitate removal of product from the active site, if binding of substrate to one subunit were to lower the affinity (or increase the rate of dissociation) of product bound at the adjacent subunit. Additional kinetic studies will be required to elucidate the relevance of cooperative interactions characterized in this study to the physiological activity of factor XIIIa.

Registry No. Factor XIII, 9013-56-3; factor XIIIa, 9067-75-8; Ca, 7440-70-2; α-thrombin, 9002-04-4.

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[6] This hypothesis is analogous to the proposal that the half-of-the-sites reactivity observed in the reaction of 6-diazo-5-oxonorleucine with the active-site thiol group of CTP synthetase is mimicked in the formation of the covalent glutamyl-enzyme intermediate formed in the CTP synthetase catalyzed reaction (Levitski et al., 1971).

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