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THE ROLE OF THE GLA DOMAIN IN THE ACTIVATION OF BOVINE COAGULATION FACTOR X BY THE SNAKE VENOM PROTEIN XCP

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SUMMARY: The activation by XCP of coagulation factor X and a factor X species lacking the Gla-domain was studied in the presence and absence of Ca^{2+}. Both proteins could be activated at low rates in the absence of Ca^{2+}. The activation of the unmodified factor X was stimulated by the addition of Ca^{2+}, whereas GD factor X activation was insensitive to Ca^{2+}. The stimulatory effect of Ca^{2+} seen with the unmodified factor X correlated strongly with a calcium-dependent change in intrinsic protein fluorescence. This conformational change required the Gla-domain as the fluorescence emission of GD factor X was the same with or without Ca^{2+}. Fluorescence changes which accompanied activation were the same for both factor X and GD factor X. This suggests that the Gla-domain does not participate in the structural changes which accompany activation.

Bovine coagulation factor X is a vitamin K dependent plasma glycoprotein (1) containing 12 Gla residues clustered within the first 40 amino acids of the light chain (2). Factor X may be activated *in vivo* by either the intrinsic (3) or extrinsic (4) coagulation pathways. This activation involves a Ca^{2+} dependent, Gla mediated binding of the vitamin K dependent factors to a negatively charged surface (5). Factor X may also be activated by a protein (XCP) isolated from the venom of *Vipera russelli* (6). The activation by XCP does not require a negatively charged surface but the activation still has a requirement for exogenous Ca^{2+} (7). However, this exogenous Ca^{2+} is apparently not necessary for the catalytic activity of XCP as the enzyme cleaves Apoprotein AI in the absence of Ca^{2+} (8). This observation, plus the fact that factor X may still be activated by XCP after the region of the

Abbreviations: Gla, γ-carboxyglutamic acid; GD factor X, Gla domainless factor X; XCP, factor X coagulant protein; TBS, 0.02 M Tris-HCl, 0.1 M NaCl, pH 7.5; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; S2222, Bz-Ile-Glu-Gly-Arg-p-Nitroanilide.

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light chain containing the Gla residues had been removed (9), led us to investigate the role of the Gla residues of factor X in its activation by XCP.

MATERIALS AND METHODS

Materials: The venom of Vipera russelli was obtained from the Miami Serpentarium, Miami, Fla. Chymotrypsin was purchased from Worthington Biochemicals, Co., S2222 was a product of Ortho Diagnostics. Chelex 100 resin was purchased from Bio-Rad.

Proteins: Bovine factor X was isolated (10) and, when assayed by the method of Bachmann et al (11), demonstrated 240 u/mg of activity. XCP was purified from the venom of Vipera russelli (12) and, when assayed by the method of Jackson et al (13), showed 4.04×10^4 u/mg of activity. GD factor X was prepared as described by Morita and Jackson (8) and showed less than 0.1 u/mg of factor X clotting activity. All proteins were dialyzed against TBS containing Chelex to remove trace concentrations of Ca^{2+}. All buffers were passed through a 1.5 x 40 cm column of Chelex prior to use to remove contaminating Ca^{2+}. The $E^{1\%}_{A280}$ values and molecular weights used for the calculation of protein concentrations were as follows: factor X; 9.6, 55,100 (14,15); XCP, 13.4, 78,500 (16,12); GD factor X, 7.9, 50,800. The extinction coefficient for GD factor X was determined by the method of Dorsey et al (17) and its molecular weight calculated from the sequence. All proteins were homogeneous by SDS polyacrylamide gel electrophoresis performed according to Studier (18). Each appeared as a single species in the absence of β-mercaptoethanol; factor X, M_r = 55,000; GD factor X, M_r = 51,000; XCP M_r = 79,000. Upon reduction, both factor X and GD factor X demonstrated 2 polypeptide bands. The bands observed for the unmodified factor X were consistent with the heavy and light chains of factor X; 40,000 and 16,000 daltons, respectively. GD factor X had an unmodified heavy chain and a light chain of 12,000 daltons. Reduced XCP showed a band at 60,000 daltons and a doublet at 20,000 and 18,000 daltons. Disc gel electrophoresis by the method of Davis (19) showed GD factor X was a less negatively charged protein than the unmodified factor X.

Initial rates of activation: Factor X or GD factor X was equilibrated with or without Ca^{2+} in TBS at 25°C. The activation of either substrate was initiated by the addition of XCP. Aliquots were removed from the reaction at time points and diluted into TBS with 0.001 M EDTA. The diluted samples were monitored for factor Xa activity by the ability of factor Xa to hydrolyze S2222 (final concentration of S2222: 0.12 mM). The rate of S2222 hydrolysis was recorded at 405 nm at 25°C with a Beckman DU8 spectrophotometer. Standard curves using purified factor Xa indicated that the rate of S2222 hydrolysis was linear to 6 nM factor Xa. Previous studies had demonstrated GD factor Xa was able to hydrolyze S2222 at the same rate as the unmodified enzyme (W.F. Skogen unpublished results).

Fluorescence measurements: All fluorescence measurements were made using a Spex Fluorolog spectrofluorimeter. A Tektronix 31 programmable calculator was interfaced to the spectrofluorimeter to provide operational control, data collection, and signal intergration. All spectral measurements were done at 25°C and the slits were kept closed except during a scan to avoid photo-degradation of the sample. Samples were excited at 280 nm. Emission intensity was quantified by integration of the uncorrected fluorescence signal at 1 nm intervals. Samples were titrated by the sequential addition to the cuvette of Ca^{2+} diluted in TBS. Changes in fluorescence intensity in response to the addition of Ca^{2+} were complete within 1 min at 25°C. However, in order to ensure that equilibrium had been reached, emission spectra were not recorded until at least 5 min after the addition of titrant.

In all experiments, background signal due to solvent was subtracted, and all data were corrected for dilution due to the addition of Ca^{2+}.

RESULTS

In the presence of 5mM Ca^{2+}, factor X was rapidly activated by XCP. Double reciprocal analysis of the initial rates of activation as a function of substrate produced a straight line and allowed the determination of Km and k_{cat} (Table I). If Ca^{2+} was not included in the incubation, no factor Xa activity was observed at this level of XCP. However, at higher concentrations of XCP, factor Xa activity could be detected. This activation of factor X in the absence of Ca^{2+} obeyed Michaelis-Menten kinetics and double reciprocal analysis showed alterations in both Km and k_{cat} (Table I). On the other hand, Ca^{2+} did not affect the rate of GD factor X activation. GD factor X was a poor substrate for XCP, however the activation obeyed Michaelis-Menten kinetics and both Km and k_{cat} were determined (Table I).

The calcium-dependence of the activation of both the unmodified factor X and GD factor X is shown in Fig. 1. For the unmodified factor X, Ca^{2+} concentrations up to 0.2 mM had no effect on rates of activation whereas higher concentrations stimulated the activation. The stimulation was sigmoidal and half maximal at 0.65 mM Ca^{2+}. As noted above, the activation of GD factor X was not sensitive to Ca^{2+}.

TABLE I

The effect of Ca^{2+} on the kinetics of factor X or GD factor X activation by XCP.

substrate	Ca^{2+} (mM)	K_m [a] (μM)	k_{cat} [a] (min^{-1})
X[b]	0	9.1	14
X[c]	5	0.37	347
GD-X[d]	0	38.4	59
GD-X[d]	5	40.3	56

[a]Kinetic parameters were determined from double reciprocal analysis of the initial rates of activation as a function of substrate.
[b]Activated by 5×10^{-8} M XCP
[c]Activated by 1×10^{-9} M XCP
[d]Activated by 5×10^{-9} M XCP

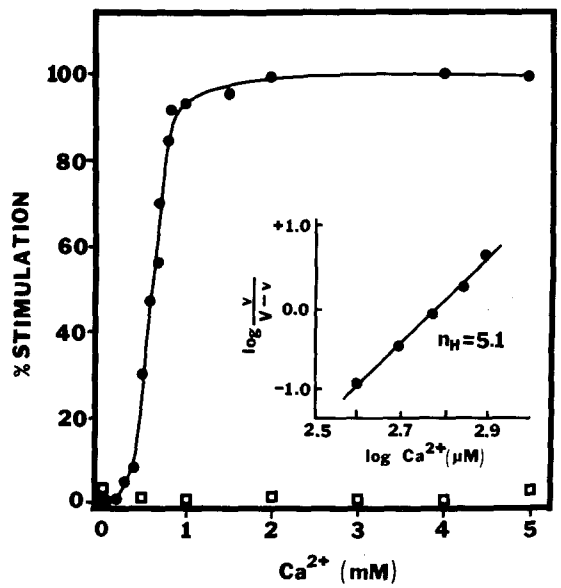

Figure 1. Calcium dependence of the rate of factor X and GD factor X activation by XCP. Incubations in TBS (25°C) contained either 1.5 X 10^{-6} M factor X (●) activated with $1x10^{-9}$M XCP or $3.6x10^{-6}$M GD factor X (□) activated with $5x10^{-8}$ M XCP. The rate of the intact factor X activation in the presence of 5 mM Ca^{2+} (310nM factor Xa/min) was considered maximal (100% stimulated). Rates of GD factor X activation were expressed as a percentage increase in the rate of activation in the presence of 5 mM Ca^{2+} (1.6nM factor Xa/min). The insert is a Hill plot analysis of data representing the stimulation of factor X activation by Ca^{2+}.

Calcium-dependent changes in the protein structures were investigated using intrinsic protein fluorescence emission. A small but reproducible increase in fluorescence intensity was observed with factor X at low Ca^{2+} concentrations. At higher Ca^{2+} concentrations the protein fluorescence was quenched substantially (Fig. 2). Half maximal quenching of fluorescence occurred at 0.7 mM Ca^{2+}. These results agree with earlier observations of a calcium-dependent conformational change in factor X (20,21). GD-factor X , however, exhibited no change in emission intensity, and hence in fluorescence-detected conformation, upon the addition of Ca^{2+} (Fig. 2). The wavelength of maximum emission intensity was constant for each protein during this titration. Activation of the intact factor X in the presence of Ca^{2+} caused an increase in intrinsic fluorescence intensity and a red shift in the emission (Fig. 3A). Interestingly, the same fluorescence change was observed upon activation of GD-factor X (Fig. 3B). The same results were obtained when these proteins were activated in the absence of Ca^{2+}. SDS electrophoresis

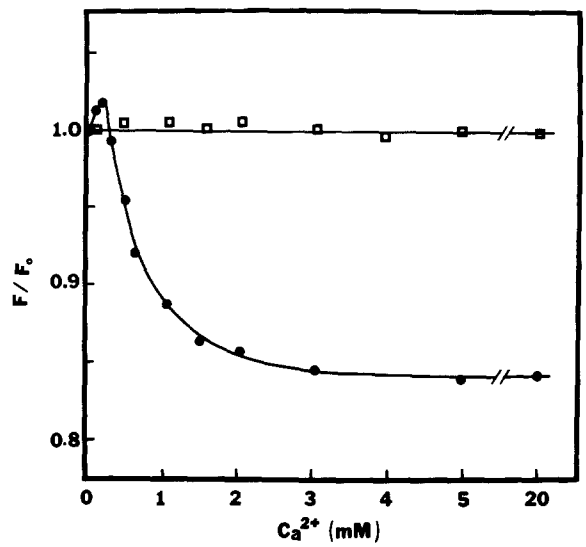

Figure 2. Calcium dependence of the intrinsic protein fluorescence of factor X and GD factor X. Emission spectra of 1.8×10^{-6} M factor X (●) or 2.0×10^{-6} M GD factor X (□) in TBS were obtained and integrated from 340 to 400 nm in the absence (Fo) or presence (F) of Ca^{2+}.

of samples removed from the cuvettes after activation demonstrated the cleavage by XCP was complete. The proteins also exhibited full hydrolase activity towards S2222.

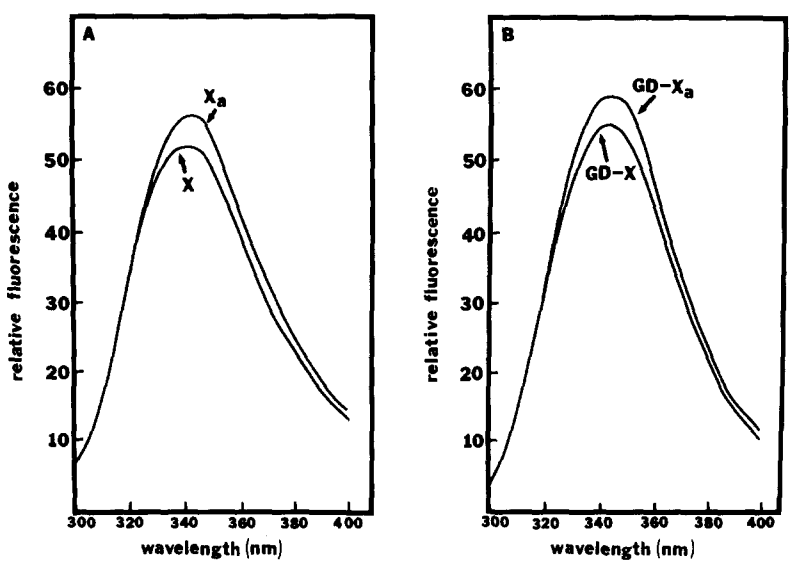

Figure 3. Uncorrected intrinsic protein fluorescence emission spectra of factor X and GD factor X prior to and after activation with XCP. Emission spectra of 1.8×10^{-6} M factor X (A) and 2.0×10^{-6} M GD factor X (B) in TBS with 5 mM Ca^{2+} were obtained at 25°C. XCP was then added to the cuvette (1×10^{-9} M to factor X; 5×10^{-8} M to GD factor X). At a time point sufficient to completely activate each sample, a second spectrum was taken. This spectrum was corrected for volume changes and any contribution to the spectrum by the XCP.

DISCUSSION

We have demonstrated that Ca^{2+} greatly stimulates the activation of coagulation factor X by XCP. This stimulation exhibits positive cooperativity as a Hill plot analysis of the data (insert Fig. 1) gives a slope (n_H) equal to 5.1. Half maximal stimulation, which occurs at 0.65 mM Ca^{2+}, correlates well with the calcium-dependent changes observed by intrinsic protein fluorescence (fluorescence transition midpoint = 0.7 mM Ca^{2+}, Fig. 2) and is in agreement with the binding of Ca^{2+} to factor X (half saturation = 0.65 mM Ca^{2+} (22)). This evidence suggests that Ca^{2+} initiates a conformational change in factor X which is a prerequisite for optimal proteolysis by XCP. This calcium-dependent conformational change requires the Gla-domain as the fluorescence emission of GD factor X is the same in the presence or absence of Ca^{2+} (Fig. 2). Thus the inability of Ca^{2+} to stimulate the activation of GD factor X is due to the inability of the protein to undergo a Gla dependent conformational change.

The similarity in the fluorescence changes observed upon activation of factor X and of GD factor X suggests that the Gla-domain does not participate in the structural changes which accompany the activation. These changes in fluorescence emission resulting from activation (fig. 3A) are consistent with observations made by Furie and Furie (23) who concluded that a single tyrosine and a single tryptophan residue are partially exposed to solvent during the activation of factor X.

These studies also revealed alterations in the Km and k_{cat} of the activation of GD factor X by XCP (Table I). The GD factor X kinetic parameters were not altered by addition of Ca^{2+} suggesting there is an interaction between XCP and factor X that is Ca^{2+} independent. The high Km and low k_{cat} determined for the activation of intact factor X in the absence of Ca^{2+} may explain why it has been thought exogenous Ca^{2+} was obligatorily required for activation.

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