

The role of γ -carboxyglutamyl residues in the positive cooperative binding of Ca^{2+} to blood coagulation factor X

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THE ROLE OF γ -CARBOXYGLUTAMYL RESIDUES IN THE POSITIVE COOPERATIVE BINDING OF Ca^{2+} TO BLOOD COAGULATION FACTOR X

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Summary

1. The calcium binding properties of factor X and its analogous decarboxy-protein have been compared with the aid of flow rate dialysis and ultraviolet difference spectroscopy.

2. Factor X binds approx. 20 mol of calcium per mol of protein. The first four sites exhibit positive cooperativity.

3. Changes in the ultraviolet difference spectrum when Ca^{2+} is bound suggest a conformational change.

4. In decarboxyfactor X low affinity of Ca^{2+} and no ligand-induced conformational change was observed. It is concluded that the presence of γ -carboxy-glutamate residues is a prerequisite for positive cooperative Ca^{2+} binding.

Introduction

The binding of Ca^{2+} to prothrombin requires the presence of γ -carboxylated glutamate residues [1–3]. Carboxylation of 10 glutamates in the aminoterminal (fragment 1) region of prothrombin has been shown to occur by action of vitamin K at a postribosomal stage [4–6].

The Ca^{2+} -mediated binding of prothrombin to phospholipid surfaces, a prerequisite of normal thrombin generation, is thought to occur via these residues [7,8]. It was reported that an abnormal prothrombin, isolated from the plasma of cattle treated with vitamin K antagonists and lacking residues does not bind to phospholipid vesicles [9].

Calcium binding to prothrombin [3,10–14] and prothrombin fragment 1 [3,13] has been investigated in a number of laboratories. It is shown that prothrombin can bind 10–14 mol of Ca^{2+} , of which about 4 mol bind with a high affinity. Ten Ca^{2+} binding sites are observed in fragment 1. Factor X, another, vitamin K-dependent clotting factor, shows positive cooperativity until 3–5 of the 20 sites are occupied [3]. This suggests that here too Ca^{2+}

binding is mediated by γ -carboxyglutamate residues.

In this article we compare Ca^{2+} binding to factor X and to decarboxyfactor X that is obtained from the plasma of cattle under phenprocoumon treatment. In this way it will be possible to demonstrate that γ -carboxyglutamate residues are necessary for cooperative Ca^{2+} binding.

Materials and Methods

Factor X and decarboxyfactor X were purified according to the method described previously [15]. All chemicals were analytical grade and obtained from Merck. $^{45}\text{Ca}^{2+}$ was obtained from the Radiochemical Centre, Amersham, U.K. Extinction coefficient ($E_{1\text{cm}}^{1\%}$ at 280 nm) and molecular weights used for calculation of the protein concentrations were 12.4 and 55 000, respectively, for both factor X and decarboxyfactor X [16]. Standard CaCl_2 solutions in 0.05 M Tris \cdot HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.02% sodium azide were prepared from reagent grade $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The Ca^{2+} concentration in these solutions was determined by titration [17].

Flow rate dialysis

The Ca^{2+} binding properties of factor X and decarboxyfactor X were studied by the flow rate dialysis technique according to Colowick [18]. In a representative experiment the upper chamber was filled with 2 ml of a solution containing about 1.5 mg protein in 0.1 M NaCl 0.05 M Tris \cdot HCl, pH 7.5, 10 μl of $^{45}\text{Ca}^{2+}$ solution (specific activity 8 $\mu\text{Ci}/\mu\text{g}$) was added to the upper chamber (final concentration, 12 μM). The steady state was established after 12 ml of 0.05 M Tris \cdot HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.02% sodium azide passed through the lower chamber at a flow rate of 4 ml/min. After the steady state was reached, small volumes (5–15 μl) of a non-radioactive Ca^{2+} solution were added to the upper chamber to increase the total Ca^{2+} concentration stepwise.

Fractions of 3 ml were collected and a 0.5 ml aliquot was mixed with 5 ml of a gelling scintillation solution (Aquasol, New England Nuclear) and counted in a Packard Tricarb liquid scintillation spectrometer. All experiments were performed at room temperature.

In an identical experiment, the steady state concentration of $^{45}\text{Ca}^{2+}$ was determined in the absence of protein. This control value will be a measure of the concentration of 100% free Ca^{2+} in the upper chamber (designated the 100% F value).

The Ca^{2+} binding properties of factor X and decarboxyfactor X were evaluated by plotting \bar{v} (mol Ca^{2+} bound/mol protein) versus $\bar{v}/\text{free } \text{Ca}^{2+}$ concentration (F) according to the method of Scatchard [19]. The ligand-binding properties were also expressed by the equation given originally by Hill [20]:

$$\log \frac{\bar{v}}{N - \bar{v}} = \log K + n_H \log [F] \quad (1)$$

in which \bar{v}/N is the fraction of the total number of binding sites occupied by ligand, $[F]$ is the free ligand concentration and K and n_H are constants. When $\log \bar{v}/(N - \bar{v})$ is plotted versus $\log [F]$, the slope (n_H , Hill coefficient) indicates

the presence of any site-site interaction. That is, binding is cooperative ($n_H \neq 1$) or non-cooperative ($n_H = 1$) [21].

Ultraviolet difference spectroscopy

Ultraviolet difference spectra were measured on an Aminco DW-2 double beam spectrophotometer equipped with a thermostated cell holder maintained at 25°C. A set of matched, rectangular quartz cells (Hellma 104 QS) with light path of 1.0 cm were used. To study the protein- Ca^{2+} interaction a Ca^{2+} solution was added in the sample cell and equal volume of buffer in the reference cell, using Hamilton microsyringes. Both cells contain approx. 1.5 mg factor X or decarboxyfactor X in 0.1 M NaCl 0.01 M Tris · HCl at pH 7.5. Difference spectra were recorded from 240 nm to 340 nm on a full scale of 0.05 absorbancy unit.

Results and discussion

Ca^{2+} binding by factor X and decarboxyfactor X

The flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence and absence of factor X are shown in Fig. 1A. The initial addition of $^{45}\text{Ca}^{2+}$ to the factor X solution

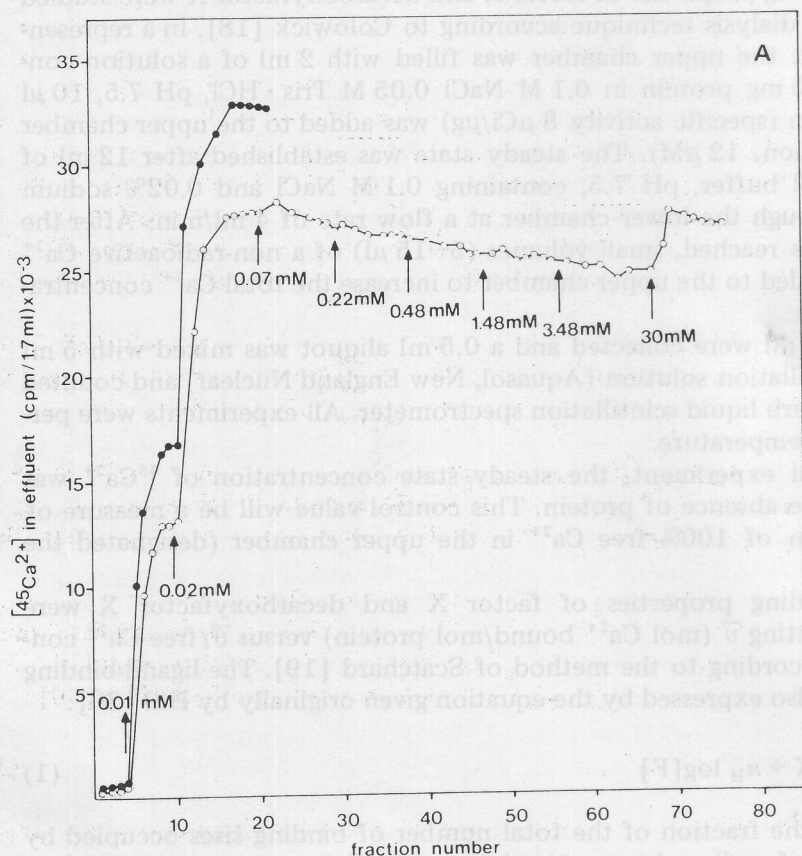


Fig. 1A.

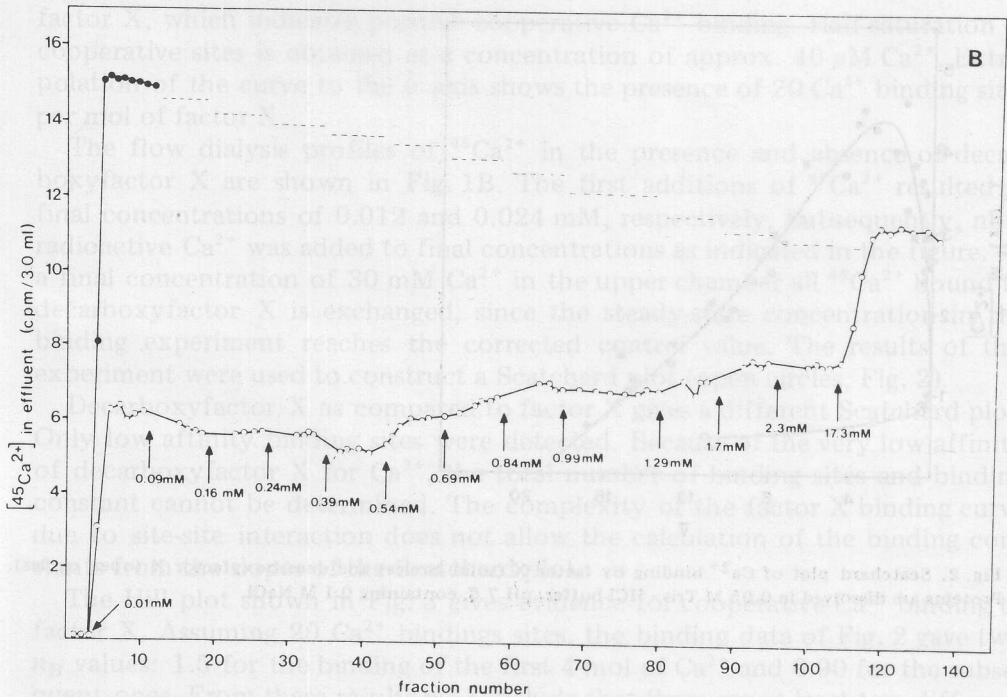


Fig. 1. A. Flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence (open circles) and absence (solid circles) of factor X. The first arrow indicates $^{45}\text{Ca}^{2+}$ introduced into 2.0 ml of 0.05 M Tris · HCl/0.1 M NaCl, pH 7.5, at a concentration of 0.012 mM. Subsequent additions of non-radioactive Ca^{2+} were made at concentrations indicated by the arrows. The corrected 100% F control values for each step in the experiment with factor X are represented by the dashed lines. For further experimental details see Materials and Methods. B. Flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence (open circles) and absence (solid circles) of decarboxy-factor X. The first two arrows indicate $^{45}\text{Ca}^{2+}$ introduced into 1.5 ml of 0.05 M Tris · HCl/0.1 M NaCl, pH 7.5, at concentrations of 0.012 and 0.024 mM, respectively. Subsequent additions of non-radioactive Ca^{2+} were made at concentrations indicated by the arrows. The corrected 100% F control values for each step in the experiment with decarboxyfactor X are represented by the dotted lines. For further experimental details see Materials and Methods.

(0.036 M) in the upper chamber resulted in a final Ca^{2+} concentration of 0.012 mM. Subsequent additions of non-radioactive Ca^{2+} are indicated by the arrows. The concentration of Ca^{2+} shown represents the final Ca^{2+} concentration in the upper chamber. Up to sample no. 10 the control curve (solid circles) represents experimental values. The dotted part of the curve is the calculated control curve compensated for dilution and cpm loss. Addition of excess Ca^{2+} (17 mM) resulted in a rise of the steady-state concentration of $^{45}\text{Ca}^{2+}$ to the level of the corrected control value. The radioactivity in the effluent, therefore, is indeed a measure for the free Ca^{2+} concentration. It also indicates that the steady state concentration in the effluent does not depend on the total Ca^{2+} concentration in the upper chamber. The absence of a spike of radioactivity in the effluent after addition of excess non-radioactive Ca^{2+} to the protein containing solution indicates that no $^{45}\text{Ca}^{2+}$ was bound to the membrane at this stage [22]. The binding curve for factor X (solid circles) obtained by plotting $\bar{v}/[\text{Ca}^{2+}]_F$ versus \bar{v} according to Scatchard [19] is shown in Fig. 2. The Scatchard plot shows a positive slope between 0 and 3–4 mol Ca^{2+} bound per mol

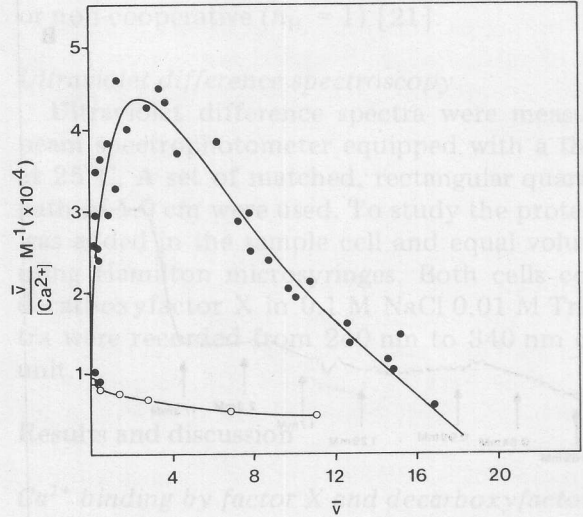


Fig. 2. Scatchard plot of Ca^{2+} binding by factor X (solid circles) and decarboxyfactor X (open circles). Proteins are dissolved in 0.05 M Tris · HCl buffer, pH 7.5, containing 0.1 M NaCl.

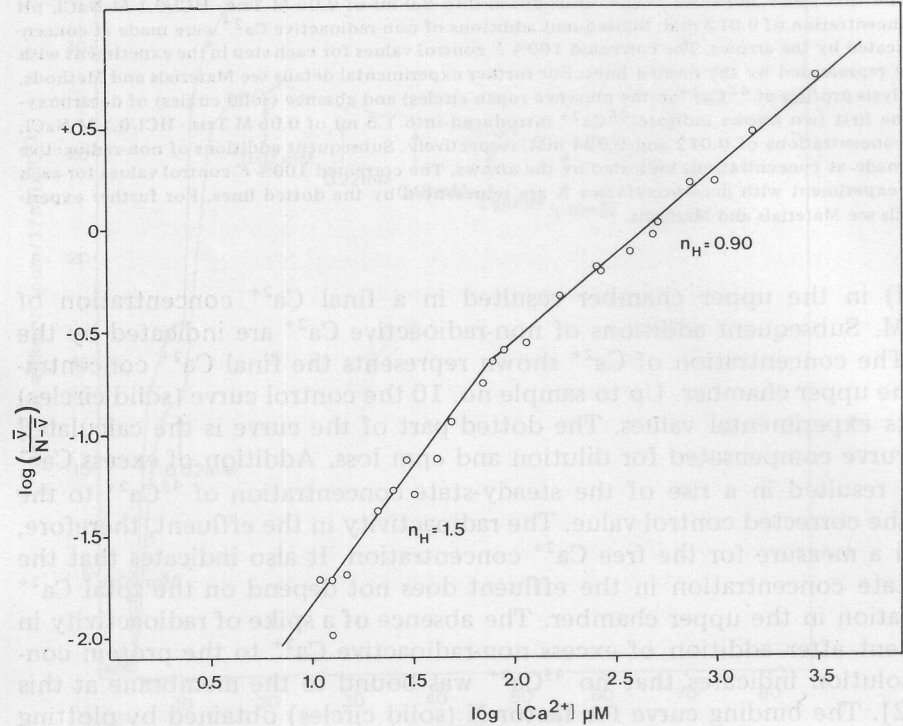


Fig. 3. Hill plot of the factor X Ca^{2+} binding data. Data are those of Fig. 2 which were obtained with factor X.

factor X, which indicates positive cooperative Ca^{2+} binding. Half saturation of cooperative sites is obtained at a concentration of approx. $40 \mu\text{M}$ Ca^{2+} . Extrapolation of the curve to the $\bar{\nu}$ axis shows the presence of 20 Ca^{2+} binding sites per mol of factor X.

The flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence and absence of decarboxyfactor X are shown in Fig. 1B. The first additions of $^{45}\text{Ca}^{2+}$ resulted in final concentrations of 0.012 and 0.024 mM, respectively. Subsequently, non-radioactive Ca^{2+} was added to final concentrations as indicated in the figure. At a final concentration of 30 mM Ca^{2+} in the upper chamber all $^{45}\text{Ca}^{2+}$ bound to decarboxyfactor X is exchanged, since the steady-state concentration in the binding experiment reaches the corrected control value. The results of this experiment were used to construct a Scatchard plot (open circles, Fig. 2).

Decarboxyfactor X as compared to factor X gives a different Scatchard plot. Only low affinity binding sites were detected. Because of the very low affinity of decarboxyfactor X for Ca^{2+} , the total number of binding sites and binding constant cannot be determined. The complexity of the factor X binding curve due to site-site interaction does not allow the calculation of the binding constants from the slopes of the Scatchard plot.

The Hill plot shown in Fig. 3 gives evidence for cooperative Ca^{2+} binding to factor X. Assuming 20 Ca^{2+} binding sites, the binding data of Fig. 2 gave two n_{H} values: 1.5 for the binding of the first 4 mol of Ca^{2+} and 0.90 for the subsequent ones. From these results we conclude that there are at least two different classes of binding sites, e.g., positive cooperativity in the binding of the first 3–4 calcium ions and about 16 sites showing no site-site interaction or mildly attenuating interactions, as would be expected for ions binding to a macromolecule.

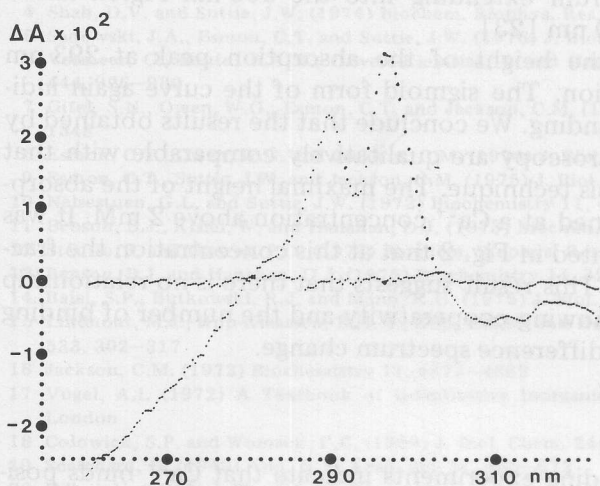


Fig. 4. Ultraviolet difference spectrum produced by the interaction of factor X with Ca^{2+} . Experimental conditions: 1.5 mg factor X dissolved in 0.01 M Tris · HCl/0.1 M NaCl buffer, pH 7.5. Final Ca^{2+} concentration in the sample cuvette: 5 mM.

cooperative Ca^{2+} binding. This is strongly supported by the results obtained with the ultraviolet difference spectroscopy experiments with factor X and decarboxyfactor X. Indeed, only in the case of factor X evidence for perturbation of a tryptophane environment and, hence, at least a minor conformational change on binding of Ca^{2+} is obtained. It is further shown that this Ca^{2+} -induced conformational change exhibits cooperativity. Although several models for the geometry of the Ca^{2+} binding to γ -carboxyglutamate are proposed [24–26], no conclusion can be put forward about the relationship between the different sets of sites (positive cooperative and non-cooperative) and the number of γ -carboxyglutamate residues involved in Ca^{2+} binding.

From recent studies [27,28] on metal-ion-induced transitions of prothrombin fragment 1, it appears that prothrombin fragment 1 has two classes of metal binding sites. One set of sites (about 4), apparently non-selective with respect to the metal ion bound, probably triggers a conformational transition, which leads to unmasking of a subsequent set of sites. This second set of sites must be filled in order to permit binding of fragment 1 to phospholipids and to obtain Ca^{2+} -mediated self-association of prothrombin fragment 1.

The question arises of whether the phenomenon of self-association causes the changes in the ultraviolet difference spectrum. This would provide a reasonable explanation for the observed difference between the number of sites showing positive cooperativity (3–4 calcium ions bound per mol factor X) and the number of sites which have to be filled in order to obtain a maximal change in the ultraviolet spectrum (about 10). Perturbation of the tryptophanyl residues by self-association may be superimposed on the perturbation due to site-site interaction of the initial binding sites.

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