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Calcium-Induced Dissociation of Human Plasma Factor XIII and the Appearance of Catalytic Activity

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1. The Ca^{2+} dependence of the activity of plasma Factor $XIII_a$ was studied by using the continuous assay based on the incorporation of dansylcadaverine into dephosphorylated acetylated β-casein (β-substrate). The K_m for Ca^{2+} is about 0.170mM. 2. At low concentrations of Ca^{2+} there was a lag in attaining the steady-state rate. The size of the lag was decreased and eventually abolished if the enzyme was preincubated with a high concentration of Ca^{2+} before assay. The concentration of Ca^{2+} required to decrease the lag phase by 50% in 10min depended on the protein concentration: at 0.87mg of protein/ml it required 17mM-Ca^{2+} and at 0.44mg/ml it needed 10mM-Ca^{2+}. 3. The concentrations of Ca^{2+} required either to abolish the lag phase in the appearance of enzyme activity or to activate the essential thiol for reaction with 5,5′-dithiobis-(2-nitrobenzoate) in 10min incubation were similar at the same protein concentration. This indicated that Ca^{2+} induces a conformation change that is responsible for both phenomena. A model is proposed that links this conformation change to the dissociation of the tetrameric enzyme. 4. This was supported by the observation that the addition of excess of *b* chains to the Factor $XIII_a$ (a'_2b_2) increased the concentration of Ca^{2+} required to expose the reactive thiol, and inhibited the Ca^{2+}-dependent aggregation of *a′* chains. 5. Platelet Factor $XIII_a$ (a'_2) was inhibited by 5,5′-dithiobis-(2-nitrobenzoate) in the absence of Ca^{2+}, and no lag phases were observed in attaining the steady-state rate at low Ca^{2+} concentrations, thus confirming the model for the activation of the plasma enzyme. 6. The Ca^{2+} dependence of platelet Factor $XIII_a$ indicated that Ca^{2+} has an additional role in the enzyme mechanism of the plasma enzyme, perhaps being involved in substrate binding. 7. The dependence of the stability of plasma Factor $XIII_a$ on Ca^{2+} and protein concentration indicates that the decay in activity is related to the tetramer dissociation. 8. β-Substrate decreased the Ca^{2+} concentration required for (1) abolition of the lag phase and (2) enzyme inhibition by thiol reagents. The effect on the former is greater than on the latter. 9. The role of the *b* chains of the plasma Factor and the evolutionary significance of the plasma and platelet Factors are considered.

Human plasma Factor XIII is thought to consist of two different subunits arranged as a tetramer of composition a_2b_2 (Schwartz *et al.*, 1973). The thrombin-activated enzyme (Factor $XIII_a$) catalyses the cross-linking of fibrin by forming γ-glutamyl-ε-lysyl amide bonds in a strictly Ca^{2+}-dependent transamidation reaction (Pisano *et al.*, 1968). The enzyme can be assayed by following the incorporation of radioactive glycine ethyl ester into β-substrate (Cooke & Holbrook, 1974*a*); the K_m value for Ca^{2+} is less than 1mM. The high Ca^{2+} concentrations used by other workers (Dvilansky *et al.*, 1970; Tyler, 1970; Sheltawy *et al.*, 1972) are necessary because of Ca^{2+}–substrate interactions that occur when Hammarsten casein is the acceptor substrate (Cooke & Holbrook, 1974*a*).

Ca^{2+} causes Factor $XIII_a$ to dissociate into subunits, the *a′* subunits subsequently aggregating into a misty precipitate (Cooke & Holbrook, 1974*b*), and is also required for inhibition of the essential thiol of plasma Factor XIII (Cooke *et al.*, 1974). The Ca^{2+} concentrations required for both these processes are very much higher than that required to observe maximum assay rates, indicating that they may be non-physiological reactions. The present paper reports a re-examination of the Ca^{2+} dependence of plasma Factor $XIII_a$ by using the continuous fluorimetric assay involving dansylcadaverine and β-substrate (Cooke & Holbrook, 1974*a*). The Ca^{2+} dependence of enzyme reactivity was found to be markedly dependent on protein concentration because of its relation to the dissociation of the tetramer. Experiments are reported that indicate that this dissociation is necessary for the expression of catalytic activity.

The catalytic activity of plasma Factor $XIII_a$ is associated only with the *a′* chains (Schwartz *et al.*,

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1973; Cooke *et al.*, 1974). McDonagh *et al.* (1969) observed that platelets contribute up to 50% of the pro-transglutaminase activity present in human blood. Schwartz *et al.* (1971, 1973) showed that the subunit of platelet Factor XIII (a_2) is very similar to or identical with the *a* subunits of the plasma zymogen. The common antigenic determinant, reported by Bohn (1970), can be attributed to the common *a* chain that is responsible for the catalytic activity of both enzymes. The kinetic data of Chung & Folk (1972) agree with this concept of a common subunit, which also explains the observation that patients with a congenital deficiency of plasma Factor XIII also lack platelet Factor XIII (Loewy, 1968; McDonagh *et al.*, 1969), since this could be a single genetic defect. The fluorescent-antibody studies of Kiesselbach & Wagner (1972) indicated that *a* chains are synthesized in the platelets (and megakaryocytes).

Decreased Factor XIII concentrations are associated with hepatic damage (Nussbaum & Morse, 1964; Mandel & Gerhold, 1969). This could indicate that the liver is the site of synthesis of the structural *b* chains of plasma Factor XIII (alternatively, the liver could be a secondary site for *a*-chain synthesis). The Ca^{2+} dependence of the platelet Factor $XIII_a$ reactivity was investigated to test the model proposed for the Ca^{2+}-dependent activation of the plasma enzyme. The role of the plasma *b* chains is discussed together with the evolutionary significance of the platelet and plasma Factor XIII.

Materials and Methods

Human plasma Factor XIII was prepared by ion-exchange chromatography followed by Sepharose 6-B resolution of component A (plasma zymogen: a_2b_2) and component B (b_2 subunits) as described by Cooke & Holbrook (1974*b*). Factor $XIII_a$ was prepared by thrombin cleavage as described by Cooke & Holbrook (1974*a*). The thrombin contaminant was inactivated by 1 mM-di-isopropyl phosphorofluoridate (BDH Chemicals Ltd., Poole, Dorset, U.K.) before storage of the Factor $XIII_a$ at 0°C for up to 4h. Factor $XIII_a$ activity was measured by the continuous fluorimetric assay (Cooke & Holbrook, 1974*a*); one enzyme unit gives a 1% increase in the starting fluorescence/min at 25°C.

Out-dated, platelet-rich plasma was a generous gift from the South-West Regional Blood Transfusion Centre. This platelet concentrate was centrifuged at 2000*g* for 7min (10°C) to remove residual erythrocytes and the supernatant was removed by aspiration. The platelets were then sedimented at 6000*g* (10°C) for 45min, and washed by resuspending in 0.9% NaCl and re-sedimenting. These washed platelets were suspended in 50mM-Tris–HCl, pH7.5, containing 1mM-EDTA, and sonicated in a plastic tube for 10s at 0°C [Branson Soniprobe type 1130A (Dawe Instruments, London W3 0SD, U.K.), used on maximum setting, level 8]. The platelet membranes were then removed by centrifugation at 105000*g* for 1h at 4°C, and the supernatant was dialysed for 12h at 4°C against 2×50vol. of the Tris–HCl buffer described above. This diffusate was then chromatographed on DEAE-cellulose, as described by Schwartz *et al.* (1971). The platelet zymogen is much less stable than that of the plasma, and much lower yields were recorded during the ion-exchange-chromatography stage of the preparation. The half-life of the plasma Factor XIII in 0.1M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA at 4°C is approx. 7–8 weeks (Cooke & Holbrook, 1974*a*); the platelet Factor XIII retained 65% of its initial activity after 11 days under the same conditions (half-life about 19 days).

Results

Ca^{2+} dependence of the incorporation of dansylcadaverine into β-substrate

The rate of incorporation of dansylcadaverine into β-substrate is invariant in the Ca^{2+} concentration range 5–50mM, in agreement with the results of Cooke & Holbrook (1974*a*) by using the radioactive assay method. Fig. 1 shows six assay traces at different Ca^{2+}

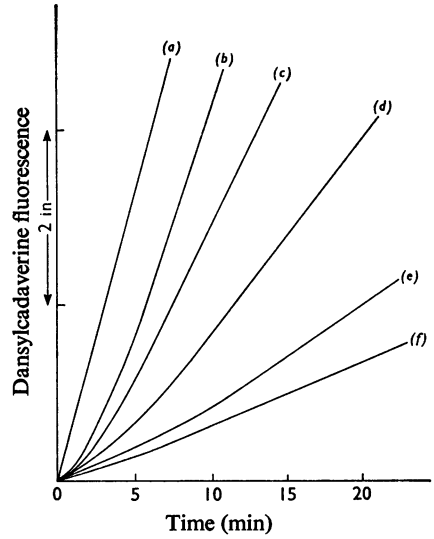


Fig. 1. *Ca^{2+} dependence of the rate at which Factor $XIII_a$ incorporates dansylcadaverine into β-substrate*

Assays were performed as described by Cooke & Holbrook (1974*a*). Factor $XIII_a$ (1.7 units, assayed under optimum conditions) was used at the following Ca^{2+} concentrations: curves (*a*) 5–60mM, (*b*) 1mM, (*c*) 0.2mM, (*d*) 0.125mM, (*e*) 0.075mM and (*f*) 0.050mM. A 1-in increase in the fluorescence scale in 5min corresponds to an activity of 0.46 unit.

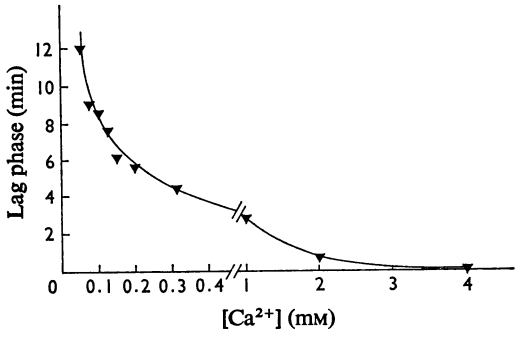


Fig. 2. *Ca^{2+} dependence of the lag in attaining the steady-state assay rates*

The lag is the time that elapses subsequent to enzyme addition, before the steady state is achieved. The assay conditions are described in Fig. 1.

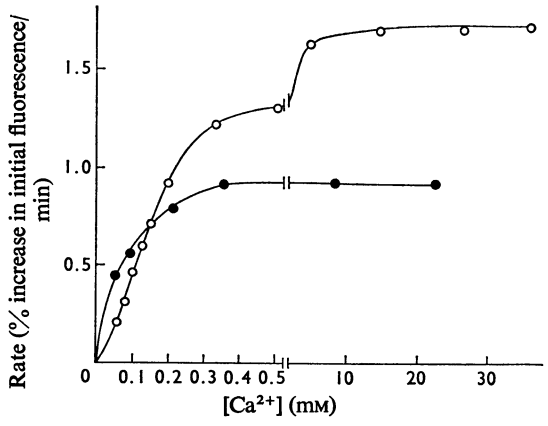


Fig. 3. *Ca^{2+} dependence of the incorporation of dansylcadaverine into β-substrate catalysed by plasma Factor $XIII_a$ (○) or platelet Factor $XIII_a$ (●)*

Assay conditions are described in the Materials and Methods section.

concentrations. An unexpected feature of these traces is the lag phase at low Ca^{2+} concentrations. This lag, i.e the time that elapses subsequent to the addition of enzyme before the steady-state rate is achieved, is longer at low Ca^{2+} concentrations (Fig. 1), suggesting that there is a Ca^{2+}-dependent rate-determining process that precedes the establishment of the steady-state rate. Fig. 2 shows the lengthening of the lag phase as the Ca^{2+} concentration is decreased. Fig. 3 shows steady-state rates and indicates that the K_m has a value of about 0.170mM-Ca^{2+}. However, at low Ca^{2+} concentrations the relationship does not follow a simple saturation curve; this may be related to the associated lag phases. The assay traces depicted in Fig. 1 are similar to those obtained while studying the thrombin optimum required in the linked assay of the Factor XIII zymogen (Cooke & Holbrook, 1974*a*). At low thrombin concentrations, a longer time is required for cleavage of the zymogen and thus longer lag phases were observed. The corresponding steady-state rates were less than maximum, the suggested explanation being that an appreciable fraction of Factor $XIII_a$ is denatured before activation is complete. This agrees with the observation that the linearity of the steady-state rate under optimum conditions is approximately independent of the rate and hence is not determined by the consumption of substrates (or the generation of products). This implies that assay rates that display long lag phases are not a true representation of the rates at these sub-optimum Ca^{2+} (or thrombin) concentrations. A thrombin-dependent lag phase of 15min was associated with a 40% decrease in the steady-state rate (Cooke & Holbrook, 1974*a*), whereas a 4–5min lag incurred little decrease (5–10%) of the rate observed under optimum conditions. Corrections of this order applied to Fig. 3 would produce an approximate saturation-curve relationship. The irregular shape of the curve at low Ca^{2+} concentrations could also be partially explained if the assay medium sequesters Ca^{2+} (up to 0.03mM). This is unlikely, since the dephosphorylation of β-casein (involved in the preparation of β-substrate) decreases its affinity for Ca^{2+} (Cooke & Holbrook, 1974*a*). The final EDTA concentration present (added with the enzyme sample) is 2.5μM.

The size of the lag phase at low Ca^{2+} concentrations was decreased and eventually abolished if the enzyme was preincubated with a high concentration of Ca^{2+} and then diluted into the assay system. This is shown in Fig. 4(*a*); the Ca^{2+} concentration in the assay, after addition of the enzyme sample, was 0.2mM; a 10min preincubation in 17mM-Ca^{2+} is required to decrease the lag by 50% and 40mM-Ca^{2+} would be required for total abolition. This is not a non-specific ionic-strength effect, since preincubation in 20–50mM-Mg^{2+} did not alter the lag. The steady-state rates recorded in Fig. 4(*a*) are almost constant probably because any increase that might result from a decrease in the lag is masked by the increasing loss in Factor $XIII_a$ activity incurred by preincubation with higher Ca^{2+} concentrations (see below).

The high Ca^{2+} concentrations required for the abolition of the lag are not compatible with the Ca^{2+} optimum of the Factor $XIII_a$ activity (Figs. 1 and 3). However, if the Ca^{2+}-dependent conformation change involved is related to the dissociation of the tetrameric enzyme (Cooke & Holbrook, 1974*b*), the conformation change might be dependent on protein concentration. This was tested by repeating the preincubations at half the protein concentration. The lag phase

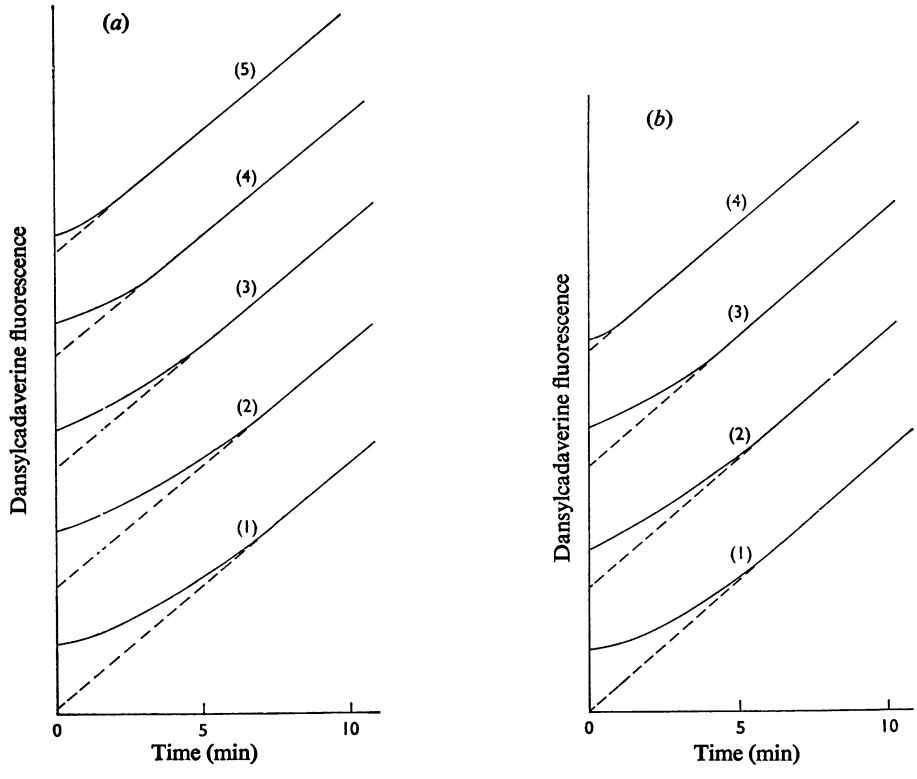


Fig. 4. *Abolition of the lag phase in Factor XIII$_a$ assays by preincubating Factor XIII$_a$ with Ca^{2+}*

(*a*) Samples of Factor XIII$_a$ (0.87mg/ml) in 0.1M-Tris–HCl buffer, pH7.5, were preincubated for 10min with (1) 0mM-Ca^{2+}, (2) 10mM-Ca^{2+}, (3) 15mM-Ca^{2+}, (4) 20mM-Ca^{2+} and (5) 30mM-Ca^{2+} at 25°C. Portions (10μl) were then added to the assay medium containing 0.2mM-Ca^{2+} (concentration after mixing). (*b*) Samples of Factor XIII$_a$ (0.44mg/ml) were incubated for 10min with (1) 0mM-Ca^{2+}, (2) 4mM-Ca^{2+}, (3) 10mM-Ca^{2+} and (4) 15mM-Ca^{2+}. Portions (20μl) were then added to the assay medium containing 0.2mM-Ca^{2+} (concentration after mixing).

was halved by preincubation in 10mM-Ca^{2+} (Fig. 4*b*). This indicates that the Factor XIII$_a$ mechanism involves a Ca^{2+}-dependent conformation change that is dependent on protein concentration. The protein-concentration dependence of this phenomenon was not investigated at lower protein concentrations because of the difficulties involved in assaying larger portions containing Ca^{2+}, while maintaining the Ca^{2+} concentration in the assay in the range required to observe lag phases.

Cooke *et al.* (1974) established that the Ca^{2+} concentration required for inhibition of Factor XIII$_a$ by 5,5′-dithiobis-(2-nitrobenzoate) decreased sharply as the protein concentration was decreased. Fig. 5 shows that the concentrations of Ca^{2+} required either to abolish the lag phase in the appearance of enzyme activity or to activate the thiol for reaction with 5,5′-dithiobis-(2-nitrobenzoate) in 10min incubation are similar at the same protein concentrations. This indicates that Ca^{2+} induces a conformation change that is responsible for both phenomena. The protein concentration dependence of these two reactions suggests that the conformation change alters the equilibrium between the a'_2b_2 tetramer and the forms containing only *a*′ or *b* subunits.

Stability of Factor XIII$_a$ in the presence of Ca^{2+}

Factor XIII$_a$ was quite stable in the absence of Ca^{2+}; no detectable decrease in activity occurs during a 4h incubation in 0.1mM-EDTA. Addition of Ca^{2+} to the incubation medium led to a decay in the activity (Fig. 6). $MgSO_4$ (45mM) led to only a 15% decay after 400min, indicating that the effect is not simply due to ionic strength. Higher concentrations of Factor XIII$_a$ are more stable when exposed to corresponding Ca^{2+} concentrations. For instance 6mg of Factor XIII$_a$/ml loses only 26% of its activity after 300min incubation in 20mM-Ca^{2+}, whereas 0.4mg of enzyme/ml lost 83% of its activity under the same conditions (Fig. 6). The Ca^{2+} and protein-concentra-

tion dependence of the stability of Factor $XIII_a$ suggests that the inactivation depends on the dissociation described above. It is possible that the inactivation is the result of oxidation of the exposed thiol, since the ability of reducing agents to preserve enzyme activity has been known for several years (Loewy *et al.*, 1961).

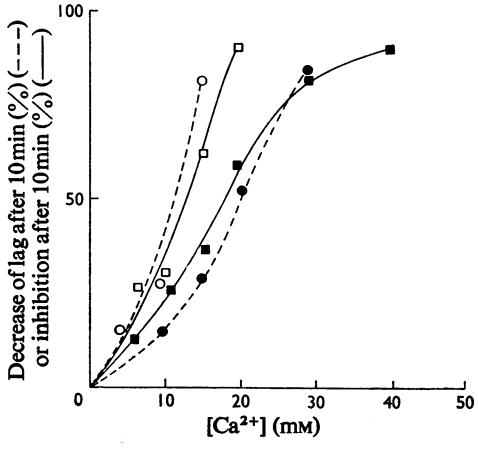


Fig. 5. *Comparison between the protein-concentration dependence of the inhibition of Factor $XIII_a$ by 5,5'-dithiobis-(2-nitrobenzoate) and the abolition of the lag phase in Factor $XIII_a$ assays at 0.2mM-Ca^{2+}*

The conditions for the enzyme inhibition by 5,5'-dithiobis-(2-nitrobenzoate) were described by Cooke *et al.* (1974). The protein concentrations used were 0.38mg/ml (□) and 0.73mg/ml (■). The protein concentrations used to study the decrease of the lag were 0.44mg/ml (○) and 0.87mg/ml (●) (as described in Fig. 4).

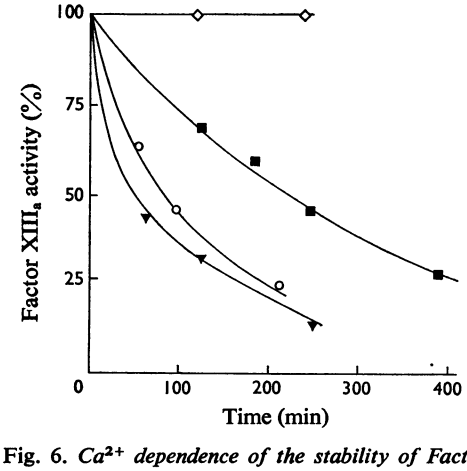


Fig. 6. *Ca^{2+} dependence of the stability of Factor $XIII_a$*

Factor $XIII_a$ (0.4mg/ml) was incubated in the presence of the following Ca^{2+} concentrations: ◇, 0mM; ■, 2.5mM; ○, 8.5mM; ▼, 20mM, at 25°C. Samples from each incubation were assayed periodically.

Effect of component B (excess of b subunits) and β-substrate on the Ca^{2+}-dependent behaviour of Factor $XIII_a$

The hypothesis that the Ca^{2+}-dependent conformation change is linked to the dissociation of the tetrameric enzyme (a'_2b_2) can be tested by monitoring the Ca^{2+}-dependent behaviour of the enzyme in the presence of excess of *b* subunits. The effect would depend on the dissociation constant of the tetramer at the Ca^{2+} concentration used (see the Discussion section). Fig. 7 shows that the rate of enzyme inhibition by 5,5'-dithiobis-(2-nitrobenzoate) at a fixed Ca^{2+} concentration is decreased in the presence of excess of *b* subunits.

The aggregation of the *a'* chains becomes more pronounced as Factor $XIII_a$ is exposed to higher Ca^{2+} concentrations (Fig. 8). This is consistent with the Ca^{2+} dependence of the dissociation of the enzyme; Fig. 8 indicates that excess of *b* subunits inhibits this aggregation of *a'* chains.

Cooke *et al.* (1974) reported that β-substrate enhanced the rate of inhibition of Factor $XIII_a$ in Ca^{2+} by either 5,5'-dithiobis-(2-nitrobenzoate) or a mercurial, i.e. at 0.73mg of enzyme/ml the Ca^{2+} concentration required for 50% reaction in 10min decreased

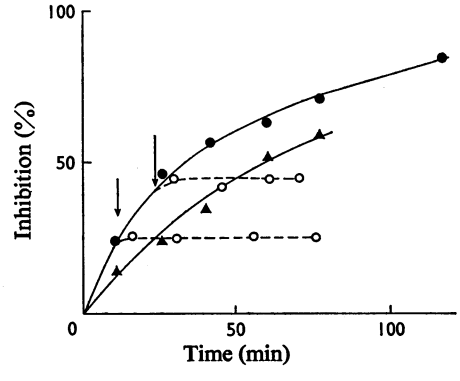


Fig. 7. *Effect of excess of b_2 protein on the rate of inhibition of Factor $XIII_a$ by 5,5'-dithiobis-(2-nitrobenzoate) in 10mM-Ca^{2+}*

Factor $XIII_a$ (0.5mg/ml) was incubated in the presence of 5,5'-dithiobis-(2-nitrobenzoate) (0.5mM) and Ca^{2+} at 25°C (●). A parallel incubation was carried out in the presence of 4.5mg of component B/ml (▲). Portions from each incubation mixture were assayed periodically. The effect of Ca^{2+} chelation was demonstrated by the addition of 20mM-EDTA (pH adjusted to 7.5 with 5M-NaOH) at the times indicated by the arrows, and by monitoring the subsequent rate of inhibition (○).

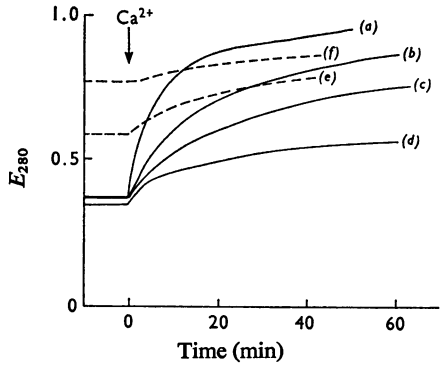


Fig. 8. *Effect of Ca^{2+} and component B on the rate of turbidity formation of Factor $XIII_a$ (0.22mg/ml in 0.1M-Tris–HCl buffer, pH7.5, at 25°C)*

The extinctions of the solutions were monitored at 280nm by using a Hilger–Gilford recording spectrophotometer. Ca^{2+} concentrations used were: (*a*) 30mM, (*b*) 15mM, (*c*) 7.5mM and (*d*) 3.75mM. The effect of component B on turbidity formation at 15mM-Ca^{2+} is shown by (*e*) 0.2mg of component B and (*f*) 0.5mg of component B/ml.

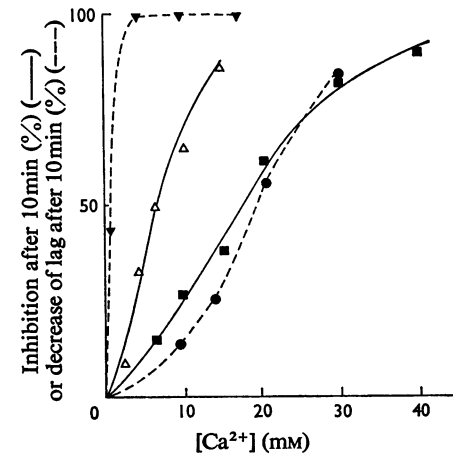


Fig. 9. *Effect of β-substrate on the abolition of the lag phase in Factor $XIII_a$ assays and the inhibition of Factor $XIII_a$ by 5,5′-dithiobis-(2-nitrobenzoate)*

(*a*) Samples of Factor $XIII_a$ (0.8mg/ml) were incubated with various Ca^{2+} concentrations at 25°C. After 10min, samples (10μl) were assayed with 0.2mM-Ca^{2+} in the assay medium, after mixing (●). A parallel set of incubations was done in the presence of 10mg of β-substrate/ml (▼). (*b*) Enzyme (0.73mg/ml) was incubated with 5,5′-dithiobis-(2-nitrobenzoate) (0.5mM) for 10min (■) as described by Cooke *et al.* (1974). The percentage inhibition was then determined. A parallel experiment was performed in 20mg of β-substrate/ml (△).

from 18 to 7mM in the presence of 20mg of β-substrate/ml. The effect of β-substrate on the abolition of the lag phase in assays performed at low Ca^{2+} concentrations is much more marked (Fig. 9).

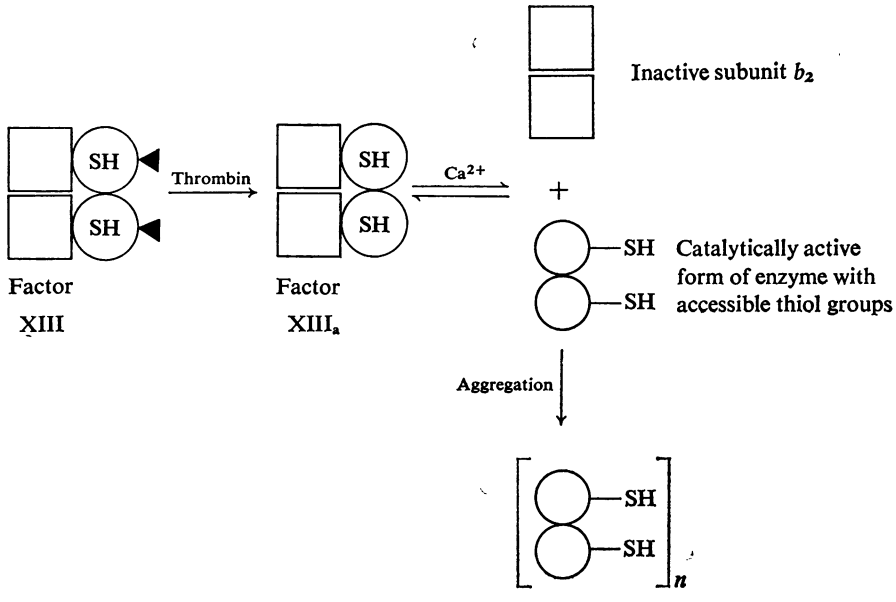
Ca^{2+} dependence of platelet Factor $XIII_a$

The zymogen, platelet Factor XIII, was not inhibited by preincubation with 5,5′-dithiobis-(2-nitrobenzoate) for 10min and assaying a portion by the thrombin-linked assay described by Cooke & Holbrook (1974*a*). However, unlike the plasma enzyme, the platelet Factor $XIII_a$ was 100% inhibited in less than 5min in the absence of Ca^{2+} (or in 3mM-EDTA). Similarly, no lag phases were observed in the attainment of the assay steady-state rates at low Ca^{2+} concentrations, in sharp contrast with the behaviour of the plasma enzyme. The platelet Factor $XIII_a$ stored at 0°C in 0.1M-Tris–HCl buffer, pH7.5, containing 1mM-EDTA has a relatively short half-life of 2–3h (no loss in activity of the plasma enzyme occurred during a 4h storage under identical conditions). This provides further evidence that the essential thiol group of the platelet enzyme is accessible in the absence of Ca^{2+}.

Fig. 3 shows a comparison of the Ca^{2+} dependence of the steady-state rates of the plasma and the platelet enzyme. The K_m for the two enzymes are about 0.17 and 0.07mM respectively. This difference may be a consequence of the plasma enzyme activity recorded at low Ca^{2+} concentrations, being decreased by the long lag phases (discussed above), resulting in an erroneously high K_m value.

Discussion

The simplest scheme that will explain the protein-concentration dependence of the enzyme reactivity reported in the present paper is shown below. The important feature is that the Ca^{2+}-dependent conformation change is linked to the equilibrium between a'_2b_2 tetramer and forms containing only a' or b subunits. Dissociation of the tetramer results in the appearance of 1 reactive thiol group/a' chain and the appearance of catalytic activity. Dilution of such an equilibrium system results in a greater proportion of the protein being present in the dissociated form. The non-physiological Ca^{2+} concentrations used in the thiol inhibition and lag-phase experiments are a direct consequence of the high, non-physiological protein concentrations used. The model (Scheme 1) predicts that the steady-state rate at a particular Ca^{2+} concentration is proportional to the ratio a'_2/a'_2b_2 (ignoring subsequent aggregation of the dimer), and that the steadily accelerating kinetics of the lag phase is a reflexion of the slow attainment of this equilibrium. It is not possible to speculate about the mechanism involved in the linked equilibrium, since all the information presented here pertains to equilibria rather than kinetics.



Scheme 1. *Model for the activation of plasma Factor $XIII_a$ by Ca^{2+}*

The model (Scheme 1) indicates that the rate of *a'*-chain aggregation depends on the Ca^{2+} concentration (i.e. concentration of *a'* chains). This is consistent with the data in Fig. 8, which also shows the inhibitory effect of excess of *b* subunits on the *a'*-chain aggregation, presumably because of the effect on the dissociation of the tetrameric enzyme. The aggregation of the *a'* chains is responsible for the fractionation into insoluble *a'* aggregates and soluble *b* chains reported by Cooke & Holbrook (1974*b*). The aggregate is still active (though it is possible that it dissociates under assay conditions), but it is possible that steric factors impose some limitations on its activity. This is perhaps why the thiol-inhibition curves are biphasic (e.g. Fig. 7), the thiol becoming less accessible as the *a'* chains aggregate. This slow sequestering of the *a'* chains is a complicating feature of the linked equilibrium, especially at high protein and Ca^{2+} concentrations where its effect will be most noticeable. The extent of inhibition by 5,5'-dithiobis-(2-nitrobenzoate) is decreased in the presence of excess of *b* chains (Fig. 7), which is compatible with the effect of *b* chains in suppressing the dissociation of the enzyme. The present finding that the *b* chains must be removed from contact with the *a'* chains for the expression of catalytic activity confirms the structural role of the *b* chains. The *b* chains must either mask the active site of the *a'* chains or inhibit a conformation change of the *a'* chains required for exposure of the active site.

Human platelet Factor XIII (a_2) is closely related to the plasma Factor XIII (discussed in the introduction). The experiments described here show that the phenomena associated with the dissociation of the plasma enzyme are not observed with the platelet enzyme, i.e. thiol inhibition is not dependent on Ca^{2+}, and there is no lag in attainment of the steady-state assay rate at low Ca^{2+} concentration. This is consistent with the model proposed for the Ca^{2+} activation of the plasma enzyme. The model shows that Ca^{2+} is required to dissociate plasma Factor $XIII_a$, but this does not exclude the possibility that Ca^{2+} has an additional role in the enzyme mechanism. Indeed, the Ca^{2+} dependence of the platelet enzyme (Fig. 3) indicates that Ca^{2+} is involved in the mechanism of the *a'* subunits. It is conceivable that Ca^{2+} is required for substrate binding in a similar manner to the mechanistically related guinea-pig liver transglutaminase. Ca^{2+} binding to this monomeric enzyme (mol.wt. 80000) causes a conformation change necessary for substrate binding (Folk *et al.*, 1967; Folk, 1969), though there is no gross change in size or shape of the molecule (Connellan *et al.*, 1971). The existence of at least two roles (and hence probably sites) for Ca^{2+} in the mechanism of the plasma enzyme

may be responsible for the sigmoidal Ca^{2+} dependence of its reactivity (Figs. 5 and 9).

An important difference between the guinea-pig liver enzyme and plasma Factor $XIII_a$ is that glutamine substrates protect the essential thiol of the former (Folk & Cole, 1966), whereas the susceptibility to inhibition was enhanced in the latter (Cooke *et al.*, 1974). This can be explained on the basis of the model if the association between β-substrate and the a' chains leads to further dissociation of the tetramer. Alternatively, substrate binding to the enzyme may decrease the Ca^{2+}-binding constant, promoting the dissociation of the tetramer. The effect of β-substrate on the abolition of the lag phase is more pronounced (Fig. 9). This differential substrate effect can most simply be explained by suggesting that the liganded a' subunits are in fact protected from thiol inhibition, but that this protection is much less effective than that conferred by the b subunits in the tetrameric form of plasma Factor $XIII_a$.

Preliminary experiments showed that β-substrate (3mg/ml) greatly inhibited the Ca^{2+}-dependent aggregation of the a' subunits of Factor $XIII_a$ (plasma or platelet factors at about 0.3mg/ml). One can conclude that a'-chain aggregation probably does not occur *in vivo*, because of the large excess of fibrin substrate that is present. The concentration of a' chains present under physiological conditions is very much less than that used in these experiments; this also militates against the formation of aggregates.

Evolutionary significance of platelet and plasma Factor XIII and the role of the plasma b subunits

Hawkey (1970) has summarized the evolutionary significance of cells in blood coagulation. There is a progressive increase in the number of plasma components of the clotting mechanism from *Limulus*, in which there are no pre-existing plasma components, to mammals. Heilbrunn (1961) suggested that this progressive increase in the complexity of the plasma system is due to an evolution from basic cellular and protoplasmic coagulation reactions to plasma clotting. This evolution might have been achieved by a transfer of these haemostatic functions from cells (platelets) to the plasma (Macfarlane, 1961). Alternatively, mutations in enzymes of other plasma systems could have been interposed in the existing stages of the plasma coagulation system. Factor XIII could be an example of the partial evolution of a clotting function from being present only in platelets to also existing in the plasma. The catalytic subunit is still synthesized in the platelet but at some stage in the cell cycle it is released into the plasma, where it combines with the stabilizing b subunits (probably synthesized in the liver). Bohn (1972) and Schwartz *et al.* (1973) have shown that a chains from platelet Factor XIII will spontaneously combine with b chains to give a species identical with plasma Factor XIII. Final proof of the protective role of the b chains requires a study *in vivo* of the half-lives of the a_2 (platelet) and plasma forms of Factor XIII in plasma. An alternative hypothesis can be proposed based on the fact that the sialic acid content of the b chains is much greater than that of the a chains (Bohn *et al.*, 1973). Morell *et al.* (1971) have suggested that removal of sialic acid residues provides a signal for rapid catabolism of plasma glycoproteins; if this mechanism operates for the plasma coagulation factors, the combination with b chains could prevent the rapid catabolism of the a chains. The b chains may fulfil both the role of enhancing the chemical stability of the Factor XIII a chains, and the role of preventing their rapid catabolism.

This degeneracy of functions in the platelet and plasma is also found with fibrinogen (Castaldi & Caen, 1965; Nachman *et al.*, 1967). The plasma fibrinogen, which is synthesized in the liver (Straub, 1963), is very similar to or identical with the platelet fibrinogen (Bezkorovainy & Rafelson, 1964; Solum & Lopaciuk, 1969; James & Ganguly, 1973). There is no information yet about the physicochemical relationship between plasma Factors XII and XI, and the platelet molecules responsible for a similar coagulation activity reported by Walsh (1972) and Schiffman *et al.* (1973). This degeneracy of Factor XIII and fibrinogen is consistent with divergent evolution from platelet to plasma. It is noteworthy that the transglutaminase cross-linking system that is the essential haemostatic mechanism of the crustaceans is entirely cellular (Duchateau & Florkin, 1954; Doolittle & Lorand, 1962; Fuller & Doolittle, 1971).

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