WHEAT GERM LECTIN, A TOOL TO INVESTIGATE METAL ION-INDUCED STRUCTURAL CHANGES OF BOVINE BLOOD COAGULATION FACTOR X_1

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1. Introduction

Blood coagulation factor X, a plasma glycoprotein produced by the vitamin K-dependent pathway [1], consists of a light chain (M_r 16 000) that contains 12 γ-carboxyglutamic acid residues [2] and a heavy chain (M_r 39 000) that bears two carbohydrate moieties [3]. Factor X is the precursor of a serine protease, Factor Xa, that activates prothrombin by specific limited proteolysis in the last stages of blood coagulation [1]. Like prothrombin, factor X undergoes a cation-catalyzed protein transition which is a prerequisite to protein–phospholipid binding [4].

Conformation-specific antibodies have been used as probes of the γ-carboxyglutamic acid-rich region of bovine prothrombin to assess metal ion-induced structural changes [5,6]. Here, we report that the wheat germ lectin can also be used as a macromolecular probe to investigate metal ion-induced conformational changes of bovine factor X_1. The classical precipitin reaction between lectin and glycoprotein only occurs when certain metal ions are present. Ca^{2+} and Mn^{2+} are able to induce a precipitin reaction between wheat germ lectin and factor X. The reaction is slow at 2.5 mM metal ion but becomes more rapid when higher divalent cation concentrations are used. Mg^{2+} is ineffective in promoting such a precipitin reaction. The various conditions that give rise to a precipitin reaction demonstrate that the factor X_1 molecule must undergo a transition catalyzed by metal ions to allow binding of lectin to its specific sugar(s). This leads to suggestions on the nature of this structural change.

2. Materials and methods

Bovine factor X_1 was a kind gift from Dr E. W. Davie (University of Washington, Seattle) and was purified according to [7]. The factor X_1 preparation used in this study had a specific clotting activity of 60–70 units/mg. Activated factor X (factor Xa) was prepared according to [8] using Russel's viper venom (RVV). Diisopropyl fluorophosphate (DFP)-inactivated Factor Xa was obtained by incubating factor Xa (1 mg/ml) with 10 mM DFP for 2 h at 37°C; excess reagent was then eliminated by gel filtration on Sephadex G-25 or by dialysis. RVV and DFP were purchased from Sigma Chemical Co, St Louis. Factor Xa amidolytic activity was measured using *N*-benzoyl–L-isoleucyl–L-glutamyl–glycyl–L-arginine–*p*-nitroanilide HCl (S-2222), a chromogenic substrate from Kabi, Stockholm, in [9]. 2-Acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glycosamine), *N,N'*-diacetylchitobiose, lectin from wheat germ (*Triticum vulgaris*) prepared according to [10] and lectin insolubilized on Ultrogel AcA 22 (2% acrylamide, 2% agarose) were purchased from Réactifs IBF-Pharmindustrie, Villeneuve-la-Garenne. Lectin from wheat germ (WGA) was electrophoretically pure. All other materials were of the highest quality available.

Unless specified, all our experiments were performed in 10 mM morpholinopropane sulfonic acid buffer, containing 0.15 M NaCl and 0.01% (w/v) NaN_3, adjusted to pH 7.35 using 5 N NaOH. All the solutions were filtered before use. Hemagglutination assays of WGA were carried our according to [10] using a 3% suspension of human red blood cells (A group) washed ≥5-times in the above buffer. Protein concentrations were determined from A_{280} using $\epsilon^{1\%}_{280} = 12.4$ for factor X_1 [7] and $\epsilon^{1\%}_{280} = 15.0$ for WGA [10]. Sodium

dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis analysis was performed according to [11]; gels were stained for protein with Coomassie brilliant blue. Precipitin reactions between factor X_1 and WGA were followed by recording turbidity at 330 nm using a Beckman Acta MVI double beam spectrophotometer equipped with an automatic stirrer allowing vigorous stirring just after rapid addition of the component triggering the reaction. Stirring was stopped 2 s after addition and turbidity was immediately recorded using time constants ranging from 0.5–8 s. All our measurements were carried out at 22°C with a slit-width of 1 nm and were made in quartz semi-micro-cells of 1 cm optical pathlength, 0.4 cm width, containing 1 ml final vol.

3. Results

When bovine factor X_1 and wheat germ lectin were mixed at 1 mg/ml final conc. each, a visible precipitin reaction could be obtained only in the presence of Ca^{2+} at >1 mM. In the presence of 1 mM EDTA the protein solution remained limpid and it was not possible to detect any significant increase of turbidity even after prolonged incubation. Fig.1 shows an SDS–polyacrylamide gel electrophoresis analysis of the respective incubation products with 10 mM Ca^{2+} of bovine factor X_1 (1 mg/ml), WGA (1 mg/ml) and factor X_1 + WGA (1 mg/ml each). After 2 h incubation the precipitate formed in the last sample was recovered by centrifugation; the supernatant contained ~80% of the total protein and ~70% of the total factor X_1 activity. After several washes, the precipitate was dissolved in buffer containing 20 mM EDTA and 0.15 M *N*-acetyl-D-glucosamine or 2.5 mM *N,N'*-diacetylchitobiose as inhibitors of WGA (review [12]). Fig.1 shows that the precipitate was composed of factor X_1 and WGA and it reveals that some degradation of factor X_1 occurred during its exposure to calcium either in the presence or in the absence of WGA, it also indicates that WGA was not degraded when incubated with factor X_1 in the presence of calcium. Under reducing conditions WGA and the factor X_1 light chain migrate almost together.

When factor X_1 was added without incubation to WGA in the presence of 10 mM Ca^{2+} at 0.1 mg/ml final conc. for each of the proteins, the precipitin reaction between the two proteins could be followed by recording turbidity at 330 nm. At these protein con-

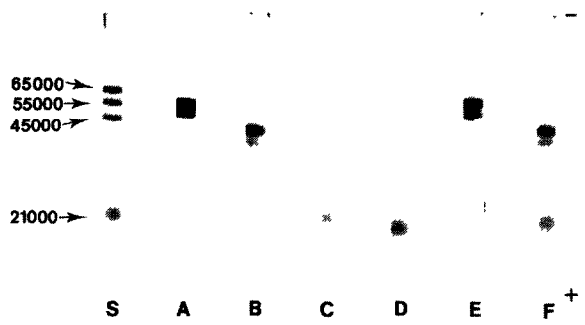

Fig.1. SDS–polyacrylamide gel electrophoresis analysis of the respective incubation products with 10 mM Ca^{2+} of bovine factor X_1, wheat germ lectin and factor X_1 + wheat germ lectin. Incubations were performed at room temperature (22°C) during 60 min. Gels are 7.5% (w/v) polyacrylamide.When mentioned, reduction of disulfide bridges was achieved by addition of 5% (v/v) β-mercaptoethanol: (S) M_r markers; (A) 30 μg bovine factor X_1 (M_r 55 000; the 50 000 M_r species reveals slight degradation of factor X_1 upon incubation with Ca^{2+}); (B) 30 μg reduced factor X_1 (heavy chain M_r 39 000; light chain M_r 16 000; the 35 000 M_r species results from degradation of the heavy chain of factor X_1 upon incubation with Ca^{2+}); (C) 30 μg wheat germ lectin (at neutral pH, WGA is a non-covalent dimer-a_2 M_r 36 000 which is dissociated in SDS and urea); (D) 30 μg reduced wheat germ lectin; (E) precipitate resulting of the incubation of factor X_1 + WGA in the presence of 10 mM Ca^{2+}, dissolved as in the text, ~30–40 μg protein (factor X_1 and its slightly degraded form) and WGA can be easily identified when compared to gels A and C, respectively); (F) as sample E but reduced (from this gel it is possible to identify factor X_1 heavy chain (and its slightly degraded form) and WGA when compared to gels B and D, respectively; under reducing conditions factor X_1 light chain and WGA migrate almost together).

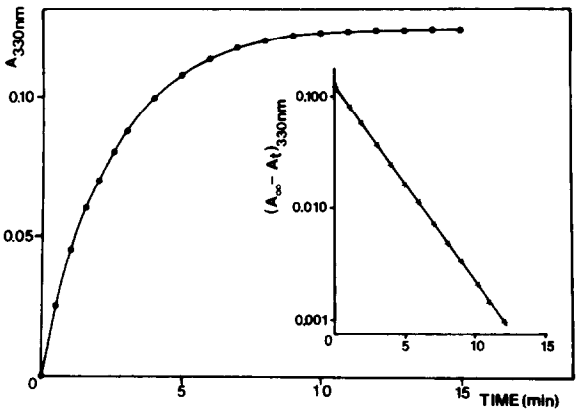

Fig.2. Typical precipitin reaction between bovine factor X_1 and wheat germ lectin in the presence of 10 mM Ca^{2+} (monitored by recording A_{330}) and its first order analysis. Factor X_1 and WGA were at 0.1 mg/ml each. Reactants were added without preincubation. Measurement was performed at 22°C.

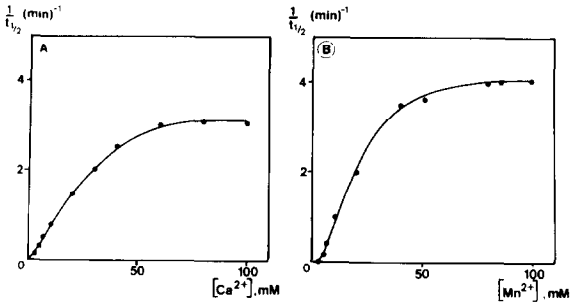

Fig.3. Variations of the inverse of the $t_{1/2}$ of precipitin reaction between bovine factor X_1 and wheat germ lectin with divalent cation concentrations: (A) Ca^{2+}; (B) Mn^{2+}. (For conditions see legend of fig.2.)

centrations the increase of turbidity had a pseudo-first-order profile (fig.2). To preserve these pseudo-first-order conditions we kept final protein concentrations constant at 0.1 mg/ml each and we determined the variations of the inverse of the halftime of the precipitin reaction as a function of $[Ca^{2+}]$ (fig.3A) and $[Mn^{2+}]$ (fig.3B) over 0–100 mM. From fig.3 it can be seen that the variations of the inverse of the half-time with metal ion concentrations have a slight sigmoidal profile, but our measurements were not precise enough at low cation concentration to allow interpretation of this tendancy. Mg^{2+} was totally ineffective in promoting such a precipitin reaction. The presence of 2 mM DFP did not affect the precipitin reaction. At >100 mM Ca^{2+} or Mn^{2+}, increasing metal ion concentration slowed down the reaction; increasing the ionic strength had a similar effect (table 1). Although very slow and weak, the precipitin reaction could still occur at high ionic strength up to 0.8 M. In each case, both an excess of EDTA and an inhibitory concentration of specific sugar of WGA were necessary to allow dissolution of the protein precipitate.

Table 1
Effect of ionic strength on the rate of the precipitin reaction between factor X_1 and WGA in the presence of Ca^{2+}

Ca^{2+} (mM)	Mg^{2+} (mM)	NaCl (mM)	$t_{1/2}$ (min)
100	0	150	0.32
150	0	150	0.45
100	50	150	0.45
100	0	300	0.4
10	0	150	1.4
20	0	150	0.7
10	10	150	2.2
10	0	300	1.8
10	0	800	~10.0

To determine whether divalent metal ions would have had any effect on lectin activity we verified that WGA hemagglutinating activity remained unchanged when assayed in the presence of 10 mM of either EDTA, Ca^{2+}, Mn^{2+}, Mg^{2+}, or Ca^{2+} + Mg^{2+}. Furthermore, when WGA was incubated with various concentrations of Ca^{2+} or Mn^{2+} at 22°C for ⩾30 min prior to the addition of factor X_1, the precipitin reaction was still a pseudo-first-order reaction with the same half-time as that measured without incubation (fig.4A). However when factor X_1 was incubated with various $[Ca^{2+}]$ or $[Mn^{2+}]$ at 22°C for 1–60 min, the precipitin reaction following the addition of WGA no longer had a first-order profile (fig.4B), the longer the incubation the more pronounced the effect. This last behaviour can be partially explained by activation or slight degradation of factor X_1 when incubated with Ca^{2+} (see fig.1) since when this incubation was performed in the presence of 5 mM DFP the deviation from pseudo-first-order reaction was attenuated but not totally abolished. Calcium-induced auto-aggregation of factor X_1 [8] could also prevent it from interacting with WGA.

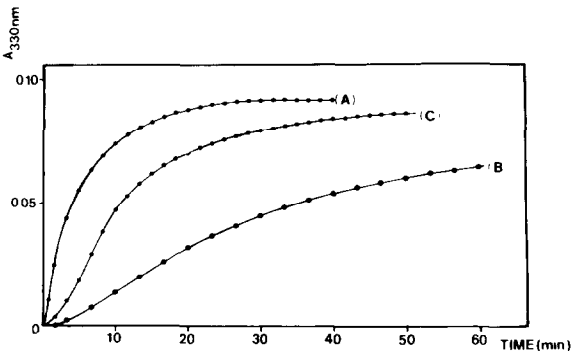

Fig.4. Various conditions of obtaining a precipitin reaction between bovine factor X_1 and wheat germ lectin in the presence of 5 mM Ca^{2+}: (A) WGA (to give 0.1 mg/ml final conc.) was preincubated with 5 mM Ca^{2+} during 30 min before triggering the reaction by the addition of factor X_1 (0.1 mg/ml final conc.); (B) factor X_1 (to give 0.1 mg/ml final conc.) was preincubated during 30 min with 5 mM Ca^{2+} before triggering the reaction by the addition of WGA (0.1 mg/ml final conc.); (C) factor X_1 and WGA (to give each 0.1 mg/ml final conc.) were preincubated during 30 min together with 1 mM EDTA before triggering the reaction by the addition of Ca^{2+} to give 6 mM final conc. (5 mM effective final conc.). All measurements were performed at 22°C.

When factor Xa was used instead of factor X_1 we could not obtain a precipitin reaction with WGA in the presence of Ca^{2+} or Mn^{2+} nor when we used DFP-inactivated factor Xa. Factor Xaβ, the final activated form of factor X, has no carbohydrate [1] thus explaining the lack of reaction with WGA.

When factor X_1 and lectin were incubated in the presence of 1 mM EDTA, under the same physical conditions as above, prior to the triggering of the precipitin reaction by addition of an excess of Ca^{2+}, the reaction was not of pseudo-first-order either in the presence or in the absence of 2 mM DFP (fig.4C) and, in this case also, the longer the incubation the more pronounced the effect. To explain this observation it is reasonable to assume that, in the presence of EDTA, WGA might be involved in a weak and non-precipitin interaction with a saccharide of one of the factor X_1 carbohydrate chains, but as soon as an excess of Ca^{2+} or Mn^{2+} is added more specific sugars become accessible to the lectin, so that the weak interaction would be gradually replaced by a stronger one giving a precipitin reaction, thus justifying the appearance of a lag phase (fig.4C).

Since the presence of Ca^{2+} or Mn^{2+} is absolutely necessary to induce a precipitin reaction between bovine factor X_1 and wheat germ lectin and since preincubation of WGA with Ca^{2+} or Mn^{2+} has no influence on this reaction, we conclude that factor X_1 must undergo a cation-catalyzed transition which allows interaction with lectin to take place. Nowhere in the literature is action of Ca^{2+} or Mn^{2+} on WGA lectin activity mentioned (review [12]).

Finally, we were not able to detect any complexation of bovine factor X_1 by insolubilized WGA even in the presence of Ca^{2+} or Mn^{2+}. This absence of interaction could be due to the density of the crosslinks between lectin and the matrix which would prevent the factor X_1 molecule from attaching to the lectin.

4. Discussion

The results presented here demonstrate that a metal ion-catalyzed conformational change affecting factor X_1 is a prerequisite to glycoprotein–lectin precipitin interaction. When Ca^{2+} or Mn^{2+}, but not Mg^{2+}, are present, specific sugars become accessible to WGA on the carbohydrate chains of factor X_1. Whether this cation–glycoprotein interaction takes place through a direct interaction between carbohydrate moieties and cation or a direct interaction between protein moiety and cation or both remains an open question. It has been reported that calcium is required for two roles in factor X–phospholipid interaction: catalysis of a protein transition and protein–phospholipid binding, the first reaction being necessary to allow the second one [4]. These authors found that, although Mn^{2+} is more potent to induce protein transition, Ca^{2+} is the only cation able to catalyze both protein transition and protein–phospholipid binding in a physiological concentration range (2.5 mM); Mg^{2+} is only poorly effective. In our study Ca^{2+} and Mn^{2+} have comparable efficiency and have a detectable effect only at $\geqslant$2 mM (fig.3A,B); Mg^{2+} is totally ineffective, and more important still there is no precipitin reaction between factor X_1 and WGA in the presence of EDTA. Thus, the differences of divalent cation specificity and active concentration towards catalysis of structural changes of bovine factor X_1 in [4] and factor X_1–WGA precipitin reaction in our study point out that the conformational changes involved in both of these studies have to be different. Physiologically, during the blood clotting process, platelet calcium [13] becomes available after platelet release reaction, possibly causing local high calcium concentrations.

The structures of the carbohydrate moieties of bovine blood coagulation factor X_1 are now known [3]: it contains both asparagine-linked and threonine-linked oligosaccharides; the carbohydrate moieties of the factor X subgroups, factors X_1 and X_2, are identical. Asparagine-linked sugar chains only contain GlcNAc which WGA can bind [12] and if factor X_1 has only two carbohydrate moieties [3], this implies that in the presence of Ca^{2+} or Mn^{2+} the interaction with wheat germ lectin has to arise only with this particular asparagine-linked carbohydrate moiety. Since this carbohydrate moiety has a limited size and if one assumes that, having comparable M_r-values (36 000 and 55 000, respectively), WGA and factor X_1 have comparable dimensions, one can reasonably explain the relatively low affinity of lectin for glycoprotein by the difficulty to form an extended lattice between lectin and glycoprotein.

Lectins are widely used in the field of cellular biology and extensive data on their specificity are now available [12]. At a molecular level, our study shows that they might be potent tools to assess structural changes occurring in glycoproteins, especially to study those whose glycan primary structure is known ([14]).

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