Role of γ-Carboxyglutamic Acid

CATION SPECIFICITY OF PROTHROMBIN AND FACTOR X-PHOSPHOLIPID BINDING*

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Divalent cations are required for two roles in prothrombin-phospholipid interaction. The first role, catalysis of a prothrombin protein transition has a reaction half-life of 100 min at 0° and is a prerequisite to phospholipid binding. The binding sites required for this transition are very low in cation specificity. All di- and trivalent cations tested were effective in this role with the exception of beryllium. Barium catalyzed the transition but only at high concentrations (6.6 mM was required for half-reaction). Blood-clotting Factor X, another γ-carboxyglutamic acid-containing protein, also undergoes a catalysis-catalyzed protein transition which is a prerequisite to Factor X-phospholipid binding. In both proteins, the transition can be monitored by a decrease in the protein's intrinsic fluorescence. Compared to prothrombin, the Factor X transition occurs much more rapidly, has a somewhat greater specificity for cations, and requires higher concentrations of cations. This indicates that the cation binding sites provided by γ-carboxyglutamic acid are not completely uniform in all proteins.

The second role of divalent cations in prothrombin-phospholipid interaction is in the actual protein-phospholipid binding. This interaction was studied by protein fluorescence quenching resulting from excitation energy transfer to a chromophore attached to the phospholipid membrane. Only strontium and barium satisfactorily replaced calcium in this role. A number of other cations form protein-phospholipid complexes but of the wrong structure. These cations inhibit the prothrombin complex (Factor X, calcium, phospholipid, Factor V). The cation specificity for Factor X-phospholipid binding is the same as for prothrombin except that higher concentrations of cations are required. Factor X, generated by action of Russell's viper venom on Factor X, displayed the same calcium requirements for the protein transition and phospholipid interaction as Factor X.

The cation requirements of the prothrombinase complex correlate with the cation requirements of prothrombin and Factor X-phospholipid binding. Strontium is the only cation that will singly replace calcium. Barium is ineffective alone because the concentrations required to catalyze the protein transition cause precipitation of the phospholipid. Combination of certain other cations with barium will, however, substitute for calcium. The other cations (specifically magnesium or manganous ion) can catalyze the protein transitions and barium forms the correct protein-phospholipid complexes.

The vitamin K-dependent structures in the blood-clotting proteins, γ-carboxyglutamic acid residues (1–3), are involved in calcium and phospholipid binding by prothrombin (4–6). Recent evidence indicates that in addition to γ-carboxyglutamic acid, native protein structure is required for tight calcium binding and for phospholipid binding by prothrombin (7, 8). Furthermore, it is clear that there are different types of calcium binding sites. Prothrombin binds a maximum of about 10 calcium ions with half-saturation at room temperature occurring at about 0.6 mM calcium (6–10). Based on studies of a calcium-dependent prothrombin transition, it was concluded that 3 or 4 calcium ions are required to catalyze a protein transition which is a prerequisite for prothrombin-phospholipid interaction (11, 12). After the protein transition, calcium is further involved in formation of the prothrombin-phospholipid complex (11, 12). Due to the homology of the vitamin K-dependent blood clotting proteins (13), exemplified by the similarity of the γ-carboxyglutamic acid-containing regions of prothrombin and Factor X (14, 15), it might be expected that these other proteins display calcium and phospholipid binding properties similar to prothrombin. The present study examines the cation requirements for the prothrombin transition and prothrombin-phospholipid complex formation. These two processes are shown to have different cation specificities which allow each process to be studied independently. This study also demonstrates that factor X-phospholipid binding has requirements similar but...
not identical to prothrombin-phospholipid binding. The specificity provides some insight into the nature of the binding sites provided by γ-carboxyglutamic acid and is correlated with the cation requirements of the prothrombinase complex (blood-clotting Factors X and V, Ca²⁺, phospholipid).

METHODS

Bovine Factor X was produced from purified bovine Factor X by incubation with Russell's viper venom (Sigma). Typically, 0.6 mg of highly purified Factor X (15, 16), which contains no Factor X activity assayed according to Bachmann et al. (17), was incubated with 30 μg of Russell's viper venom for 30 min at 37°. The product contained 30 to 100 units of Factor X/mg. Bovine serum, used as a source of Factor V, was incubated with the clot for 3 days at room temperature, centrifuged, and stored frozen. The hydrolysis of TosArgOMe by thrombin was measured according to Hummel (18). Unless specified, all experiments were carried out in 0.05 M Tris buffer (pH 7.5) containing 0.1 M NaCl. All metal ions are of reagent grade and were provided as the chloride salts.

Phospholipid vesicles were formed by dissolving the phospholipids in chloroform:methanol (2:1) and evaporating to dryness followed by addition of buffer, sonication, and finally centrifugation to remove excess turbidity. DNP-PE was synthesized according to procedures for similar derivatives (20) using 1,2-dipalmitoyl-sn-glycerol-3-phosphorylethanolamine and 2,4-dinitro-1-fluorobenzene purchased from the Sigma Chemical Co. Phospholipid composition is expressed as the ratio of phospholipids before sonication. The two phospholipid compositions used in this study are: 

**Equilibrium titrations** were performed by a method described as the “one-step EDTA addition” method (12). In this procedure, the protein (plus or minus phospholipid) is incubated in the presence of a known metal ion concentration until equilibrium is reached and the fluorescence of this sample is measured. EDTA is added in sufficient quantity to completely reverse the metal-ion-dependent fluorescence change and the fluorescence is again measured. The fluorescence in the presence of the metal ions relative to the fluorescence in the absence of metal ions is calculated. A titration is performed by varying the metal ion concentration. In all experiments, the fluorescence emission from the system in the absence of cations is assigned the value of 100 and relative changes brought about by the presence of cations are studied. This method was used for metal ion titration of the Factor X and Fragment I protein transitions by measuring intrinsic protein fluorescence change. This method was also used for studying the binding of Factor X and prothrombin to phospholipid. In the latter case, the fluorescence quenching due to energy transfer to the phospholipid membrane can be calculated by knowing the concentration of protein and the concentration of membrane. Since the distance through which fluorescence energy transfer can occur is restricted, quenching due to this process will be low in dilute solutions and due to random location of the molecules. If the protein becomes bound to the membrane surface, the average distance between the protein and the chromophore decreases and quenching of protein fluorescence by energy transfer will increase. It is not necessary to know accurately the distance involved since increased quenching indicates a closer relationship of protein and phospholipid which can only come about by protein-phospholipid binding.

In the absence of calcium ions, the fluorescence emission of prothrombin (80 μg in 2.5 ml) is decreased by 58% upon the addition of phospholipid (200 μg) vesicles containing 50% DNP-PE. This decrease is the same as that observed when the phospholipid preparation is mixed with a similar concentration of pure tryptophan. This fluorescence quenching is due primarily to absorption of incident or emitted light by the DNP-PE and to some energy transfer resulting from random location of the molecules. The fluorescence yield from tryptophan is not further affected by the addition of calcium ions. The fluorescence emission of prothrombin, however, is further decreased by addition of calcium as shown in Fig. 1. Assigning the fluorescence yield for a solution of protein-phospholipid in the absence of calcium a value of 100%, the addition of calcium decreases fluorescence emission by a maximum of about 37%.

The addition of calcium to a sample of prothrombin plus phospholipid membranes which contain no chromophore capable of accepting excitation energy from protein results in a decrease of fluorescence emission by only 5 to 8% (Fig. 1). This decrease has been shown to be due to an intrinsic protein fluorescence change resulting from a calcium-dependent protein transition (12). The difference between the per cent fluorescence change in these two systems (Fig. 1) is due to fluorescence energy transfer from protein to the DNP-PE as the protein binds to the phospholipid in a calcium-dependent process. As illustrated by the method used in Fig. 1,
The fluorescence of the sample was measured, EDTA was added, and measurement as a percentage of the second. Data for phospholipid buffer were incubated at 35° at the calcium concentrations indicated. Data for phospholipid containing 20% DNP-PE (1:1) (O—O) are shown.

Prothrombin (50 μg) plus phospholipid (200 μg) in 2.5 ml of buffer were incubated at 35° at the calcium concentrations indicated. The fluorescent of the sample was measured, EDTA was added, and the resulting fluorescence was measured. The graph presents the first measurement as a percentage of the second. Data for phospholipid consisting of both Folch Fraction III (O—O) and of Folch Fraction III:DNP-PE (1:11) (●—●) are shown.

![Graph](image1)

**Fig. 1.** Measurement of prothrombin-phospholipid binding by fluorescence energy transfer. The one-step EDTA addition method was used. Prothrombin (50 μg) plus phospholipid (200 μg) in 2.5 ml of buffer were incubated at 35° at the calcium concentrations indicated. The fluorescent of the sample was measured, EDTA was added, and the resulting fluorescence was measured. The graph presents the first measurement as a percentage of the second. Data for phospholipid consisting of both Folch Fraction III (O—O) and of Folch Fraction III:DNP-PE (1:11) (●—●) are shown.

![Graph](image2)

**Fig. 2.** Factor X protein transition and phospholipid interaction. The intrinsic Factor X (50 μg in 2.5 ml) fluorescence change in the presence of calcium was monitored by the "one-step EDTA addition technique" in the absence (Δ—Δ) and presence (●—●) of phospholipid (200 μg of Folch Fraction III). Titration of 50 μg of Factor X in the presence of phospholipid containing a chromophore (200 μg of Folch Fraction III:DNP-PE (1:1)) is also given (●—●). Factor Xa (O—O), produced from Factor X a as described under "Methods," was titrated by the "one-step EDTA addition method" using the same conditions as for Factor X.

This quenching is reversed upon dissociation of the protein from the phospholipid, accomplished by the addition of EDTA to remove calcium from the complex.

The lipid composition of 50% DNP-PE (Fig 1) then produces about a 50% reduction in fluorescence emission due to energy transfer. In other experiments, a lipid composed of 20% DNP-PE yielded only a 6% reduction in fluorescence emission attributable to energy transfer. A 50% DNP-PE lipid was used in subsequent experiments to obtain a large change in fluorescence when the protein binds to the phospholipid.

Changes in the fluorescence yield from Factor X was also studied with the results shown in Fig. 2. Upon addition of calcium to Factor X there is a change in intrinsic fluorescence indicating a change has occurred in the environment of one or more fluorescent amino acids. The fluorescence change is reversed by addition of EDTA. Unlike the prothrombin transition which has a half life of about 100 min at 0° (12), the Factor X transition is complete in less than 0.2 min at 0°. The calcium dependence of the Factor X transition is shown in Fig. 2 (Δ—Δ) with a maximum intrinsic fluorescence change of about 10% and half-reaction occurring at about 1.0 mM calcium. If phospholipid containing no DNP-PE is added (Fig. 2, Δ—●), the calcium concentration at half-reaction is decreased but no substantial change occurs in the maximum fluorescence change. If phospholipid containing DNP-PE is used, the fluorescence change is about 38% (Fig. 2, ●—●). The greater quenching is due to fluorescence energy transfer as described for prothrombin in Fig. 1. The calcium concentration required for Factor X-phospholipid binding is about twice that of prothrombin-phospholipid binding (compare Figs. 1 and 2).

The results of binding of Factor Xa to phospholipid are also shown in Fig. 2 (O—O). A difference in the maximum quenching of Factor X and Xa fluorescence is anticipated since the Xa preparation contains proteins from Russell's viper venom and an activation peptide, neither of which will bind to the phospholipid and induce fluorescence quenching by energy transfer. The half-reaction and degree of cooperativity for Factor Xα-phospholipid binding appear the same as those for Factor X-phospholipid binding.

**Direct Measurement of Calcium Dependence of Protein-Phospholipid Binding**—There are two calcium-dependent processes in prothrombin-phospholipid binding. One is a protein transition which is a prerequisite to the second process, the actual protein-phospholipid binding. Previous studies examined the protein transition alone or the combined protein transition plus phospholipid binding (12). The calcium dependence for the prothrombin-phospholipid binding shown in Fig. 1 is also due to a combination of the transition and phospholipid binding steps. More accurate study of the protein-phospholipid interaction depends on the measurement of the protein-phospholipid interaction independent of the transition.

Many metal ions will substitute for calcium in the protein transition step but only a few will substitute for calcium in binding prothrombin to phospholipid (see below). The best example is manganous ion which catalyzes the prothrombin transition at low concentration (0.05 mM) but does not form a protein-phospholipid complex. This allows the study of the cation requirements of prothrombin-phospholipid binding in the absence of transition effects by the method shown in Fig. 3A. At zero calcium the fluorescence change is due to intrinsic protein fluorescence changes associated with the protein transition catalyzed by manganous ion. As the calcium concentration is increased, prothrombin binds to the phospholipid and the fluorescence emission decreases due to excitation energy transfer to DNP-PE. Attainment of a maximum energy transfer at higher calcium reflects a condition where all of the prothrombin is phospholipid-bound and further calcium does not bring about further quenching of fluorescence.

If it is assumed that prothrombin-phospholipid binding is a two-state process consisting of free prothrombin and one form of phospholipid-bound prothrombin, the intermediate fluorescence values can be used to calculate the per cent of protein which is phospholipid-bound. Treating the data in Fig. 3A as such a two-state equilibrium, a Hill plot can be constructed as shown in Fig. 3B from which the calcium concentration at half-reaction (0.08 mM) and the Hill coefficient estimated at
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Fig. 3. Measurement of protein-phospholipid binding in the absence of protein transition effects. A gives the fluorescence changes determined by the “one-step EDTA addition” method (35) for prothrombin (50 μg), phospholipid (200 μg of Folch Extract III:DNP-PE (1:1)), and MnCl₂ (0.05 mM) in 2.5 ml of buffer containing the calcium concentrations shown. B gives a Hill plot of the data from A (Δ—Δ) calculated by assigning the maximum fluorescence change at high calcium (see A) a value of 100% prothrombin bound and the fluorescence at zero calcium a value of 0% prothrombin bound. Intermediate levels were calculated by simple ratios. Also included in B are data obtained under identical conditions but with 0.2 mM Mn⁺⁺ (O—O) and with 0.2 mM Mn⁺⁺ plus twice the normal protein and phospholipid, 100 μg of prothrombin, and 400 μg of phospholipid (●—●). The slope of the line drawn is 2.2 and the intercept is at 0.08 mM Ca⁺⁺.

half-reaction (2.2) can be obtained. Also shown are experiments conducted at higher manganous ion concentration (0.2 mM). These data are experimentally indistinguishable from those obtained at 0.05 mM manganous ion indicating that these concentrations of manganous ion do not measurably interfere with the calcium binding sites involved in prothrombin-phospholipid binding. At 1 mM and 2 mM manganous ion the half-reactions occurred at about 0.2 and 0.35 mM calcium, respectively. Therefore, at high concentrations, manganous ion interferes with phospholipid binding. Magnesium ion also inhibits prothrombin-phospholipid binding. When 1 mM magnesium was used to catalyze the transition, the half-reaction for prothrombin-phospholipid binding occurred at 0.13 mM calcium which is higher than that observed in Fig. 3 and indicates an inhibition by magnesium ion.

A third type of experiment shown in Fig. 3B involves the use of twice as much prothrombin and phospholipid. Calcium is bound during prothrombin-phospholipid complex formation and, since total calcium is plotted in Fig. 3, it is necessary to determine whether the bound calcium represents a measurable portion of the calcium added to the cuvette. Since the results of this experiment are similar to the other experiments, it can be concluded that the change in free calcium concentration due to bound calcium is negligible.

Factor X-phospholipid binding was also studied by the method shown for prothrombin in Fig. 3. The presence of 0.2 mM manganous ion resulted in a 10% decrease in the Factor X fluorescence due to the protein transition described in Fig. 2. Further addition of calcium resulted in binding of Factor X to the phospholipid with half-reaction occurring at 0.99 mM calcium. The fact that manganous ion causes about the same change in intrinsic Factor X fluorescence as calcium and lowers the calcium requirement for Factor X-phospholipid binding (compare the 0.22 mM value here with data in Fig. 2) indicates that the Factor X transition is a prerequisite to Factor X-phospholipid binding. This is supported by several other observations below and is the same order of events observed for prothrombin (12).

Cation Specificity of Protein Transition and Phospholipid Binding (Table I)—As exemplified by the group II cations (magnesium, calcium, strontium, and barium), catalysis of the prothrombin/Fragment 1 transition has a low cation specificity. The differing ionic radii of these cations (Mg⁺⁺ = 0.66 Å, Ca⁺⁺ = 0.99 Å, Sr = 1.12 Å, and Ba⁺⁺ = 1.34 Å) indicate that the binding sites involved in the transition are not highly restricted. All other metal ions tested with the exception of beryllium also catalyzed the transition. For these latter ions, no attempt was made to determine the metal ion concentration at half-reaction but the concentrations required are lower than the group II cations.

The ability of various metal ions to catalyze the Factor X transition was also determined. The results (Table I) indicate a greater specificity for calcium and higher concentration requirements than the prothrombin transition. While the Factor X transition is a cooperative process (Fig. 2, Δ—Δ) indicating that more than one cation is required for the transition, determination of the Hill coefficient was not attempted due to the high percentage errors in the measurements at the extremes of the reaction.

The ability of metal ions to participate in protein-phospholipid binding was examined with the results shown in Table I. For both prothrombin and Factor X, magnesium is ineffective at all concentrations studied. Calcium is the most effective, followed by strontium and barium. The specificity for magnesium and barium is the opposite of that observed for the transition and it can be concluded that the binding sites for the two processes are different. Several other metal ions (Table I) did form a prothrombin-phospholipid complex but of different structure. This was evidenced by 60 to 70% quenching of fluorescence due to energy transfer. The group II metal ions shown in Table I (Ca⁺⁺, Sr⁺⁺, Ba⁺⁺) all formed prothrombin phospholipid complexes which resulted in about 30% fluorescence quenching by energy transfer to phospholipid (see Fig. 3).

Rates for Prothrombin Transition Catalyzed by Different Cations—Potential differences in the binding of different cations to prothrombin can be studied by examination of the rate constants for the prothrombin transition catalyzed by these ions. The transition may be affected by the size or type of cation at the binding site and the result will be a change in
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Catalysis of the protein transition

|          | Mg²⁺ | Ca²⁺ | Sr²⁺ | Ba²⁺ | Mn²⁺ | Be²⁺ | Fe³⁺ | Zn²⁺ | La³⁺ | Th⁴⁺ | Pb²⁺ |
|----------|------|------|------|------|------|------|------|------|------|------|------|
| Prothrombin | 0.40 | 0.40 | 0.85 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| Fragment 1 | 0.43 | 0.40 | 0.99 | 6.6  | 6.6  | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| Factor X  | 1.00 | 1.00 | 1.00 | >4   | >4   | >4   | >4   | >4   | >4   | >4   | >4   |

Formation of the protein-phospholipid complex

|          | Prothrombin | Factor X |
|----------|-------------|----------|
|          | >10         | >10      |
|          | 0.08        | 0.22     |
|          | 0.18        | 0.50     |

"Values determined as described under "Methods" at 35°.

"Indicates that this value was not determined.

"Be²⁺ was ineffective in this capacity but appeared to bind tightly to the protein and prevented calcium from catalysis of the transition.

"Values determined with DNP-PE:Folch fraction III (1:1) and a protein:phospholipid ratio of 1:4 (w:w) by the method described in Fig. 3.

A protein phospholipid complex was formed but the fluorescence quenching due to energy transfer was about twice that observed for Ca²⁺, Sr²⁺, and Ba²⁺.

Catalysis of the protein transition (6890) to the protein and prevented calcium from catalysis of the transition. The intrinsic fluorescence of Fragment 1 transition. The intrinsic fluorescence of Fragment 1 is almost complete at about 0.05 

The activation energy. The rate constants for reaction of Fragment 1 with group II cations (magnesium, calcium, strontium, and barium) were determined from 15-41. The Arrhenius plot of these data detected no difference in the rate constants or activation energy (18 ± 2 kcal/mol) for the transition catalyzed by these different ions. When calcium was added to a prothrombin-phospholipid mixture, the rate constants for binding prothrombin to phospholipid, measured by fluorescence energy transfer, were the same as those obtained for the fragment 1 transition catalyzed by the group II cations. These data are in agreement with the previous report (19) which indicated that a slow protein transition necessarily precedes rapid phospholipid binding.

Examination of the rates for the prothrombin transition catalyzed by cations other than group II, however, revealed some considerable differences characterized by a more rapid transition which is concentration-dependent. The transition catalyzed by manganous ion is present in more detail in Fig. 4. The time required to reach 80% of maximum fluorescence change is plotted as the measure of the rate of the transition. The rate constant for the fragment 1 transition catalyzed by calcium is independent of concentration (12) and these results are given in Fig. 4 at zero manganous ion concentration. The transition catalyzed by low concentrations of manganous ion are characterized by rates similar to the calcium-catalyzed transition. As manganous ion concentration increases, the rate also increases until, at 2 mM manganous ion, the reaction is more than 80% complete in less than 0.1 min and cannot be measured by the procedures used here. Since 0.05 mM manganous ion catalyzes the complete transition, it is obvious from Fig. 4 that the change in the rate of the transition is brought about by manganous ion(s) binding to sites other than those required for the transition. If the manganous ion(s) responsible for the faster rate are bound to calcium binding site(s) (prothrombin binds about 10 calcium ions, only 3 or 4 of which are required for the transition (12)), calcium should compete with and increase the manganous ion concentrations required for the more rapid transition. As shown in Fig. 4, the presence of 3 mM calcium actually decreases the manganous ion concentration needed to change the reaction rate. It is concluded that manganous ion binds to non-calcium sites on prothrombin and increases the rate of the transition.

Of the cations given in Table I, all of those outside of the group II cations gave faster rates for the prothrombin transition. Based on the concentration required for the faster transition, most appeared much more effective in this respect than manganous ion. Detailed analyses of the other ions were not performed here but such studies may provide information on the nature of the activation complex which they are affecting.

Substitution of Metal Ions in Prothrombinase Complex—The conversion of prothrombin to thrombin requires a protease (blood-clotting Factor Xa) an accelerator protein (blood-clotting Factor Va), calcium, and phospholipid. Factor Va may function by facilitating the binding of Factor Xa to prothrombin (22, 23) which occurs on a phospholipid membrane surface (24-26). Calcium is necessary for binding prothrombin and Factor Xa to the phospholipid (24-26, see Ref. 27).

In examination of the fragment 1 protein transition of several different preparations, it appears that care must be exercised to eliminate metal ions. Some preparations displayed a biphasic fluorescence change with a rapid change immediately upon addition of calcium followed by a slow change displaying the normal kinetics of the prothrombin transition (12). Dialysis against 50 mM EDTA eliminated the rapid fluorescence change. The apparent explanation is that some protein molecules contain contaminating metals bound to the sites titrated in Fig. 4 and undergo a rapid transition when calcium is added. Metal-free protein molecules undergo the normal transition.
Table I.

Strontium will substitute for calcium but barium, magnesium (Fig. 5), and manganous ion (not shown) are ineffective. This is consistent with the metal ion requirements of prothrombin and Factor X-phospholipid binding (Table I). Combinations of cations will substitute for calcium as seen for magnesium plus barium (Fig. 5, $\Delta-\Delta$) or barium plus manganous ions (Fig. 5, $\Delta$--$\Delta$). The magnesium or manganous ions catalyze the Factor X and prothrombin protein transitions while barium forms the correct protein-phospholipid complexes. The combination of manganous ion plus barium is just as effective as calcium in this system. The lower yield of thrombin generation in the presence of 1.5 mM calcium.

In other experiments it was determined that neither terbium (0.2 mM) nor beryllium (1 mM) would substitute for calcium in the prothrombinase complex. Furthermore, these cations inhibited thrombin generation in the presence of 1.5 mM calcium.

For a review) The results of activation of prothrombin by the complete system are shown in Fig. 5 ($\bullet--\bullet$). Elimination of calcium greatly diminishes thrombin generation ($\bullet--\bullet$). If any component of the prothrombinase system was deleted, a similar decrease in thrombin generation was observed. For instance, no detectable thrombin was generated if Factor X was omitted. If phospholipid was omitted, the thrombin generated at 5 min was 10% of that of the complete system. If serum (Factor V) was eliminated, the activity at 5 min was 5% of that of the complete system shown in Fig. 6. Similar requirements for the complete system were observed for the activations using other metal ions.

Strontium will substitute for calcium but barium, magnesium (Fig. 5), and manganous ion (not shown) are ineffective. This is consistent with the metal ion requirements of prothrombin and Factor X-phospholipid binding (Table I). Combinations of cations will substitute for calcium as seen for magnesium plus barium (Fig. 5, $\Delta-\Delta$) or barium plus manganous ions (Fig. 5, $\Delta$--$\Delta$). The magnesium or manganous ions catalyze the Factor X and prothrombin protein transitions while barium forms the correct protein-phospholipid complexes. The combination of manganous ion plus barium is just as effective as calcium in this system. The lower yield of thrombin from magnesium plus barium as well as from strontium reflect the cation specificity of Factor X shown in Table I.

Under the conditions given in Fig. 5, 0.2 mM TbCl$_3$ inhibition thrombin production by 30%. At pH 7.0 (0.2 M imidazole buffer), the inhibition of thrombin production by 0.2 mM TbCl$_3$ or 1.0 mM BeCl$_2$ was over 60%. This inhibition is presumably due to formation of the “incorrect” protein-phospholipid complex as indicated above by greater fluorescence energy transfer. It is likely that all of the metal ions which form the incorrect structure (Table I) will inhibit the prothrombinase complex.

Correlation of prothrombinase activity with Factor X-phospholipid binding is shown in Fig. 6. Under these conditions, thrombin generation is not directly proportional to Factor X$_a$ concentrations (determined separately) and Factor X rather than X$_a$ is used to study the protein-phospholipid binding. Nevertheless, a close association between prothrombinase activity and Factor X-phospholipid binding is indicated. As measured by turbidity, the phospholipid vesicles aggregate at about 4.5 mM strontium. This corresponds to a discontinuity in the thrombin generation and fluorescence quenching curves. Prothrombin binds to phospholipid at much lower strontium concentration (Table I) and therefore does not affect these results. The concentrations of strontium required to bring about Factor X-phospholipid binding (Fig. 6) are a function of the very high strontium needed to catalyze the Factor X transition (Table I) and the much lower strontium concentrations needed for actual Factor X-phospholipid binding (Table I, in the presence of 0.2 mM Mn). Equations describing these combined reactions have been presented (12). These data provide good evidence that the Factor X transition must precede Factor X-phospholipid binding.

**DISCUSSION**

The results presented here demonstrate that the calcium binding sites provided by $\gamma$-carboxyglutamic acid residues have a low cation specificity and do not have exactly the same...
cation affinity or specificity in all proteins. Calcium is required for two distinct steps in both Factor X and prothrombin-phospholipid binding. The first step, a protein transition, is catalyzed by nearly all divalent or trivalent cations. Factor X requires higher cation concentrations and is more specific for calcium than is prothrombin. The calcium binding sites involved in the second step, the actual protein-phospholipid complex formation, show greater selectivity for cations than those required for the protein transition. Strontium and barium are the only cations observed to satisfactorily replace calcium in this role. A number of others will form a protein-phospholipid complex, but of different structure, resulting in inhibition rather than stimulation of the coagulation reactions. It seems possible that the cation specificity of this process is at least partially a function of the phospholipid.

Other workers have reported that Factor Xa binds to phospholipid in the presence of calcium but that Factor X does not (24). The results presented here are in direct conflict with that report. We have observed no substantial difference between the interaction of Factor X and Xa with phospholipid as measured by the calcium concentrations required for catalyzing the protein transition (data not shown) or for binding the protein to the phospholipid (Fig. 2). When factor X-phospholipid binding was tested by a gel filtration technique similar to that used previously (7, 24), Factor X bound to the phospholipid at 2 mM calcium and clearly separated from the phospholipid in 2 mM EDTA which is in conflict with the earlier report. Differences in Factor X resulting from methods of preparation may be possible but seem unlikely. The preparations used here were devoid of Factor Xa activity but could be readily activated by Russell’s viper venom which indicates that it is a native protein. The results of sodium dodecyl sulfate gel electrophoresis also indicate a normal Factor X (15, 28). The facts that phospholipid is required for physiological conversion of Factor X to Xa (27) and that Factor X conversion to Xa involves only subtle changes in the protein structure (29) are consistent with both proteins binding to phospholipid.

In these studies, the association of prothrombin with phospholipid has been assumed to be an equilibrium between free protein and a single form of phospholipid-bound protein:

$$m\text{Ca} + n\text{CaPT} + P\cdot L \rightleftharpoons n + m\text{CaPT} - P\cdot L \quad (1)$$

In this reaction $n$ represents the 3 or 4 calcium ions required to catalyze the protein transition (12), $P\cdot T$ is prothrombin, $P\cdot L$ is a phospholipid membrane, and $d_{PL}$ is the activity of the phospholipid. Upon binding to phospholipid, additional calcium ($m$) is bound. By holding phospholipid constant and varying calcium concentration, a Hill plot was constructed (Fig. 3B). The Hill coefficient of 2.2 agrees with the Hill coefficient for prothrombin-phospholipid binding estimated previously by a different method (12). A similar Hill coefficient was obtained for Factor X-phospholipid binding. Based on the fact that the Hill coefficient must be equal to or less than the number of ligands bound (30), this indicates that at least 2 calcium ions ($m$) are involved in prothrombin-phospholipid complex formation. The affinity of the complex for these calcium atoms, measured by the calcium concentration at half reaction (0.08 mM, Fig. 3) establishes that these sites are either provided by the phospholipid or are generated during protein-phospholipid association. This is seen from the fact that the free protein has no binding sites with such high affinity. At room temperature, free prothrombin binds a total of about 10 calcium ions in a cooperative manner with half-saturation occurring at 0.6 mM calcium (7–10). At 0.08 mM calcium, less than 1 ion is bound by prothrombin. Based on the usual model for calcium function in prothrombin-phospholipid binding, it seems likely that these sites represent calcium atoms which bind between the protein and the phospholipid. Further studies will be required to establish the exact nature and number of these binding sites.

**Note Added in Proof**—A recent publication has carefully documented the binding of lanthanide ions to prothrombin (31). These workers also reported that lanthanide ions will substitute partially (up to 25% of maximum activity) for calcium in the prothrombinase complex. Since the cation concentrations used were much lower than those reported here where an inhibition of the prothrombinase complex (when supplemented with calcium) is observed, it follows that very low concentrations of lanthanide ions (e.g. 20 μM) form a protein-phospholipid complex which at least partially resembles the correct structure for the prothrombinase complex but that the higher lanthanide ion concentrations used in our studies (200 μM) form an incorrect protein-phospholipid complex. Furie et al. actually observed that, under certain conditions, >20 μM lanthanide ion caused an inhibition of thrombin generation. They did not report studies at concentrations higher than 40 μM.

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