An Equilibrium Study of Metal Ion Binding to Human Plasma Coagulation Factor XIII

By BARBARA A. LEWIS, JEAN-MARIE FREYSSINET and J. JOHN HOLBROOK
Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

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1. The binding of Ca^{2+} to plasma coagulation Factor XIII from man and from cow caused a small decrease in the intrinsic fluorescence of the protein with a dissociation constant of 0.1 mM. A similar decrease was observed with the thrombin-activated Factors (Factors XIIa). The decrease in protein fluorescence was also caused by both Ni^{2+} and Mn^{2+} but not by Mg^{2+} . 2. $^{45}\text{Ca}^{2+}$ binding was directly demonstrated by equilibrium dialysis. Ca^{2+} at 0.2 mM bound to Factor XIII (a_2b_2) and Factor XIIIa (a'_2b_2) but not to isolated b_2 -protein. A tight-binding site for Ca^{2+} is associated with the a -subunits. 3. The Ca^{2+} essential for the enzyme activity of Factor XIII from man, pig and cow can be replaced by Ni^{2+} , Cu^{2+} , La^{3+} , Mn^{2+} , Fe^{3+} , Y^{3+} , Co^{2+} , Sr^{2+} or Tb^{3+} , but not by Mg^{2+} .

Factor XIII is the zymogen of the enzyme responsible for the stabilization of the fibrin gel in the final stage of the blood-clotting process. The enzyme has been shown to modify fibronectin in a reaction that has been associated with fibroblasts and wound healing (Keski-Oja *et al.*, 1976). Human plasma Factor XIII consists of two different subunits arranged as a tetramer of composition a_2b_2 (Schwartz *et al.*, 1973; Cooke & Holbrook, 1974b). Thrombin cleaves a 36-residue peptide from each a -subunit and converts the zymogen into Factor XIIIa (a'_2b_2). Factor XIIIa is, in the presence of Ca^{2+} , the active enzyme and catalyses the cross-linking of fibrin (Doolittle, 1973). Systematically the enzyme is fibrin-glutamine-fibrin-lysine endo- γ -glutamyltransferase. The steady-state kinetics of the reaction are consistent with a substituted double-displacement acyl-enzyme mechanism (Chung & Folk, 1972; Curtis *et al.*, 1974) characteristic of a number of endo- γ -glutamyltransferases. Examples of this type of enzyme with less restricted substrate specificity have been found in guinea-pig hair follicle, where it cross-links keratin (Asquith *et al.*, 1970; Harding & Rogers, 1972), the guinea-pig liver (Folk & Chung, 1973) and the ox non-hairy epidermis (Buxman & Wuepper, 1976). The enzymes found in blood platelets, the placenta and the uterus, like plasma Factor XIII, exist in zymogen form and require activation by thrombin. All of the glutamyltransferases are absolutely dependent on Ca^{2+} for their activity and have a K_m for Ca^{2+} less than 1 mM [see the review by Folk & Chung (1973) and also Buxman & Wuepper (1976) and Cooke (1974)]. Plasma Factor XIIIa is an

exception to the above generalization in that many of its properties, such as the presence of a lag phase in the protein assay (Cooke & Holbrook, 1973), the dissociation and precipitation of a' -subunits (Cooke & Holbrook, 1974b), the activity in a small-substrate assay (Stenberg *et al.*, 1975) and the appearance of thiol-group reactivity (Cooke *et al.*, 1974), respond to Ca^{2+} concentrations well above 1 mM, sometimes as high as 50 mM. This unique and complex dependence on Ca^{2+} correlates with the unique occurrence in the plasma Factor XIII of b -subunits (Cooke, 1974). Changes of Ca^{2+} concentration in a low and a high concentration range induce different responses from plasma Factor XIII, and in the present paper we investigate the Ca^{2+} -binding sites on Factor XIII and its derivatives with a view to eventually distinguishing Ca^{2+} -binding sites required for catalysis from those associated with major changes in the tertiary and quaternary structure of the protein. We show that 'tight' Ca^{2+} -binding sites are always present, whether or not the zymogen has been cleaved by thrombin, and that the sites are present only on a -subunits.

Experimental

Cohn-I pastes from cryosupernatant of out-dated human plasma were generously given by the Protein Fractionation Centre of the Scottish National Blood Transfusion Association, Edinburgh, Scotland, U.K. Phenylmethanesulphonyl fluoride and dansyl-cadaverine were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Hammarsten casein was obtained from BDH Chemicals, Poole, Dorset, U.K. Bovine thrombin (95 N.I.H. units/mg) was from

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Leo Pharmaceutical Products, Ballerup, Denmark. Sephadex G-50 (coarse grade) was from Pharmacia Fine Chemicals, Uppsala, Sweden. $^{45}\text{CaCl}_2$ (sp. radioactivity 20mCi/mg of Ca) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Butyl-PBD scintillator [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Human plasma Factor XIII was isolated from Cohn-I paste essentially as described by Cooke & Holbrook (1974b). To suppress the generation of large amounts of Factor XIIIa we introduced two modifications which kept the proportion of Factor XIIIa below 3%: (i) the Cohn-I paste was dissolved in 0.5M-KCl/50mM-Tris/10mM-EDTA/1mM-phenylmethanesulphonyl fluoride adjusted to pH7.5 with 10M-HCl. The sulphonyl fluoride was used to inhibit any residual trace of serine proteinases [see Magnusson (1974) for general references], which could have been responsible for the activation of Factor XIII; (ii) 5% (w/v) of BaSO_4 was added to the Cohn-I solution, which was stirred for 15min at 20°C, then centrifuged at 2000g for 15min to remove the BaSO_4 . This step, used in the preparation of prothrombin and Factor X (Esnouf *et al.*, 1973), was introduced in an attempt to remove traces of these two zymogens and did not affect the specific activity of the Factor XIII, which remained in the supernatant.

Factor XIII from cow plasma was prepared by the method of Takagi & Konishi (1972). Factor XIII from pig plasma was prepared by the same method except that the DEAE-cellulose column was equilibrated in a buffer of lower ionic strength (0.02M-Tris/1mM-EDTA adjusted to pH7.5 with 10M-HCl).

Factor XIIIa was prepared by incubation of Factor XIII with thrombin (10 N.I.H. units/ml) at room temperature for 20min. The activated enzyme was stored at 0°C for up to 8h without loss of activity.

b_2 -Protein was prepared from Factor XIIIa (20mg/ml) by incubation with 33mM- CaCl_2 in 0.1M-Tris, adjusted to pH7.5 with 10M-HCl, overnight at room temperature. The white precipitate containing α' -subunits was removed by centrifugation at 12000g for 10min. CaCl_2 was removed from the supernatant containing b_2 -protein by passage through a column (1cm $\times$ 30cm) of Sephadex G-50 (coarse grade) equilibrated in 0.1M-Tris/HCl buffer, pH7.5 (in the protein fluorescence experiments), or by dialysis for 24h against 3 $\times$ 30vol. of the same buffer (in the equilibrium-dialysis experiments). Factor-XIIIa activity was assayed by the method described by Cooke & Holbrook (1974a), which continuously measures the increase in fluorescence as dansyl-cadaverine is incorporated into dephosphorylated acetylated β -casein in the presence of 5mM-

CaCl_2 . One unit of enzyme activity is the amount of enzyme that gives 1% increase in the starting fluorescence/min at 25°C. In some experiments CaCl_2 was omitted from the assay medium and replaced by chlorides of various other metals.

The dansyl-cadaverine incorporated into casein in the presence of Factor XIIIa was directly measured: the fluorescence assay was stopped after 5min by the addition of trichloroacetic acid to a final concentration of 15% (w/v). The cuvette contents were centrifuged at 1000g for 5min and the protein precipitate was washed with 4 $\times$ 5ml of 6% trichloroacetic acid. The washed precipitate was dissolved in 5ml of 1% sodium dodecyl sulphate in 0.1M-Tris/HCl buffer, pH7.5. The dansyl fluorescence was measured with reference to standard solutions of 10 μM - and 5 μM -dansyl-cadaverine. The trace fluorescences of controls in which Factor XIIIa was replaced by the zymogen Factor XIII were subtracted.

In the presence of 5mM- CaCl_2 , 7.5 fluorescence units of Factor XIIIa catalysed the incorporation of 30nmol of dansyl-cadaverine into casein/min, i.e. 1 fluorescence unit of Factor XIIIa represents the amount of enzyme that catalyses incorporation of 4nmol of dansyl-cadaverine/min.

Equilibrium dialysis

All solutions were prepared in distilled de-ionized water. CaCl_2 solutions (10 μM –3mM) containing a constant proportion (66 μM) of $^{45}\text{CaCl}_2$ were prepared in 0.1M-Tris/HCl buffer, pH7.5, and stored in polythene bottles at 4°C. Protein solutions (9–20mg/ml), routinely stored in the presence of 2mM-EDTA, were dialysed overnight against 0.1M-Tris/HCl buffer, pH7.5 (3 $\times$ 30vol.), to remove the chelating agent. Visking 36/32 dialysis tubing was boiled in 0.2M-EDTA, washed in deionized distilled water and stored in 0.1M-Tris/HCl buffer, pH7.5. The equilibrium dialysis was carried out in micro-dialysis cells as described by Walsh *et al.* (1976). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. ^{45}Ca radioactivity was assayed by liquid-scintillation spectrometry in toluene-based scintillator (4g of butyl-PBD/litre of toluene).

Protein fluorescence

Measurements were carried out with the dual-wavelength titrating spectrophotometer and fluorimeter described by Shore *et al.* (1975), with a 200W Xe-Hg arc (90/B00/1; Hanovia Inc. Oriel Scientific, P.O. Box 136, Kingston-on-Thames, U.K.), The excitation monochromator was set at 296nm and the protein fluorescence was observed through a Wratten 18A filter.

Results

Ca²⁺ binding monitored by protein-fluorescence changes

The active-site thiol peptide of Factor XIII (-Gly-Gln-Cys-Trp-) contains a tryptophan residue (Holbrook *et al.*, 1973) and we first tried to monitor Ca²⁺ binding by observing whether there was a change in the tryptophan fluorescence of the protein. Fig. 1 shows a typical experiment in which the protein fluorescence of human Factor XIII (0.2 mg/ml) was monitored during titration with CaCl₂. A -2% change was observed, with a dissociation constant for Ca²⁺ (K_{Ca}) of 0.13 mM. The average result obtained from three such experiments was $K_{Ca} = 0.12$ mM. We obtained an indistinguishable result when the human Factor XIII was replaced by that from cow. We also

investigated the binding of Ni²⁺ and Mn²⁺ to human and cow Factor XIII by this technique (Fig. 2). The binding of these metals was slightly weaker than that of Ca²⁺. The average values were K_{Ni} 0.25 mM and K_{Mn} 0.32 mM. Titration of the protein with MgCl₂ (up to 1 mM) caused no change in fluorescence.

Human Factor XIIIa (in the presence of catalytic amounts of thrombin) was also titrated with Ca²⁺ (Fig. 3) and we observed the same magnitude of decrease in protein fluorescence as with the zymogen, with a similar dissociation constant for Ca²⁺ ($K_{Ca} = 0.12$ mM), Ni²⁺ and Mn²⁺ also gave similar results with Factor XIIIa to those with Factor XIII (K_{Ni} 0.25 mM, K_{Mn} 0.32). We obtained similar fluorescence changes with the activated factor from cow. In these experiments the metal-ion concentration was not

Fig. 1. Effect of Ca²⁺ on the protein fluorescence of Factor XIII

(a) The fluorescence of Factor XIII (0.2 mg/ml) was recorded as CaCl₂ was continuously added to a stirred solution. The buffer was 0.1 M-Tris adjusted to pH 7.5 with 10 M-HCl at 25°C. The fluorescence is expressed as a percentage of the fluorescence of a control to which EDTA was added instead of CaCl₂. This corrected for dilution. (b) Analysis of the above results: $\alpha = \Delta\text{Fluorescence}/\Delta\text{Fluorescence as } [Ca^{2+}] \rightarrow \infty$; $K_{Ca} = 0.13$ mM.

Fig. 2. Effects of (a) Ni²⁺ and (b) Mn²⁺ on the protein fluorescence of Factor XIII

The experimental conditions were as for Fig. 1 except that NiCl₂ or MnCl₂ was added instead of the CaCl₂.

Fig. 3. Effect of Ca^{2+} on the protein fluorescence of b_2 -protein and of Factor XIIIa

The conditions were as for Fig. 1, except that b_2 -protein was 0.1 mg/ml. The Factor XIIIa was 0.2 mg/ml.

increased beyond 1 mM, to avoid the light-scattering problems from the protein aggregation known to occur at high Ca^{2+} concentrations (Cooke & Holbrook, 1974b). Under conditions similar to those above, Ca^{2+} did not affect the protein fluorescence of b_2 -protein (Fig. 3). This result is consistent with the suggestion that it is the α - and not the β -subunits that change their fluorescence in 0.2 mM- Ca^{2+} .

Direct Ca^{2+} binding by equilibrium dialysis

The results of equilibrium dialysis of Factor XIII, Factor XIIIa (in the presence of thrombin) and of b_2 -subunits against $^{45}\text{CaCl}_2$ in micro-dialysis cells are presented in Fig. 4. Recovery of ^{45}Ca radioactivity at the end of the experiment was always over 90% and of protein was over 95%. The results are less precise than those obtained by using protein fluorescence, yet they clearly demonstrate the saturation of tight Ca^{2+} -binding sites in the same range of Ca^{2+} concentrations that perturb the protein fluorescence. We have also attempted to detect much weaker Ca^{2+} binding, and indeed at 2.5 mM-free Ca^{2+} the bound Ca^{2+} increased to about 8 ions/molecule. Whether this weak binding is competition of Ca^{2+} for non-specific cation-binding sites, or whether it reflects occupancy of those sites that give rise to 'high'- Ca^{2+} effects, or both, is open to speculation. The binding curve reaches a plateau when each molecule of Factor XIII binds 1.2–1.5 atoms of Ca^{2+} . At the low Ca^{2+} concentrations that we studied, no binding of Ca^{2+} to b_2 -subunits could be measured. At low Ca^{2+} concentrations both Factor XIII and Factor XIIIa bound similar amounts of Ca^{2+} .

Fig. 4. Binding of Ca^{2+} to Factor XIII (●), Factor XIIIa (○) and b_2 -protein (△) monitored by equilibrium dialysis. Each micro-dialysis cell contained on one side of the membrane 20 μl of protein solution in 0.1 M-Tris adjusted to pH 7.5 with 10 M-HCl and on the other side 20 μl of the same buffer containing $^{45}\text{Ca}^{2+}$. The vertical bars are the S.E.M. of the results of 5–15 experiments at each Ca^{2+} concentration.

Replacement of the Ca^{2+} cofactor by other metal ions

The results of experiments in which Ca^{2+} ions were replaced by other metals in the fluorescence assay are summarized in Table 1. A wide range of metal ions (both bi- and ter-valent) can to some extent replace Ca^{2+} ions as cofactor for Factor XIIIa activity. It was possible that fluorescence artifacts could affect the measured rates in the fluorescence assay of activity. At the pH of the assay (7.5) there was precipitation of casein in the presence of some metals. This was not a serious problem in most cases, and even when it took place the double-beam instrument automatically corrected for the scattering (Cooke & Holbrook, 1974a). With Cr^{3+} , Ce^{3+} , Al^{3+} (and Tb^{3+} at concentrations over 1.25 mM), the precipitation was too heavy to allow any fluorescence measurements. No relative fluorescence change was observed in control experiments in which (a) Factor XIIIa was omitted from the assay or (b) Factor XIII was included in the assay but the thrombin required for activation was omitted, and we conclude that the changes in fluorescence that we observed reflected enzyme activity. This was confirmed for Tb^{3+} by measurement of 32 nmol of dansyl-cadaverine covalently bound to casein after exposure to Factor XIIIa (7.5 units at optimum conditions, i.e. 5 mM- Ca^{2+}) in the presence of 0.5 mM- Tb^{3+} for 5 min.

Discussion

Using equilibrium dialysis, or by measuring perturbed tryptophan fluorescence, we have established that both Factor XIII and Factor XIIIa, but not b_2 -protein, bind Ca^{2+} tightly. The fluorescence technique does not reveal the number of Ca^{2+}

Table 1. *Effect of different metal-ion cofactors on the activity of Factor XIIIa in a fluorescence assay*

The metal ion chlorides at 5 mM replaced the 5 mM-CaCl₂ present in the normal fluorescence assay (see the text). The enzyme was from human, cow or pig plasma. n.m., Not measured. Tb³⁺ was assayed at 0.5 mM-TbCl₃. Numbers of determinations are given in parentheses.

Metal	Rate of fluorescence increase (% of that in the presence of 5 mM-CaCl ₂)			Comments
	Human	Cow	Pig	
Ca ²⁺	100 (4)	100 (2)	100 (2)	
Ni ²⁺	220 (3)	n.m.	250 (2)	Light turbidity in both cuvettes
Mn ²⁺	55 (4)	59	63 (2)	
Co ²⁺	20 (2)	n.m.	18 (2)	Turbidity
Sr ²⁺	16 (2)	n.m.	18 (2)	Turbidity
La ³⁺	57 (2)	n.m.	57 (2)	Light turbidity
Fe ³⁺	45 (2)	n.m.	43 (2)	Turbidity
Y ³⁺	24 (2)	n.m.	29 (2)	Light turbidity
Cu ²⁺	130 (2)	n.m.	150 (2)	Turbidity
Cd ²⁺	n.m.	n.m.	10 (2)	Turbidity
Tb ³⁺	16 (3)	14 (2)	n.m.	Light turbidity at 0.5 mM-Tb ³⁺ . At 5 mM-TbCl ₃ the enzyme was precipitated
Mg ²⁺	0 (2)	0 (2)	0 (2)

Scheme 1. *Binding of Ca²⁺ to the a- and a'-subunits in Factor-XIII complexes*

ions bound. The equilibrium-dialysis experiments show that the tight-binding sites of the tetramer are saturated when 1.2–1.5 g-ions of Ca²⁺ are bound. We believe this is a low estimate, as even the presence of only 30% inactive molecules would increase the value to two tight Ca²⁺-binding sites per a₂b₂ tetramer. The b₂-protein does not have the tight Ca²⁺-binding sites. We suggest that these sites are on the a- and a'-subunits in a₂b₂ and a'₂b₂ tetramers, although it is remotely possible that the sites are on the a–b interface. These results are unified in Scheme 1.

Factor XIII is absolutely dependent on Ca²⁺ for activity and has a cysteine residue and a neighbouring tryptophan residue at the active site. Yet it might have been a coincidence that the tight-binding Ca²⁺ site decreased tryptophan fluorescence, and we have attempted to provide other evidence that the tight-binding Ca²⁺ site that changes protein fluorescence is related to catalysis. We observe that Ca²⁺ required for catalytic activity can be replaced, with varying effectiveness, by other metal ions, in particular Ni²⁺ and Mn²⁺, but not Mg²⁺. Similarly Ni²⁺ and Mn²⁺, but not Mg²⁺, induce the same decrease in protein fluorescence as Ca²⁺. Taken together these results

allow the conclusion that the 'tight' Ca²⁺-binding site is associated with catalysis in the a'-subunit of Factor XIIIa.

Although we have no reason to doubt that Ca²⁺ is the normal physiological cofactor for Factor XIIIa, it was interesting to observe the apparently low specificity of the enzyme from three different sources (human, cow and pig) with respect to metal ions. Such comparisons of the properties of an enzyme from different sources are useful, as the comparison should reveal which properties of the human enzyme are fundamental to its biological role and which are species-specific details of doubtful general significance.

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