Prothrombin Requires Two Sequential Metal-dependent Conformational Transitions to Bind Phospholipid

CONFORMATION-SPECIFIC ANTIBODIES DIRECTED AGAINST THE PHOSPHOLIPID-BINDING SITE ON PROTHROMBIN*

(Received for publication, July 1, 1986)

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Prothrombin is a γ-carboxyglutamic acid-containing protein that binds to phospholipid vesicles in the presence of calcium ions after undergoing a metal ion-induced conformational transition. To integrate recent data into a scheme that is compatible with our knowledge of prothrombin-metal interaction, we have proposed a new model of prothrombin structure. In this model prothrombin undergoes two metal-dependent conformational transitions: PT → PT* → PT'. The first transition is not cation-specific, but the second transition is metal-selective for Ca(II), Sr(II), or Ba(II). Only the PT' conformer binds to phospholipid surfaces. To test this model, anti-prothrombin antibodies that only bind to prothrombin in the presence of Ca(II) but not Mg(II) (PT*-specific) were isolated, and termed anti-prothrombin-Ca(II)-specific. Half-maximal binding of antibody to prothrombin was observed at 0.1 mM CaCl₂ or 1 mM SrCl₂, but no binding was observed with Mg(II), Mn(II), or Ba(II). However, prothrombin in the presence of both Mg(II)/Ba(II) or Mn(II)/Ba(II) demonstrated significant interaction with the antibody. Prothrombin binding to phospholipid vesicles was inhibited by the anti-prothrombin-Ca(II)-specific antibody or its Fab fragment, but was not inhibited by anti-prothrombin-Mg(II) antibody or its Fab fragment directed at the PT' conformer. These results support this three-state model for prothrombin. The metal specificity characteristic of prothrombin-phospholipid interaction is a property required for the expression of the phospholipid-binding site in the binary prothrombin-metal complex.

Prothrombin is a plasma glycoprotein (M, 72,000) that participates in the final stages of blood coagulation (1, 2). Prothrombin has a known amino acid sequence (3, 4) and exhibits marked structural homology of its thrombin domain with trypsin and other trypsin-like serine proteases (5). This protein contains 10 γ-carboxyglutamic acid residues that confer the unique metal-binding properties characteristic of this class of calcium-binding proteins (6-10). Prothrombin contains two high affinity metal-binding sites and multiple lower affinity sites (9, 11, 12). The high affinity sites are defined by γ-carboxyglutamic acid residues that form an intramolecular bridge through a single metal ion (10, 13). Upon occupancy of these metal-binding sites, prothrombin undergoes a conformational transition that can be monitored by the quenching of intrinsic fluorescence (14-16), perturbation of the circular dichroism spectrum (17), and expression of novel antigenic determinants (18-21). This metal-induced conformational transition has been localized to a domain in bovine prothrombin consisting of the NH₂-terminal 42 amino acids (22).

RL1.3 is a conformation-specific monoclonal antibody that binds to prothrombin only in the presence of metal ions; Ca(II), Mg(II), and Mn(II) bound to prothrombin support antibody-prothrombin interaction (23). J01.1 is a conformation-specific monoclonal antibody directed against abnormal (des-γ-carboxy) prothrombin that binds to prothrombin only in the absence of Ca(II); however, Mg(II) and Mn(II) do not inhibit antibody-prothrombin interaction (24). These results were not compatible with the current two-state model of the effect of metal ions on prothrombin (14, 15). In this model, prothrombin undergoes a single metal-dependent conformational transition that is induced by a wide variety of metal ions. We had anticipated that the binding of RL1.3 and J01.1 to prothrombin conformers would be mutually exclusive, since J01.1 bound to the calcium-free conformer and RL1.3 bound to the metal-stabilized conformer of prothrombin. However, both conformation-specific antibodies bound to the prothrombin conformer stabilized by Mg(II) (23, 24). These results suggested that this conformer is an intermediate in the pathway to a prothrombin structure that binds to membrane surfaces. We refined the currently accepted two-state model of prothrombin structure, and proposed a three-state model to describe the effect of metal ions on prothrombin:

PT → PT' → PT".

The salient features of this model, shown in Fig. 1, include a first metal-dependent conformational transition (PT → PT') that is induced by Ca(II), Mg(II), Mn(II), and most other divalent and trivalent metal ions. This form of prothrombin, PT', is unable to bind to membrane surfaces. A second conformational transition (PT' → PT") is highly metal-selective. It is induced by Ca(II), but most other metal ions are ineffective. This conformer, PT", binds to membrane surfaces.

In the current study, we have tested several predictions of this three-state model. First, it might be possible to isolate a...
conformation-specific antibody directed against the third conformer, PT* that would not bind to the first (PT) or second (PT') conformer. This antibody would have the functional properties of binding the prothrombin Ca(I1) complex but not the prothrombin Mg(I1) complex. Second, to determine whether this antibody is directed against the phospholipid-binding site of prothrombin, the effect of this antibody on prothrombin-phospholipid binding was evaluated. We predicted that anti-prothrombin.Ca(I1)-specific antibodies, 1 directed against the PT* conformer, would inhibit prothrombin-phospholipid binding. The antibodies directed against the PT' conformer would not interfere with prothrombin-phospholipid binding.

MATERIALS AND METHODS

Proteins—Prothrombin was prepared from human plasma by barium citrate adsorption, DEAE-cellulose chromatography, and affinity chromatography using dextran-Sepharose (26, 27).

Antibodies—Anti-prothrombin.Ca(I1)-specific, anti-prothrombin.Mg(I1), and anti-prothrombin Mg(I1)/Ba(I1) antibodies were prepared from anti-prothrombin antisera by sequential immunofluorescence chromatography. Anti-prothrombin antibodies were purified from rabbit anti-human prothrombin antisera by applying antiserum to a prothrombin-Sepharose column (1.5 x 5 cm). The anti-prothrombin antibodies were eluted with 4 M guanidine-HCl, dialyzed against TBS, and concentrated by ultrafiltration (28, 29). These purified anti-prothrombin antibodies were dialyzed into TBS/1 mM MgCl2, and applied to the prothrombin-Sepharose column equilibrated with TBS/1 mM MgCl2. The antibodies which bound to the prothrombin-Sepharose column were termed anti-prothrombin.Mg(I1) antibodies. Antibodies which failed to bind to the column under these conditions were collected, concentrated by ultrafiltration, and dialyzed against TBS/1 mM CaCl2. These antibodies were applied to the prothrombin-Sepharose column equilibrated in TBS/1 mM CaCl2. Antibody bound to the prothrombin-Sepharose column in the presence of Ca(I1) or Mg(I1) was eluted with 3 mM EDTA. These antibodies were selected from anti-prothrombin antibodies on their ability to bind to prothrombin-Sepharose in the presence of 1 mM Ca(I1) but not in the presence of 1 mM Mg(I1). These antibodies were termed anti-prothrombin.Ca(I1)-specific.

The Fab fragments of anti-prothrombin.Mg(I1) and anti-prothrombin.Ca(I1)-specific antibodies were prepared by the method of Harrison and Mage (30). Affinity-purified anti-prothrombin antibodies (20 mg/ml) in 10 mM cysteine and 2 mM EDTA were digested with papain (0.2 mg/ml) at 37 °C for 2 h. The digest was dialyzed against TBS/1 mM MgCl2 and purified on prothrombin-Sepharose column, as described above for the anti-prothrombin-Mg(I1) antibodies; this preparation represents the Fab fragments of anti-prothrombin-Mg(I1) antibodies. The Fab fraction that did not bind to the column was dialyzed against TBS/1 mM CaCl2 and applied to the prothrombin-Sepharose column. The Fab antibody that eluted with 3 mM EDTA was the Fab fragment of anti-prothrombin.Ca(I1)-specific antibodies. Analysis of both Fab preparations by SDS-gel electrophoresis confirmed the molecular weight of the Fab fragments and the absence of undigested immunoglobulin. The binding activity of these fragments, including the effect of Ca(I1) and Mg(I1) on prothrombin interaction, was comparable to the intact immunoglobulin, as measured using a direct binding assay.

Anti-prothrombin.Mg(I1)/Ba(I1) antibodies were purified by the identical method except that, in place of 1 mM CaCl2, a solution of 1 mM MgCl2, 1 mM BaCl2 was employed.

Immunoassays—The interaction of anti-prothrombin.Ca(I1)-specific or anti-prothrombin.Mg(I1) antibodies with prothrombin was studied using a direct radioimmunoassay. 125I-labeled prothrombin (5.6 x 10¹⁰ M; 100 μl) in TBS was added to the antibody solution (100 μl) in TBS. The metal ions, at the indicated concentration, were present in 100 mM TBS (100 μl). Each tube was mixed and incubated overnight at 4 °C. The bound 125I-labeled prothrombin was precipitated by the addition of goat anti-rabbit Ig, and normal rabbit Ig added as carrier. The precipitate which appeared was harvested by centrifugation and the 125I assayed in a Beckman Gamma 6000 spectrometer.

Light Scattering—The interaction of prothrombin with membrane surfaces was evaluated by the relative 90° light-scattering technique (31, 32). Photolipid vesicles (phosphatidylethanolamine and phosphatidylserine in a 1:1 ratio; prepared (33) and quantitated (34) by standard methods, were used in light-scattering experiments performed in a Perkin-Elmer model MPF-3 fluorescence spectrophotometer. Proteins were added to TBS, 5 mM CaCl2, pH 7.4. Prothrombin in 3.4-μg aliquots (5 μl) was added to a fluorescence cuvette containing 0.96 ml of buffer with or without 7.4 μg of phospholipid. The light-scattering intensity of the prothrombin-phospholipid mixture in buffer. The data were plotted as light intensity versus micrograms of prothrombin per micrograms of phospholipid.

To determine whether anti-prothrombin.Mg(I1) antibodies or anti-prothrombin.Ca(I1)-specific antibodies inhibited the binding of prothrombin to phospholipid vesicles, the light-scattering experiment was repeated in the presence of either of these antibodies. Equal volumes of prothrombin (1.36 mg/ml in TBS/5 mM CaCl2) and anti-prothrombin.Mg(I1) antibodies (1.68 mg/ml in TBS/5 mM CaCl2) were mixed and incubated for 10 min. Aliquots (5 μl) of this mixture were added to a fluorescence cuvette containing 0.96 ml of buffer, with or without 7.4 μg of phospholipid, and the intensity of scattered light determined. For the experiments using anti-prothrombin.Ca(I1)-specific antibodies, prothrombin (1.36 mg/ml and anti-prothrombin.Ca(I1)-specific antibodies (0.85 mg/ml in TBS/5 mM CaCl2) were mixed in a ratio of 1 volume of prothrombin solution to 2 volumes of antibody solution. After a 10-min incubation, 7.5-μl aliquots of the mixture were added to a fluorescence cuvette containing 0.96 ml of TBS/5 mM CaCl2, 51 μg of anti-prothrombin.Ca(I1)-specific antibodies, with or without 7.4 μg of phospholipid, and the intensity of scattered light determined. For experiments using anti-prothrombin.Ca(I1)-specific antibodies, prothrombin (1.36 mg/ml and anti-prothrombin.Ca(I1)-specific antibodies (0.85 mg/ml in TBS/5 mM CaCl2) were mixed in a ratio of 1 volume of prothrombin solution to 2 volumes of antibody solution. After a 10-min incubation, 7.5-μl aliquots of the mixture were added to a fluorescence cuvette containing 0.96 ml of TBS/5 mM CaCl2, 51 μg of anti-prothrombin.Ca(I1)-specific antibodies, with or without 7.4 μg of phospholipid, and the intensity of scattered light determined. For experiments using Fab fragments, equal volumes of prothrombin (500 μg in 1 ml of TBS/5 mM CaCl2) and anti-prothrombin.Mg(I1) Fab (500 μg in 1 ml of TBS/5 mM CaCl2) or anti-prothrombin.Ca(I1)-specific Fab (700 μg in 1 ml of TBS/5 mM CaCl2) were mixed and incubated as above. Aliquots of these mixtures (20 μl) were added to a fluorescence cuvette containing 960 μl of TBS/5 mM CaCl2 and either 10 μg of anti-prothrombin.Mg(I1) Fab or 14 μg of anti-prothrombin.Ca(I1)-specific Fab. The titration was performed as described above.

Phospholipid Binding by Gel Filtration Analyses—Phospholipid vesicles (0.2 ml) were incubated with 0.2 ml of 115I-labeled prothrombin (1.2 x 10¹⁰ M) and 115I-labeled anti-prothrombin.Mg(I1) antibodies (1.6 x 10⁻⁵ M) in TBS/5 mM CaCl2. After incubation at 23 °C for
30 min, the various species were separated by size using a Bio-Gel A15 column (1 x 60 cm) equilibrated in TBS/5 mM CaCl₂. Fractions of 1 ml were collected and the elution profiles of prothrombin and antibody determined by assay of $^{125}$I and $^{131}$I, respectively, in aliquots of these fractions. The column was calibrated with prothrombin alone, antibody alone, prothrombin and phospholipid vesicles, antibody and phospholipid vesicles, and the prothrombin-antibody complex.

RESULTS

Anti-Prothrombin-Ca(II)-specific Antibodies—We have previously prepared polyclonal and monoclonal antibodies specific for the metal-stabilized structure of prothrombin (20, 22, 23). These antibodies bind to prothrombin similarly in the presence of Ca(II) and a variety of other metal ions. The prothrombin-Mg(II) antibodies, which bind to prothrombin in the presence of Ca(II) and Mg(II) but not in the absence of metal ions, have properties similar to these antibodies (Fig. 2A). However, recent observations had led us to anticipate that a subpopulation of anti-prothrombin-Ca(II) antibodies might have properties significantly different than the anti-prothrombin-Ca(II) antibodies as a whole (24). From previous work (24), we have predicted the presence of an antibody population that would bind to prothrombin in the presence of Ca(II) but not in the presence of Mn(II) or Mg(II). Anti-prothrombin antibodies were applied to prothrombin-Sepharose in the presence of Mg(II). The antibodies which failed to bind were dialyzed into Ca(II) and reapplied to a column of prothrombin-Sepharose. The antibodies which bound to prothrombin in the presence of Ca(II) (but not in the presence of Mg(II)) were eluted with EDTA and termed anti-prothrombin-Ca(II)-specific.

The interaction of the anti-prothrombin-Ca(II)-specific antibodies with prothrombin was studied using a direct binding radioimmunoassay. The anti-prothrombin-Ca(II)-specific antibodies bind to prothrombin in the presence of 1 mM CaCl₂ (Fig. 2B). No binding was observed when metal ions were eliminated. Significant binding of anti-prothrombin-Ca(II)-specific antibodies to prothrombin was not observed in the presence of 1 mM Mg(II). This is in contrast to anti-prothrombin-Ca(II) antibodies, anti-(12-44)N antibodies, and RL1.3 conformation-specific antibodies to prothrombin, which bind equivalently to prothrombin in the presence of Mg(II) or Ca(II) (19, 20, 23).

Specificity of Antibody-Prothrombin Binding—To determine which metal ions support antibody-prothrombin binding and the concentration of metal ions which yield optimal expression of the metal-stabilized antigenic determinant, the interaction of anti-prothrombin-Ca(II)-specific antibodies with $^{125}$I-labeled prothrombin was evaluated in a direct binding radioimmunoassay. In these experiments, the percentage of bound $^{125}$I-labeled prothrombin was determined as a function of metal ion concentration. As shown in Fig. 3, Ca(II) supported antibody-prothrombin interaction. Half-maximal binding was observed at 0.1 mM CaCl₂. SrCl₂ also supported significant binding, with half-maximal binding observed at about 1 mM SrCl₂. MnCl₂ had little effect on antibody-prothrombin interaction, and MgCl₂ had no effect. BaCl₂ had a small but reproducible effect. Half-maximal binding was observed at about 1 mM BaCl₂.

Effect of Metal Ion Combinations on Antibody-Prothrombin Interaction—According to our hypothetical model, most divalent and trivalent metal ions would be capable of inducing the first of the sequential conformational transitions. However, the metal requirements for the second conformational transition are highly selective. The observations of Nelsestuen (14) suggested to us that Ba(II) might be less effective in promoting the first conformational change than other metal ions. Moreover, the metal requirements for phospholipid-prothrombin interaction (15) suggested that Ca(II) and Sr(II), and, to a lesser extent Ba(II), might be effective in inducing the second conformational transition. We evaluated the interaction of anti-prothrombin-Ca(II)-specific antibodies with prothrombin in the presence of both Mn(II) and Ba(II). In principle, Mn(II) could induce the first conformational transition and Ba(II) could induce the second, thus exposing the same antigenic site that is expressed in the presence of Ca(II). As shown in Table I, minimal antibody-prothrombin interaction was observed in the presence of 2.5 mM Ba(II) (1% bound), 2.5 mM Mn(II) (2% bound), or in the absence of metal ions (0.5% bound). However, in the presence of both 2.5 mM Ba(II) and 0.1 mM Mn(II), significant antibody-antigen binding was observed (14% bound). Similar results were observed with 2.5 mM Ba(II), 1.0 mM Mn(II) (16% bound). Approximately 80% of the prothrombin antigen was bound in the presence of Ca(II). These results suggest that certain combi-
nations of metal ions are more effective in the expression of the anti-prothrombin-Ca(II)-specific antigen than these metal ions alone. However, the antigen structure of the Ba(II)/Mn(II)-stabilized prothrombin is not equivalent with that structure stabilized by Ca(II). Nonetheless, these results lend further support for the concept that the two conformational transitions differ in their metal specificity.

Although the Mn(II)/Ba(II) combinations were partially effective in expressing the anti-prothrombin-Ca(II)-specific antigen, Ca(II) was considerably more effective. We purified an antibody subpopulation from anti-prothrombin antiserum, analogous to the anti-prothrombin-Ca(II)-specific antibody, which bound to prothrombin-Sepharose only in the presence of Mg(II)/Ba(II), but not in the presence of Mg(II) alone. We called this antibody anti-prothrombin-Mg(II)/Ba(II)-specific. As shown in Table I, this antibody bound prothrombin minimally in the presence of Ba(II) or Mg(II). In combination, Ba(II)/Mg(II) supported significant binding (31%), although this level was lower than that produced by Ca(II) (75%). Although quantitative differences were observed when anti-prothrombin-Ca(II)-specific antibodies and anti-prothrombin-Mg(II)/Ba(II) antibodies were compared, it is likely that these populations are similar.

Inhibition of Prothrombin-Phospholipid Interaction with Conformation-specific Antibodies—If the anti-prothrombin-Ca(II)-specific antibodies are directed against the phospholipid-binding site on the prothrombin-Ca(II) complex, these antibodies should inhibit the interaction of prothrombin with phospholipid vesicles. The binding of prothrombin to phospholipid vesicles in the presence of various antibody populations was compared using the relative 90° light-scattering technique (Fig. 4). The conditions chosen were those in which most of the prothrombin is antibody-bound. In the absence of antibody, prothrombin demonstrates the typical binding pattern. Preincubation with anti-prothrombin-Mg(II) antibodies did not inhibit prothrombin from binding the lipid vesicles. However, the scattering intensity was similar to that of prothrombin alone, reflecting minimal change in the overall size of the antibody-antigen-liposome complex. Anti-prothrombin-Ca(II)-specific antibodies completely inhibited the binding of prothrombin to phospholipid vesicles. These results indicate that anti-prothrombin-Ca(II)-specific antibodies are directed at or near the phospholipid-binding site and interfere with the interaction of prothrombin with lipid vesicles. In addition, these results suggest that the anti-prothrombin-Mg(II) antibodies, directed against the PT' conformer, bind to an antigenic determinant sufficiently distant from this phospholipid-binding site such that bound antibody does not prevent prothrombin from binding to lipid vesicles.

To eliminate consideration that the polyclonal anti-prothrombin-Ca(II)-specific antibody inhibits prothrombin-lipid interaction nonspecifically by aggregation of prothrombin, this experiment was repeated using Fab fragments of both anti-prothrombin-Mg(II) and anti-prothrombin-Ca(II)-specific antibodies. These antibodies are univalent and are not capable of forming immune complexes. As shown in Fig. 4B, Fab fragments of the anti-prothrombin-Ca(II)-specific antibodies completely inhibited the binding of prothrombin with the phospholipid vesicles. As with the intact antibody, the Fab fragments of the anti-prothrombin-Mg(II) antibodies did not interfere significantly with prothrombin-phospholipid interaction.

These results suggest that, in the presence of CaCl₂, a ternary complex is formed among anti-prothrombin-Mg(II) antibodies, prothrombin, and phospholipid vesicles. This complex was studied directly by gel filtration analysis using a double label experiment including ¹³¹I-labeled prothrombin and ¹²⁵I-labeled anti-prothrombin-Mg(II) antibodies. In the presence of 5 mM CaCl₂, antibody and prothrombin were associated with the phospholipid vesicle fraction (middle panel, Fig. 5). Under the conditions employed, there was
excess prothrombin which also eluted as free prothrombin and a small amount of prothrombin-antibody complex. The elution profiles of prothrombin alone, antibody alone, and prothrombin bound to phospholipid vesicles are shown in Fig. 5 (upper panel). The elution profile of the antibody-prothrombin complex is shown in Fig. 5 (lower panel). Because of insufficient quantities of anti-prothrombin-Ca(I1)-specific antibodies, parallel experiments could not be performed using gel filtration analysis. However, these results directly demonstrate that anti-prothrombin-Mg(I1) antibodies bind to prothrombin when prothrombin is associated with the phospholipid vesicles.

**DISCUSSION**

Prothrombin, the principal protein model for the class of calcium-binding proteins that contain γ-carboxyglutamic acid, has been the subject of extensive study over the past 10 years. The ability of prothrombin to undergo a metal-induced conformational transition is directly related to its ability to interact with membrane surfaces (35) and to serve as substrate for the prothrombinase complex assembled on that surface (36). γ-Carboxyglutamic acid residues define the metal-binding properties of this protein. Abnormal (des-γ-carboxy)prothrombin, a form of prothrombin deficient in γ-carboxyglutamic acid, is also deficient in metal-binding properties (25, 27, 38). This protein fails to bind to phospholipid membrane surfaces in the presence of metal ions (39), does not undergo the metal-induced conformational transition (40, 25), and lacks all of the high affinity metal-binding sites and most of the lower affinity metal-binding sites characteristic of prothrombin (25). By comparison, these results have emphasized the functional role of γ-carboxyglutamic acid in prothrombin: participation in metal-dependent stabilization of the tertiary structure of the prothrombin-metal binary complex and generation of a prothrombin structure that interacts with suitable affinity with the prothrombinase complex on membrane surfaces to facilitate the proteolytic conversion of prothrombin to thrombin.

We have examined prothrombin using monoclonal antibodies conformation-specific for the metal-stabilized conformer of prothrombin (23) and monoclonal antibodies conformation-specific for the calcium-free conformer of prothrombin (24). Both antibodies bind to the prothrombin-Mg(I1) complex. These observations have defined interpretation within the context of the existing model of the effect of metal ions on prothrombin structure. According to this model, the metal-induced conformational transition observed spectroscopically or immunochemically is cation-dependent, but not cation-specific (14-17, 19). All of the many divalent and trivalent cations tested are able to induce this conformational transition; however, Ba(I1) requires relatively higher concentrations (6 mM) to be effective (14). To integrate our data with this model, we have expanded the model to suggest that prothrombin undergoes a second conformational transition which has not been observed by the spectroscopic methods that have been employed but which might be monitored by the expression of a unique antigenic determinant. Polyclonal anti-prothrombin-Ca(I1) antibodies (20), which bind to prothrombin in the presence of all of the di- and trivalent metal ions studied, should contain a small subpopulation which is specific for the antigenic determinant that is expressed on the prothrombin-Ca(I1) complex but not the prothrombin-Mg(I1) complex.

The anti-prothrombin-Ca(I1)-specific antibodies isolated in the current study fulfill these criteria. These antibodies do not bind to prothrombin in the presence of Mg(I1), Mn(I1), or Ba(I1) but do bind to prothrombin in the presence of Ca(I1) or Sr(I1). Although Mg(I1), Mn(I1), Ca(I1), and Sr(I1) can support the first conformational transition (15), only Ca(I1) and Sr(I1) also support the second conformational transition. Ba(I1), at the concentrations employed, is not effective in supporting the first conformational transition (14). Our results emphasize the sequential nature of these alterations in tertiary structure. The anti-prothrombin-Ca(I1)-specific antigen is not expressed in the presence of Ba(I1) (although Ba(I1) does promote the second transition) because Ba(I1) is not effective in supporting the first transition. However, Mg(I1)/Ba(I1) or Mn(I1)/Ba(I1) mixtures are capable of ex-

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2 Although not tested, we suspect that the trivalent lanthanide ions (e.g. Gd(III)) will support the first and second conformational transition. This is based upon the observation that these metal ions support the conversion of prothrombin to thrombin by Factor Xa (9). Because the lanthanide ions rapidly hydrolyze above pH 7, they could not be studied in the experimental systems employed for the evaluation of cation specificity.
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pressing this antigen because Mg(II) and Mn(II) support the first conformational transition and Ba(II) supports the second conformational transition. Together, these cations fulfill the requirements for the sequential changes in the three-dimensional structure to enable lipid binding. Nelsestuen et al. (15) have previously shown that Mn(II)/Ba(II) can support 100% of the prothrombinase activity observed with Ca(II), and Mg(II)/Ba(II) can support about 50% of the activity observed with Ca(II). In contrast, Mn(II), Ba(II), or Mg(II) alone did not support prothrombinase activity. Although these results differ quantitatively from our own results on antibody-prothrombin binding, the patterns of cation specificity are identical.

The data presented suggest that the antigen against which some of the anti-prothrombin-Ca(II)-specific antibodies are directed is the phospholipid-binding site of prothrombin. The feature of the PT* conformer that distinguishes it functionally from the conformer PT' is its phospholipid-binding properties. We have demonstrated that anti-prothrombin-Ca(II)-specific antibodies, conformation-specific for PT*, directly inhibit prothrombin-phospholipid interaction. These results provide direct evidence that the topographic antigenic sites associated with each of the two conformational transitions are sufficiently separated on the protein surface such that anti-prothrombin-Ca(II)-specific antibodies interfere with prothrombin-lipid binding whereas anti-prothrombin-Mg(II) antibodies do not.

The cation ion specificity of prothrombin-phospholipid interaction has been previously established. Nelsestuen et al. (15) have shown that Ca(II), Sr(II), and Ba(II) support prothrombin binding to phospholipid surfaces. These results have been interpreted within the context of the existing model. The metal specificity has been considered due to preferences in ligand geometry and coordination number of metal ions bound by oxygen ligands of γ-carboxyglutamic acid residues on prothrombin and oxygen ligands on the phosphate groups of the phospholipid micelles. This cation specificity has been thought to be a manifestation of the prothrombin-metal-phospholipid ternary complex. However, our results on the metal ion specificity of anti-prothrombin-Ca(II)-specific antibody binding to prothrombin, in the absence of phospholipid, emphasize that this cation specificity is a characteristic of the binary prothrombin-metal complex. These results suggest that metal ions are not involved directly in the prothrombin-phospholipid interface. Although in our model we have chosen to interpret the Ca(II)-dependent alteration in prothrombin as a second conformational change, independent data supporting this suggestion have not yet been obtained. The data presented here would also be consistent with a model in which, for example, the formation of a lipid-binding site after the filling of a Ca(II)-specific metal-binding site is the result of charge neutralization. The formation of the phospholipid-binding site remains, however, a property of the binary prothrombin-metal complex.

We have tested a three-state model to describe the changes in prothrombin structure that accompany the binding of metal ions to prothrombin. The first conformational transition is cation-dependent, but cation-nonspecific. It appears to be associated with occupancy of the high affinity class of metal-binding sites, and can be observed by the quenching of intrinsic fluorescence, alteration in the circular dichroism spectrum, and the expression of new antigenic determinants. This conformer, PT', does not bind to phospholipid and lacks coagulant activity. The prothrombin-Mg(II) complex shares this structure. A second conformational transition is induced by Ca(II), Sr(II), or Ba(II), leading to the expression of a phospholipid-binding site. The prothrombin-Ca(II) complex and, to some degree, the prothrombin-Mn(II)/Ba(II) and prothrombin-Mg(II)/Ba(II) complexes express the phospholipid-binding site, the anti-prothrombin-Ca(II)-specific antigen, and prothrombin coagulant activity. The conformer, PT+, binds to the surface of phospholipid vesicles. In our model, γ-carboxyglutamic acid plays an essential role in metal binding, the consequence of which is only the stabilization of prothrombin in an active form for membrane interaction. This view represents a fundamental departure from existing models of prothrombin structure. The phospholipid-binding site does

FIG. 5. Effect of anti-prothrombin-Mg(II) antibodies on prothrombin-phospholipid micelle complex formation—Evaluation by gel filtration analysis. Mixtures, as indicated, were applied to a 1 × 60-cm Bio-Gel A5 column. [3H] or [125I] in the column fractions were assayed to determine the elution fraction of antibody or prothrombin, respectively. Upper panel, phospholipid micelles, [3H]-labeled prothrombin, and 5 mM CaCl₂ (O—O); [125I]-labeled prothrombin and 5 mM CaCl₂ (A—A); [125I]-labeled anti-prothrombin-Mg(II) antibodies, 5 mM CaCl₂ (O—O). Middle panel, phospholipid micelles, [125I]-labeled prothrombin (O—O), [125I]-labeled anti-prothrombin-Mg(II) antibodies (O—O), and 5 mM CaCl₂. Lower panel, [125I]-labeled prothrombin (O—O), [125I]-labeled anti-prothrombin-Mg(II) antibodies (O—O), and 5 mM CaCl₂. In double label experiments, the contribution of [125I] to the [3H] measurements was corrected.
not include γ-carboxyglutamic acid residues which participate in metal-dependent bridging to the phospholipid surface. Four arguments have led us to conclude that the prothrombin-phospholipid interface does not include γ-carboxyglutamic acid: 1) the absence of direct evidence for the participation of γ-carboxyglutamic acid in this interface; 2) the fact that the cation specificity for prothrombin-phospholipid binding is a property of the prothrombin-metal ion complex, not the prothrombin-metal ion-phospholipid complex; 3) the work of Rhee et al. (41) demonstrating that phospholipid binding does not alter the hydration of metal ions bound to prothrombin; 4) the general observation that high concentrations of Ca(II) do not disrupt prothrombin-phospholipid binding. The three-state model presented is consistent with existing data on the effect of metal ions on prothrombin structure. Based upon this new concept of prothrombin-phospholipid interaction, we are using the anti-prothrombin-Ca(II)-specific antibodies to define and localize the phospholipid-binding site on prothrombin.

Acknowledgments—The technical assistance of Lori DeFurio is gratefully acknowledged. We also appreciate the help of Alisan Blashill in preparing several of the antibody populations.

REFERENCES
1. Nemerson, Y., and Furie, B. (1980) CRC Crit. Rev. Biochem. 9, 45–85
2. Jackson, C. M., and Nemerson, Y. (1980) Annu. Rev. Biochem. 49, 765–811
3. Waiz, D. A., Hewett-Emmitt, D., and Seegers, W. H. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1669–1972
4. Butkowski, R. J., Elion, J., Downing, M. R., and Mann, K. G. (1977) J. Biol. Chem. 252, 4942–4957
5. Furie, B., Bing, D. H., Feldmann, R. J., Robison, D. J., Burnier, J. P., and Furie, B. C. (1982) J. Biol. Chem. 257, 3875–3882
6. Nelsestuen, G. L., Zytovkovic, T. H., and Howard, J. B. (1974) J. Biol. Chem. 249, 6347–6350
7. Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2730–2733
8. Bajaj, S. P., Batkowski, R. J., and Mann, K. G. (1975) J. Biol. Chem. 250, 2150–2156
9. Furie, B. C., Mann, K. G., and Furie, B. (1976) J. Biol. Chem. 251, 3226–3241
10. Sperling, R., Furie, B. C., Blumenstein, M., Keyt, B., and Furie, B. (1978) J. Biol. Chem. 253, 3989–3996
11. Bajaj, S. P., Nowak, T., and Castellino, F. J. (1976) J. Biol. Chem. 251, 6294–6299
12. Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Fletcher, C. H., and Bloomfield, V. A. (1981) Biochemistry 20, 351–358
13. Furie, B. C., Blumenstein, M., and Furie, B. (1979) J. Biol. Chem. 254, 12521–12530
14. Nelsestuen, G. L. (1976) J. Biol. Chem. 251, 5648–5656
15. Nelsestuen, G. L., Broderius, M., and Martin, G. (1976) J. Biol. Chem. 251, 6886–6893
16. Prendergast, F. G., and Mann, K. G. (1977) J. Biol. Chem. 252, 840–850
17. Bloom, J. W., and Mann, K. G. (1978) Biochemistry 17, 4430–4438
18. Furie, B., Provest, K. L., Blanchard, R. A., and Furie, B. C. (1978) J. Biol. Chem. 253, 8980–8987
19. Furie, B., and Furie, B. C. (1979) J. Biol. Chem. 254, 9766–9771
20. Tai, M. M., Furie, B. C., and Furie, B. (1980) J. Biol. Chem. 255, 2790–2795
21. Madan, D. A., Hall, T. J., Reiner, H. M., Hisey, R. G., and Koehler, K. A. (1980) J. Biol. Chem. 255, 8599–8605
22. Tai, M. M., Furie, B. C., and Furie, B. (1984) J. Biol. Chem. 259, 4162–4168
23. Lewis, R. M., Furie, B. C., and Furie, B. (1985) Biochemistry 22, 4524–4529
24. Owens, J., Lewis, R. M., Cantor, A., Furie, B. C., and Furie, B. (1984) J. Biol. Chem. 259, 13800–13805
25. Borowski, M., Furie, B. C., Goldsmith, G. H., and Furie, B. (1985) J. Biol. Chem. 260, 9258–9264
26. Rosenberg, J. S., Beeler, D. L., and Rosenberg, R. D. (1975) J. Biol. Chem. 250, 1607–1617
27. Mileteich, J. P., Jackson, C. M., and Majerus, P. W. (1978) J. Biol. Chem. 253, 6903–6919
28. Blanchard, R. A., Furie, B. C., Kruger, S. F., Waneck, G., Jorgenson, M., and Furie, B. (1983) J. Lab. Clin. Med. 101, 242–255
29. Blanchard, R. A., Furie, B. C., Jorgenson, M., Kruger, S., and Furie, B. (1981) New Engl. J. Med. 305, 242–248
30. Harrison, E. T., and Mage, M. G. (1967) Biochim. Biophys. Acta 147, 52–59
31. Nelsestuen, G. L., and Lim, T. K. (1977) Biochemistry 16, 4164–4171
32. Bloom, J. W., Nemesis, M. E., and Mann, K. G. (1979) Biochemistry 18, 4419–4425
33. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, E., and Carlson, F. D. (1977) Biochemistry 16, 2806–2810
34. Chen, P. S., Toribara, T. Y., and Warnier, H. (1956) Anal. Chem. 28, 1756–1758
35. Gittel, S. N., Owen, W. G., Eason, C. T., and Jackson, C. M. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1344–1348
36. Mann, K. G., Nemesis, M. E., Tracy, P. B., Hibbard, L. S., and Bloom, J. S. (1982) Biophys. J. 37, 106–107
37. Nelsestuen, G. L., and Suttle, J. W. (1972) J. Biol. Chem. 247, 8176–8182
38. Stenflo, J., and Ganrot, P. O. (1972) J. Biol. Chem. 247, 8160–8166
39. Eason, C. T., Suttle, J. W., and Jackson, C. M. (1975) J. Biol. Chem. 250, 4906–4909
40. Tuhy, P. M., Bloom, J. W., and Mann, K. G. (1979) Biochemistry 18, 6842–6848
41. Rhee, M. J., Horrocks, W. D., Jr., and Kosow, D. P. (1982) Biochemistry 21, 4524–4528