The Factor IX Phospholipid-binding Site Is Required for Calcium-dependent Activation of Factor IX by Factor Xla*

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To determine the functional role of the metal-dependent conformational changes in Factor IX, two populations of conformation-specific anti-Factor IX antibodies were prepared. Anti-Factor IX-Mg(II) antibodies bind to Factor IX in the presence of Mg(II) and other metal ions, but not in the absence of metal ions. Anti-Factor IX-Ca(II)-specific antibodies bind to Factor IX in the presence of Ca(II) and Sr(II), but not in the presence of Mn(II), Mg(II), and Ba(II). In the presence of a metal ion that induces the conformational transition recognized by the anti-Factor IX•Mg(II) antibodies, the concentrations of CaCl₂ and SrCl₂ needed for the half-maximal binding of the anti-Factor IX•Ca(II)-specific antibodies to Factor IX were reduced 3- and 20-fold, respectively. Factor IX binding to phospholipid vesicles was inhibited by the Fab fragments of the anti-Factor IX•Ca(II)-specific antibodies, but was not inhibited by the Fab fragments of the anti-Factor IX•Mg(II) antibodies. Factor Xla activation of Factor IX was also inhibited by the Fab fragments of the anti-Factor IX•Ca(II)-specific antibodies, but not by the anti-Factor IX•Mg(II) antibodies. These results support the hypothesis that Factor IX undergoes two metal-dependent conformational transitions: FIX→FIX⁺→FIX*. The first transition (FIX→FIX⁺) is metal-dependent but cation-nonselective; the second transition (FIX⁺→FIX*) is metal-selective for Ca(II) or Sr(II). The second transition results in the expression of conformational determinants necessary for membrane binding and the Ca(II)-dependent activation of Factor IX by Factor Xla. These results suggest chemical similarity between a surface of a domain of Factor Xla and phospholipid vesicles, both of which interact with Factor IX in the presence of Ca(II).

Factor IX, a vitamin K-dependent glycoprotein involved in blood coagulation (1, 2), contains 12 γ-carboxyglutamic acid residues that confer metal-binding properties essential for biological activity (3, 4). In the presence of Ca(II), Factor IX is activated to its enzymatic form, Factor IXa, by Factor Xla or Factor VIIa-tissue factor via a mechanism that involves limited proteolysis (5, 6). However, unlike other examples of activation of vitamin K-dependent blood-clotting proteins, Factor Xla proteolysis of Factor IX does not require membrane surfaces. The Factor IXa-Ca(II) complex subsequently activates Factor X (1, 2, 7) in a reaction that requires Factor VIIIa and membrane surfaces (8, 9).

The interaction of metal ions with the vitamin K-dependent coagulation proteins results in spectroscopically detectable changes in their tertiary structure (10) from a metal-free, nonfunctional conformer to a metal-stabilized functional conformer (11, 12). A number of bivalent and trivalent cations can induce the spectroscopically observable transition between these two conformational states. However, all metal ions capable of supporting this transition in Factor IX are not capable of supporting the activation of Factor IX by Factor Xla; the presence of Ca(II) or Sr(II) is required for this reaction (13-15). Also, Ca(II) but not Mg(II) can support Factor IX binding to phospholipid vesicles. Thus, the conformational transition supported by many bivalent and trivalent metal ions may be necessary but not sufficient for activation of Factor IX. Some other metal-dependent process with marked metal ion specificity must be required for activation of Factor IX and binding of Factor IX to phospholipid vesicles.

We recently proposed a three-state model that describes the metal ion-dependent conformational transitions of human prothrombin (16). An initial metal-dependent intermediate conformer of prothrombin, induced in the presence of many metal ions, is unable to bind to membrane surfaces and therefore lacks functional activity. In the presence of Ca(II) or Sr(II), a second metal-dependent conformational transition occurs. The resulting prothrombin conformer can bind to membrane surfaces and is the functional form of prothrombin. Because of the functional and sequence homology between Factor IX and prothrombin, we predicted that Factor IX would also manifest two sequential metal ion-induced conformational transitions. Furthermore, this model could explain the metal ion specificities that characterize the functional properties of Factor IX. In these studies, we demonstrate a conformation-specific antibody directed against a calcium-stabilized antigenic determinant. This determinant is coincident with or adjacent to the phospholipid-binding site. These antibodies inhibit Factor IX binding to phospholipid and inhibit the activation of Factor IX by Factor Xla, indicating a role for this Factor IX domain in Factor IX-Factor Xla interaction.

**MATERIALS AND METHODS**

**Proteins—**Prothrombin, Factor X, and Factor IX were prepared as previously described (17-19). Factor Xla and Factor Xla were prepared from barium citrate supernatant by modification of the method of Mannhalter et al. (20). The Factor Xla (specific activity 142 units/
mg) was activated by incubating 500 μg of Factor XI in TBS1 (102 M Tris (pH 7.4), 0.15 M NaCl) with 0.2 ml of trypsin-Sepharose (treated with l-1-tosylamido-2-phenylethyl chloromethyl ketone, 130 units/ml of Sepharose) at 37°C for 40 min. Factor IXa was prepared by incubation of 5 μg of Factor IX with 0.05 μg of the Factor IXa preparation in TBS, 5 mM CaCl2, for 30 min at 37°C. Factor IXa and IX were separated from Factor XI by immunoaffinity chromatography (19). Factor IXa was separated from Factor IX and IXb by gel filtration on a Sepharose S-200 column (2.5 x 75 cm). Des-carboxy-Factor IX was prepared from the plasma of patients taking sodium warfarin by modification of the method of Blanchard et al. (21). Factor IXa, Factor IX, des-carboxy-Factor IX, Factor X, and prothrombin migrated as single bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (22). The concentrations of the purified proteins were estimated by their absorbance at 280 nm using A280 values of: prothrombin, 14.4; Factor X, 11.6; Factor XI, 13.4; Factor IX and des-carboxy-Factor IX, 13.3.

Preparation of Metal-free Buffers and Protein Solutions—All buffers were made metal-free by passage over a Chelex 100 column. Metal ions were removed from protein solutions by exhaustive dialysis against metal ion-free TBS (pH 7.4) which contained Chelex 100 (1 g/liter).

Preparation of Conformation-specific Antibodies—Antibodies specific for the metal-stabilized conformation of Factor IX were purified from rabbit anti-Factor IX antisera by affinity chromatography on a Factor IX-Sepharose column (1.3 x 3.4 cm) of Factor IX per ml of Sepharose equilibrated with TBS (pH 7.4), 5 mM CaCl2 (19). Anti-IX (2 ml) was applied to the column, and the anti-Factor IX antibodies that bound were eluted with 4 m guanidine HCl, dialyzed into metal-free TBS, pooled, and concentrated by ultrafiltration. After dialysis against TBS containing Chelex 100, these antibodies were made 5 mM in MgCl2 and applied to a Factor IX-Sepharose column equilibrated in TBS, 5 mM MgCl2. The antibodies specific for the Factor IX-Mg(II) complex were eluted with TBS, 7.5 mM EDTA. These antibodies, designated anti-Factor IX-Mg(II), were pooled and concentrated. The antibodies that did not bind to the Factor IX-Sepharose column in the presence of MgCl2 were pooled, concentrated, and dialyzed into metal-free TBS containing Chelex 100. These antibodies were made 5 mM in CaCl2 and applied to the Factor IX-Sepharose column equilibrated with TBS, 5 mM CaCl2. The bound antibodies were eluted with TBS, 7.5 mM EDTA. These antibodies were pooled, concentrated, and designated as the anti-Factor IX-Ca(II)-specific antibodies.

The Fab fragments of anti-Factor IX-Mg(II)- and anti-Factor IX-Ca(II)-specific antibodies were prepared by the method of Hsiao and Putnam (23). Afinity-purified anti-Factor IX antibodies (5 mg/ml) in 50 mM Tris (pH 7.4) and 0.1% NaN3 and quantitated (28) were mixed and incubated for 15 min. Aliquots (10 μl) of this mixture were added to a fluorescence cuvette containing 0.96 ml of buffer with or without 14.4 μg of phospholipid. In other experiments buffers containing either 5 mM MgCl2 or 3 mM EDTA were used. The light-scattering intensity of the Factor IX alone and the phospholipid alone were subtracted from the light-scattering intensity obtained for the Factor IX-phospholipid mixture. The light-scattering experiment was repeated in the presence of fab fragments of the conformation-specific antibodies. Factor IX (270 μg in TBS, 5 mM CaCl2) and anti-Factor IX-Mg(II) Fab fragments (315 μg in TBS, 5 mM CaCl2) or anti-Factor IX-Ca(II)-specific Fab fragments (280 μg in TBS, CaCl2) were mixed and incubated for 15 min. Aliquots (10 μl) of this mixture were added to a fluorescence cuvette containing 0.96 ml of buffer with or without 14.4 μg of phospholipid. The data were corrected for light scattering due to the protein alone, antibody alone, and the phospholipid alone.

Activation of Factor IX by Factor Xa—The activation of Factor IX by Factor Xa in the presence of different cations and the conformation-specific antibodies was studied using the Factor IX activation-peptide release assay (29). Factor IX was labeled with tritium (30). The protein was separated from the sodium borotritide by gel filtration on Sephadex G-25. The labeled protein was repurified by immunoaffinity chromatography (19) and eluted with EDTA.

H-Labeled Factor IX (500,000 cpm in 1 ml of TBS, 5 mM CaCl2, 0.1% bovine serum albumin) was incubated with or without 50 μg of anti-Factor IX-Mg(II) or anti-Factor IX-Ca(II)-specific Fab fragments for 15 min at 37°C. After 50 μl of Factor Xa (50 μg/ml in TBS, 5 mM CaCl2) was added to the H-labeled Factor IX, 100-μl aliquots were removed at varying intervals and added to 100 μl of TBS, 20 mM EDTA, 20 mM benzamidine at 0°C. Trichloroacetic acid (150 μl, 15% v/v) was added to each sample and the supernatant recovered after centrifugation and assayed for 3H.

The effect of the anti-Factor IX-Mg(II) and anti-Factor IX-Ca(II)-specific antibodies on the Factor IXa activation of Factor IX was also studied using 125I-labeled Factor IX. Anti-Factor IX-Mg(II) (6.1 X 103 M) or anti-Factor IX-Ca(II)-specific Fab fragments (5 X 10-8 M) were mixed with 125I-labeled Factor IX (1 X 108 M) in a buffer of TBS, 0.1% bovine serum albumin, 3 mM CaCl2 and incubated for 15 min at 37°C. Factor Xa (2 X 1010 M) was added to the Factor IX-anti-Factor IX Fab mixture and aliquots were removed at varying intervals. The aliquots were mixed in Laemmli gel buffer containing 5% 2-mercaptoethanol, heated, and electrophoresed on 10% polyacrylamide gels in the presence of SDS. The gels were dried and exposed to Kodak X-Omat R film. The proteolytic generation of Factor IXa by Factor Xa over time was evaluated by densitometric analysis of the autoradiographs using an EDC densitometer (Helena Laboratories).

RESULTS

Two Populations of Conformation-specific Anti-Factor IX Antibodies—The anti-Factor IX antibodies were fractionated by affinity purification into three separate subpopulations. One population, anti-Factor IX total, bound to Factor IX in the presence or absence of metal ions. The conformation-specific anti-Factor IX-Mg(II) antibodies were selected for their ability to bind to Factor IX in the presence of Mg(II) but not in the absence of metal ions. The conformation-specific anti-Factor IX-Ca(II)-specific antibodies were selected for their ability to bind Factor IX in the presence of Ca(II), but not in the presence of Mg(II) or in the absence of metal ions. The anti-Factor IX-Mg(II) antibody population comprised 10% of the anti-Factor IX antibodies. The anti-Factor IX-Ca(II) specific antibodies represented 2% of the anti-Factor IX antibodies.

1 The abbreviations used are: TBS, Tris-buffered saline; SDS, sodium dodecyl sulfate.
Anti-Factor IX-Mg(II) Antibodies—We evaluated the metal ion requirements for the binding of the anti-Factor IX-Mg(II) antibodies to Factor IX. The anti-Factor IX-Mg(II) antibodies bound to Factor IX in the presence of 5 mM MgCl₂ or 5 mM CaCl₂, whereas there was no significant binding in the absence of divalent metal ions (Fig. 1A). Mn(II), Sr(II), and Ba(II) also supported the binding of anti-Factor IX-Mg(II) antibodies to Factor IX (Fig. 1B). The metal concentrations necessary for half maximal binding of antibody were 0.01 mM MnCl₂, 0.2 mM CaCl₂, 0.2 mM MgCl₂, 1 mM SrCl₂, and 2.5 mM BaCl₂. The antigenic specificity of the anti-Factor IX-Mg(II) antibodies was studied using a competition radioimmunoassay. Both Factor IX and Factor IXa inhibited the binding to [125I]-labeled Factor IX, indicating comparable concentrations of metal ions as high as 20 and 40 mM, respectively.

Anti-Factor IX-Ca(II)-specific Antibodies—The anti-Factor IX-Ca(II)-specific antibody population bound to Factor IX in the presence of 5 mM CaCl₂, but had no significant binding in the presence of 5 mM MgCl₂ (Fig. 2A). The binding of these antibodies to Factor IX also occurred in the presence of SrCl₂ (Fig. 2B). However, there was no significant binding of antibody in the presence of MgCl₂ or BaCl₂, even at concentrations of metal ions as high as 20 and 40 mM, respectively. There was a small, but reproducible, binding of antibody in the presence of high concentrations of MnCl₂. The concentrations of CaCl₂ and SrCl₂ required for half-maximal binding of antibody were 0.2 and 2 mM, respectively. The antigenic specificity of the anti-Factor IX-Ca(II)-specific antibodies was studied using the competition radioimmunoassay. Both Factor IX and Factor IXa inhibited the binding of the anti-Factor IX-Ca(II)-specific antibodies to [125I]-Factor IX but there was no inhibition of binding by des-γ-carboxy-Factor IX, Factor X, or prothrombin.

Effect of Metal Ion Combinations on Factor IX Antigen Expression—We evaluated the metal ion selectivity and the sequence of conformer formation by a metal ion mixing experiment. We predicted that Factor IX must form the conformer that is divalent cation-nonselective for subsequent formation of the conformer specifically stabilized by Ca(II). Therefore, in the presence of a metal ion supportive of the first conformational transition (e.g. Mg(II)), the concentrations of CaCl₂ or SrCl₂ necessary for the expression of the second conformer would be significantly reduced. To study this hypothesis, we performed a binding assay of the anti-Factor IX-Ca(II)-specific antibodies in the presence of increasing concentrations of CaCl₂ and SrCl₂ using a buffer which also contained 0.1 mM MnCl₂. This concentration of MnCl₂ can support maximal binding of anti-Factor IX-Mg(II) to Factor IX, whereas the anti-Factor IX-Ca(II)-specific antibodies will bind less than 5% of the Factor IX (Fig. 3). The presence of 0.1 mM MnCl₂ reduced the concentration of CaCl₂ necessary for half-maximal binding of the anti-Factor IX-Ca(II)-specific antibodies to 0.08 from 0.2 mM. Similarly, 0.1 mM MnCl₂ lowered the concentration of SrCl₂ necessary for half-maximal binding to 0.08 from 2 mM. This represents a 3- and 20-fold reduction in the concentrations of CaCl₂ and SrCl₂, respectively, needed for expression of the anti-Factor IX-Ca(II)-specific antigen. When a similar experiment was performed with increasing concentrations of MgCl₂ in the

![Fig. 1](image-url)

A, effect of CaCl₂ and MgCl₂ on the interaction of anti-Factor IX-Mg(II) antibodies with Factor IX. The binding of anti-Factor IX-Mg(II) antibody to Factor IX was studied in the presence of 5 mM CaCl₂ (A), 5 mM MgCl₂ (B), and metal-free buffer (C). B, metal requirements for the interaction of Factor IX with anti-Factor IX-Mg(II) antibody. The binding of anti-Factor IX-Mg(II) antibody (1 x 10⁻⁴ M) and [125I]-Factor IX (1 x 10⁻¹⁰ M) was studied as a function of CaCl₂ (A), MgCl₂ (B), MnCl₂ (C), SrCl₂ (D), and BaCl₂ (E) concentration.
Fig. 2. A, effect of CaCl2 and MgCl2 on the interaction of anti-Factor IX-Ca(II)-specific antibodies and Factor IX. The binding of anti-Factor IX-Ca(II)-specific antibody to Factor IX was studied in the presence of 5 mM CaCl2 (●), 5 mM MgCl2 (○), and metal-free buffer (□). B, metal requirements for the interaction of Factor IX with anti-Factor IX-Ca(II)-specific antibody. The binding of anti-Factor IX-Ca(II)-specific antibody (7.2 × 10⁻⁸ M) was studied as a function of CaCl2 (●), MgCl2 (○), MnCl2 (■), SrCl2 (Δ), and BaCl2 (□) concentration.

Fig. 3. Effect of MnCl2/CaCl2 or MnCl2/SrCl2 on the binding of the anti-Factor IX-Ca(II)-specific antibodies to Factor IX. The binding of anti-Factor IX-Ca(II)-specific antibody (6 × 10⁻⁸ M) to Factor IX (1 × 10⁻¹⁰ M) was studied as a function of varying concentrations of CaCl2 (●), SrCl2 (Δ), and MnCl2 (○). In the experiments indicated by the broken line (---), the buffer contained 0.1 mM MnCl2.

presence of 0.1 mM MnCl2, there was no detectable binding of the anti-Factor IX-Ca(II)-specific antibodies to Factor IX (data not shown).

Inhibition of Factor IX-Phospholipid Interaction with Anti-Factor IX-Ca(II)-specific Antibodies—The metal ion specificity of Factor IX binding to phospholipid vesicles was studied using the 90° light-scattering technique (Fig. 4A). In the presence of Ca(II), Factor IX binds to lipid vesicles. No Factor IX binding occurred in the presence of EDTA. Factor IX did not bind to the phospholipid vesicles in the presence of 5 mM MgCl2. In other experiments, concentrations of MgCl2 as high as 15 mM also failed to support Factor IX binding to phos-
The binding of Factor IX to phospholipid vesicles in the presence of mM Fab fragments of phospholipid vesicles (data not shown). Therefore, the conformational transition supported by Mg(II) does not result in the binding of Factor IX to phospholipid vesicles in the presence of CaCl₂, Factor IX alone, or Factor IX and anti-Factor IX-Ca(II) Fab fragments. However, the binding of des-y-carboxy-Factor IX, which cannot undergo the metal ion-dependent conformational transitions, does not bind to the phospholipid vesicles in the presence of Ca(II).

If the Ca(II)-dependent antigenic determinants recognized by the anti-Factor IX-Ca(II)-specific antibodies are near the phospholipid-binding site on the Factor IX-Ca(II) complex, then they should inhibit the interaction of Factor IX with phospholipid vesicles. To exclude the possibility that the conformation-specific antibodies might inhibit phospholipid vesicle binding by aggregation of Factor IX, the binding studies were performed using Fab fragments of the antibodies. The conditions of the experiment were selected such that most of the Factor IX was bound to the Fab fragments. Preincubation of Factor IX with anti-Factor IX-Mg(II) Fab fragments did not inhibit Factor IX binding to phospholipid vesicles (Fig. 4B). The anti-Factor IX-Ca(II)-specific Fab fragments completely inhibited the binding of Factor IX to the phospholipid vesicles. These results suggest that the antigenic determinants recognized by the anti-Factor IX-Mg(II) antibodies are far enough from the phospholipid-binding site of Factor IX such that they do not prevent Factor IX from binding to phospholipid vesicles. However, the anti-Factor IX-Ca(II)-specific antibodies appear to be directed at an antigenic determinant near or including the phospholipid-binding site.

Inhibition of Factor XIa Activation of Factor IX by the Anti-Factor IX-Ca(II)-specific Antibodies—The effect of the anti-Factor IX-Mg(II) and anti-Factor IX-Ca(II) antibodies on the Factor XIa activation of Factor IX was studied using the ³H-labeled activation peptide release assay (Fig. 5). Preincubation of ³H-labeled Factor IX with the Fab fragments of the anti-Factor IX-Mg(II) antibodies resulted in minimal inhibition of Factor XIa activation of Factor IX. In contrast, the Fab fragments of the anti-Factor IX-Ca(II)-specific antibodies inhibited the initial rate of activation of Factor IX by about 93%. The initial rate of activation of Factor IX in the presence of the anti-Factor IX-Ca(II)-specific Fab fragments was approximately 2-fold greater than the initial rate of activation of Factor IX observed in the presence of 3 mM EDTA.

The ³H-labeled Factor IX activation peptide release assay quantitates the amount of activation peptide released by the successful two-step cleavage of Factor IX by Factor XIa. The first cleavage, scission of an Arg-Ala bond at residues 145-146, results in a nonfunctional two-chain intermediate, Factor IXₐ. A second cleavage of Factor IX between residues 180 and 181 (Arg-Val) results in the formation of the enzymatically active Factor IXₐₐ. We therefore studied the effect of the anti-Factor IX-Mg(II)- and anti-Factor IX-Ca(II)-specific antibodies on the pattern of Factor IX activation by Factor XIa using ³H-labeled Factor IX in the presence and absence of the anti-Factor IX conformation-specific antibodies. The progressive proteolytic cleavage of Factor IX by Factor XIa with the subsequent appearance of Factor IXₐ, Factor IXₐₐ, and Factor IXₐₐ was studied by SDS gel electrophoresis and autoradiography. When ³H-labeled Factor IX is activated by Factor XIa in the presence of 3 mM CaCl₂, there is the rapid conversion of Factor IX to Factor IXₐₐ with little formation of Factor IXₐ. However, the ³H-labeled peptide release assay for CaCl₂ in the activation mixture resulted in a slow proteolysis of Factor IX with minimal formation of Factor IXₐₐ (Fig. 6B). In the presence of 3 mM MgCl₂, a pattern of Factor XIa cleavage of Factor IX identical to that observed with EDTA is observed (data not shown). The major proteolytic product of human Factor IX in the presence of Factor XIa and either MgCl₂ or EDTA is Factor IXₐₐ.

Preincubation of ³H-labeled Factor IX with the anti-Factor IX-Mg(II) Fab fragments had minimal effect on Factor IX activation by Factor XIa in the presence of 3 mM CaCl₂ (Fig. 7A). The Factor XIa proteolysis of Factor IX resulted in the formation of Factor IXₐₐ, with little accumulation of Factor IXₐ.
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**FIG. 6.** Effect of CaCl$_2$ and EDTA on the proteolysis of Factor IX by Factor XIa. Factor XIa proteolysis of $^{125}$I-labeled Factor IX in the presence of 3 mM CaCl$_2$ (A) or 3 mM EDTA (B) was studied as described under "Materials and Methods." The concentrations of Factor IX ($\bullet$), heavy chain of Factor IX$_a$ (C), and heavy chain of Factor IX$_b$ ($\bigtriangleup$) versus time were determined by densitometric scanning of radiographs.

IX$_a$. However, the addition of the anti-Factor IX-Ca(I)I-specific Fab fragments to the activation mixture significantly inhibited the proteolysis of Factor IX (Fig. 7B). The major proteolytic product of Factor IX in the presence of the anti-Factor IX-Ca(I)-specific Fab fragments was Factor IX$_a$.

**FIG. 7.** Effect of conformation-specific antibodies on the proteolysis of Factor IX by Factor XIa. Factor XIa proteolysis of $^{125}$I-labeled Factor IX in the presence of 3 mM CaCl$_2$ and the anti-Factor IX-Mg(II) Fab fragments (A) or the anti-Factor IX-Ca(II)-specific Fab fragments (B) was studied as described under "Materials and Methods." The concentrations of Factor IX ($\bullet$), heavy chain of Factor IX$_a$ (C), and heavy chain of Factor IX$_b$ (A) versus time were determined by densitometric scanning of radiographs.

**DISCUSSION**

Both immunocchemical methods employing conformation-specific antibodies and physical spectroscopic techniques have shown that the vitamin K-dependent coagulation proteins undergo a conformational transition upon binding metal ions (10). These observations have been interpreted in terms of a two-state model for the effect of metal ions on the vitamin K-dependent proteins. In this model, first proposed for prothrombin (11, 12), the vitamin K-dependent proteins undergo a single metal ion-dependent conformational transition upon binding any of a number of metal ions. The resulting metal-stabilized conformer represents the functional form of the protein capable of membrane binding. Unexplained by these studies is the Ca(II) ion specificity of prothrombin-phospholipid binding. Divalent metal ions such as Mg(II), Mn(II), and Ba(II), which can induce the metal ion-dependent conformational transition, cannot support prothrombin-phospholipid binding (12).

During studies of the metal-stabilized conformation of prothrombin using conformation-specific monoclonal antibodies, we observed a pattern of antibody binding which did not fit this two-state model (31, 32). The conformation-specific monoclonal antibody RL1.3 binds to a metal-stabilized antigenic determinant expressed on the prothrombin-metal complex, but will not bind to prothrombin in the absence of metal ions (31). The conformation-specific monoclonal antibody J01.1 to abnormal prothrombin binds to the calcium-free conformer of prothrombin, but does not bind to prothrombin in the presence of Ca(II) (32). Both antibodies bound to the prothrombin-Mg(II) complex, an observation inconsistent
with the current two-state model for prothrombin. We proposed the existence of an intermediate metal-stabilized conformation which could explain this observation: \((PT \rightarrow PT^* \rightarrow PT')\). Recent immunochemical studies employing affinity-prepared polycional conformation-specific antibodies have confirmed our hypothesis (16). In this model, prothrombin undergoes two sequential metal-dependent conformational transitions: the formation of a metal-protein complex in which a number of metal ions suffice \((PT^*)\) and the expression of a prothrombin conformer that is selectively stabilized by Ca\((II)\). Because of the sequence and functional homology among the vitamin K-dependent coagulant proteins, we predicted that the two metal-induced transitions would occur on the other vitamin K-dependent proteins. The current study supports a similar three-state model for Factor IX-metal ion interaction.

Factor IX is a vitamin K-dependent blood coagulation protein whose metal-binding properties allow for its activation by Factor Xla or Factor VIIa-tissue factor and confer membrane-binding properties on the protein (33). The activation of Factor IX to Factor Xla by Factor Xla requires Ca\((II)\) ions. The interaction of Factor IX or Factor Xla with membrane surfaces is also Ca\((II)\)-dependent. We have investigated the effect of different metal ions on the metal-induced conformational transitions of human Factor IX. For these studies, we have employed two conformation-specific antibodies against the Factor IX-metal complex. Using these antibodies, we have demonstrated that Factor IX and Factor IX undergo two sequential metal-stabilized conformational transitions: \(FIX \rightarrow FIX^* \rightarrow FIX'\). When Factor IX binds a number of different bivalent cations including Mn\((II)\), Mg\((II)\), Ca\((II)\), Sr\((II)\), and Ba\((II)\), it expresses conformational antigenic determinants which are recognized by the anti-Factor IX-Mg\((II)\) antibodies. When Factor IX binds Ca\((II)\) or Sr\((II)\), a second conformational antigenic determinant is expressed which is recognized by the anti-Factor IX-Ca\((II)\)-specific antibodies. This antigenic determinant is not expressed in the Factor IX-Mg\((II)\) or Factor IX-Mn\((II)\) complex. To express either of these metal-stabilized conformers, Factor IX must have \(\gamma\)-carboxyglutamic acid residues, since des-\(\gamma\)-carboxy-Factor IX in the presence of saturating concentrations of Ca\((II)\) will not bind either the anti-Factor IX-Mg\((II)\)- or anti-Factor IX-Ca\((II)\)-specific antibodies.

The expression of the anti-Factor IX-Mg\((II)\)- and anti-Factor IX-Ca\((II)\)-specific conformational determinants appears to occur in sequence, since the anti-Factor IX-Mg\((II)\) antigen can be present independent of the expression of the anti-Factor IX-Ca\((II)\)-specific antigen, but the converse has not been observed. Also, in the presence of a metal ion that supports the expression of the anti-Factor IX-Mg\((II)\) antigenic determinant, there was a significant reduction in the concentrations of Ca\((II)\) and Sr\((II)\) necessary for binding of the anti-Factor IX-Ca\((II)\)-specific antibodies.

An important feature of the FIX* conformer that distinguishes it functionally from the conformer FIX' is its phospholipid-binding properties. We have demonstrated that neither des-\(\gamma\)-carboxy-Factor IX nor Factor IX in the presence of EDTA can bind phospholipid, an observation consistent with the known importance of the metal ion-induced conformational transition in Factor IX-phospholipid interaction (33). In addition, Mg\((II)\) failed to support Factor IX binding to phospholipid surfaces. The phospholipid-binding site of Factor IX is therefore not expressed on the Factor IX-Mg\((II)\) complex. Furthermore, the anti-Factor IX-Mg\((II)\) antibodies, which bind to antigenic sites associated with the FIX* conformer, did not inhibit Factor IX-lipid binding in the presence of Ca\((II)\). Apparently, the antigenic sites associated with the FIX* conformer are far enough from the phospholipid-binding site of Factor IX not to interfere with Factor IX-lipid binding.

The anti-Factor IX-Ca\((II)\)-specific antibodies do inhibit Factor IX-lipid interaction, providing direct evidence that the Ca\((II)\)-dependent antigenic determinants expressed on the FIX* conformer are in proximity to or include the phospholipid-binding site.

Factor IX activation by Factor Xla is highly metal-selective (13-15). This proteolytic activation of Factor IX occurs independent of phospholipid. Byrne et al. (16) found that Sr\((II)\) can substitute for Ca\((II)\) in the Factor Xla activation of bovine Factor IX. However, with saturating concentrations of Sr\((II)\), the maximal activation rate of Factor IX was only 15% of that obtained with Ca\((II)\). In their studies, Mn\((II)\) could not support Factor IX activation, but when suboptimal concentrations of Ca\((II)\) were combined with 0.5 mxt MnCl\(_2\), the activation rate of Factor IX was equal to that observed with saturating concentrations of Ca\((II)\). Since these studies on the metal ion specificity and the kinetics of Factor IX activation parallel our findings on the expression of the anti-Factor IX-Ca\((II)\)-specific antigenic determinants, we predicted that the expression of the Factor IX* conformer is necessary for Factor IX activation. Our observation that the anti-Factor IX-Ca\((II)\)-specific antibodies inhibited Factor Xla activation of Factor IX are consistent with this hypothesis. The inability of Factor IX to be activated by Factor Xla in the presence of Mg\((II)\) and failure of the anti-Factor IX-Mg\((II)\) antibodies to inhibit activation suggest that the intermediate conformer (FIX') does not express the Factor Xla-binding site.

Although the proteolytic site of Factor Xla is present on the light chain of the molecule, recent evidence suggests that the heavy chain of Factor Xla is necessary for the Ca\((II)\)-dependent acceleration of Factor IX activation (34-36). The anti-Factor IX-Ca\((II)\)-specific antigenic determinant may therefore represent or lie in close proximity to the Factor Xla heavy chain-binding site on Factor IX that allows for optimal positioning of the Factor Xla light chain active site for Factor IX proteolysis. This positioning of the proteolytic site of Factor Xla appears to be of particular importance in successful cleavage of the arginine 180-valine 181 bond. Masking the Factor Xla-binding site on Factor IX by the Fab fragments of the anti-Factor IX-Ca\((II)\)-specific antibodies results in nearly complete inhibition of the Arg-Val cleavage, mimicking the pattern of Factor IX proteolysis observed in the absence of Ca\((II)\). These results indicate an essential difference between the activation of Factor IX by Factor Xla and the activation of a vitamin K-dependent zymogen (e.g. prothrombin) by a vitamin K-dependent enzyme (e.g. Factor Xa). The molecular assembly of the enzyme-substrate complex, Factor Xa-prothrombin, requires Ca\((II)\)-dependent membrane interaction of both components and an organizational role for the cofactor, Factor Va (37). In contrast, Ca\((II)\)-dependent Factor Xla-Factor IX interaction occurs in solution, but the activation by Factor Xla of Factor IX requires that Factor IX be in the Factor IX* conformation.

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REFERENCES
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