An investigation of the antigenicity of bovine prothrombin and several of its fragments: fragment 1 (NH$_2$-terminal 156 amino acid residues of bovine prothrombin released by thrombin digestion) and NH$_2$-terminal residues 1–39, employed a rabbit antiserum probe. The prothrombin fragment 1 antibody which had previously been fractionated by affinity chromatography and which shows an absolute specificity for the metal ion-induced conformation of prothrombin and fragment 1.

This study indicates that in the presence of calcium ions, 1–39 self-association, the formation of (1–39)/fragment 1 complexes, and prothrombin/phospholipid binding all mask the antigenic site. We hypothesize that in the presence of calcium ions a conformational change in the previously metal ion-free protein generates a hydrophobic region which is co-extensive with the antigenic region or whose conformational integrity is closely linked to the antigenic site and that this hydrophobic region includes at least the NH$_2$-terminal 39 residues of prothrombin. We further hypothesize that this hydrophobic region is involved in the protein/protein and protein/lipid interactions noted above.

Implicit in the interpretation of the protein-binding data has been the development of thermodynamic equations consistent with appropriate conceptual model systems. Application of these models has allowed the estimation of several association constants: 1) $10^8$ M$^{-1}$ for (1–39)/fragment 1 complex formation; 2) $10^{-18}$ M$^{-1}$ for (1–39)/(1–39) dimer formation; and 3) $10^6$ M$^{-1}$ for (1–39)/antibody complex formation. While the absolute values of these association constants may vary when this work is repeated using homospecific antibodies, the ability of the models to predict the observed relative changes in antigenicity is the important factor in generating the hypotheses presented. The derivation of the equations is independent of the number of antibody populations present and therefore applicable to any similar system. Further application of the model predicts that the antigenicity of the 1–39 dimer, the fragment 1/(1–39) complex, and the prothrombin/phospholipid complex is low or zero with regard to the antibody population used.

The presence of calcium ions has long been observed to cause changes in the prothrombin/phospholipid system that result in the enhanced ability of prothrombin to interact with the phospholipid surface. Structural requirements for the bovine prothrombin-calcium ion-phospholipid interaction include the presence of Gla, y-carboxyglutamic acid residues and preservation of normal disulfide bridges. In addition, the calcium ion interaction with bovine prothrombin has been shown to influence a number of intrinsic properties of the bovine prothrombin molecule which have been demonstrated to reflect a common fundamental process. These properties, which are related by a similar rate-limiting step and a similar calcium ion dose-response behavior, include: 1) metal ion-induced quenching of protein intrinsic fluorescence and metal ion-dependent changes in a variety of other spectroscopic systems; 2) acquisition of phospholipid-binding ability; 3) development of a metal ion-dependent antigenic site in fragment 1 and prothrombin; and 4) positively cooperative calcium ion-binding behavior. It has become increasingly apparent that the common event relating these processes is a metal ion-induced conformational change in the fragment 1 region of the bovine prothrombin molecule. The conformational change is sensitive to both the nature of the metal ion employed and to the concentration of the metal ion utilized; calcium ions are presumably the biologically effective metal ions. Calcium ions have been shown to be effective for inducing phospholipid binding in either prothrombin or fragment 1 at calcium ion concentrations of approximately 0.1 mM.

Two hypotheses have been advanced to explain the molecular events that enable calcium ion to facilitate the prothrombin/phospholipid interaction and to provide conceptual frameworks for the development of experimental work. The formation of mixed chelates of calcium ion interacting with ionized Gla carboxyl groups and negatively charged phospholipid head groups has been advanced (1). In this model, the calcium ion would function as a bridge between protein and...
phospholipid. Our working hypothesis, on the other hand, has involved the notion of calcium binding to the fragment 1 region to produce an amphipathic helix or a related type of secondary structure which contains a hydrophobic domain in the NH₂-terminal region of the protein. Matthes (17, 18) has pointed out the similarities between the primary structure of the Gla-containing regions of the blood-clotting proteins and the apolipoproteins. While this latter group of molecules does not contain \( \gamma \)-carboxyglutamic acid, there are areas of extensive homology with the coagulation proteins. Since the apolipoproteins are known to form amphipathic helices (19, 20), consideration of the structure-function relationship of the homologous areas leads to the hypothesis of similar conformations in the coagulation proteins.

It has become an important conceptual as well as experimental point to determine whether the phospholipid-binding characteristics of the prothrombin molecule as well as its metal ion-binding properties can be assigned to a circum-scribed, and hence characteristic, region of the protein. A logical step in this direction has been the attempt in several laboratories to localize the metal ion-induced antigenic changes known to occur in the prothrombin or fragment 1 molecule to smaller isolated regions of the prothrombin molecule. The studies of Furie et al. (21) have indicated the existence of competition between prothrombin residues 12-44 and prothrombin for a population of anti-prothrombin antibodies. Our studies, reported here, similarly indicate the existence of competition between residues 1-39 and fragment 1 for an anti-fragment 1 antibody population. However, the direct (1-39)/antibody-binding studies, also reported here, indicate that 1-39 is a relatively weak antigen compared to fragment 1 itself. Thus, comparison between direct and indirect measures of the affinity of residues 1-39 for anti-fragment 1 antibody suggests that something besides simple competition occurs.

An additional level of complexity has been introduced in the consideration of the forces regulating the interactions of blood-clotting proteins with metal ions and phospholipid surfaces by the observations of metal ion-induced antigenic changes noted to occur in the prothrombin or fragment 1 molecule. The studies of Furfey et al. (21) have indicated the existence of competition between prothrombin residues 12-44 and prothrombin for a population of anti-prothrombin antibodies. Our studies, reported here, similarly indicate the existence of competition between residues 1-39 and fragment 1 for an anti-fragment 1 antibody population. However, the direct (1-39)/antibody-binding studies, also reported here, indicate that 1-39 is a relatively weak antigen compared to fragment 1 itself. Thus, comparison between direct and indirect measures of the affinity of residues 1-39 for anti-fragment 1 antibody suggests that something besides simple competition occurs.

The experiments reported here were designed, in part, to explore changes in the antigenic behavior in these associating systems toward the purified, conformation-specific antibodies to fragment 1 used in our earlier work (13). Such antigenic changes are compared to similar changes observed in prothrombin complexed to phospholipid in the presence of calcium ions. These experiments not only provide insight into the apparent lack of direct NH₂-terminal peptide-antibody binding observed by several laboratories but also provide support for the hypothesis of hydrophobic binding between prothrombin and phospholipid. We may hypothesize from these results that the metal ion-induced hydrophobic region of prothrombin is localized within the approximately 50 NH₂-terminal residues of the protein.

MATERIALS AND METHODS

Bovine prothrombin and prothrombin fragment 1 were radiola-beled with \(^{125}\)I via a modification of the procedure of Hunter and Greenwood as previously described (13). The NH₂-terminal 1-39 residues of bovine prothrombin fragment 1 was isolated as previously described (23). The peptide was radiola-beled using the Bolton-Hunter reagent. Staphylococcus aureus was purchased as IgG-sorb from the Enzyme Center (Boston, MA). The reconstituted material was dialyzed into 0.05 M Tris, 0.14 M sodium chloride, pH 7.5, prior to use. Rabbit antiserum to bovine prothrombin fragment 1 antibody was raised and an antibody population specific for the conformation of fragment 1 induced by calcium ions was isolated using affinity chromatography as previously described (13). All immunological reactions were conducted in a 0.05 M Tris, 0.14 M sodium chloride buffer, pH 7.4, containing 2 mg of ovalbumin (Sigma)/ml. The specificity of the isolated antibody was estimated from a Scatchard plot constructed from fragment 1/ASC5 titer curves. As noted earlier (13), the data are linear at values of bound antibody less than 70%, with a y intercept indicating a maximum association constant of \(10^{9} M^{-1}\). Immunologic experiments were performed at antibody dilutions such that less than 70% of antibody would be used in binding, thereby minimizing the heterogeneity of the antibody system.

Phospholipid vesicles were prepared from a 1:1 (w/w) mixture of bovine phosphatidylcholine (Sigma) and bovine phosphatidylglycerylcerol (Sigma). A chloroform solution of the phospholipids was evaporated to dryness under nitrogen, the sample was placed in a vacuum for 4 h at room temperature following which it was hydrated by the addition of TSO buffer. Vesicles were prepared by sonication (Branson Inc. bath sonicator) of the aqueous phospholipid solution to near clarity followed by centrifugation at 2000 rpm for 10 min in order to remove large vesicles. The lipid concentration was determined from the amounts of phospholipid in the chloroform stock corrected for material lost in large vesicle formation. The phospholipid concentration employed in the titer study was 0.34 mg/ml.

Competition Binding Studies—The ability of fragment 1 residues 1-39 or fragment 1 to inhibit the binding of radiolabeled fragment 1 to ASC5 was evaluated employing a competitive radioimmunoassay. The amount of labeled fragment 1 added to each tube within a series was constant, while the amount of residues 1-39 or unlabeled fragment 1 added was such that the mole ratio of unlabeled material to radiolabeled fragment 1 varied between 1:10 and 10:1. Equal aliquots (100 µl) of calcium ion-containing TSO buffer were placed in triplicate tubes, followed by the same volume of a 15,000-fold dilution of ASC5 and equal volumes of the appropriate dilution of unlabeled material. The final concentration of calcium ions employed in the 1-39 studies was 0.38, 0.75, or 1.5 mM, while the final calcium concentration in the fragment 1 study was 1.5 mM. The tubes were incubated for 3 h at 37 °C, after which time radiolabeled fragment 1 (0.01 µCi, 0.0 µCi) was added. The tubes were then incubated for a further 3 h at 37 °C before addition of excess S. aureus to separate antibody-bound from free fragment 1. The bound material was sedimented after 10 min at room temperature by centrifugation at 2000 rpm for 2 min in an International microcentrifuge. The supernatant material containing antibody and free fragment 1 was carefully aspirated and the pellets were rinsed once with TSO buffer prior to counting.

Direct Antibody Binding of 1-39—Direct demonstration of the antigenicity of 1-39 vis-a-vis the ASC5 antibody was accomplished employing radiolabeled residues 1-39. Equal aliquots (100 µl) of calcium ion-containing TSO buffer were placed in triplicate tubes, followed by the same volume of varying dilutions of ASC5 in either a 1 or 30 mM calcium ion-containing TSO buffer. The ASC5 dilutions ranged from 100- to 10,000-fold. The tubes, assayed in triplicate, were incubated overnight at room temperature prior to the addition of excess S. aureus. The bacteria were sedimented by centrifugation and washed once prior to counting.

Solubility of 1-39 and Fragment 1 in the Presence of Various Metal Ions—The solubility of tracer amounts of residues 1-39 was investigated as a function of calcium, magnesium, and europium ion concentrations. The concentration of residues 1-39 present in each tube was approximately 2 × 10^{-10} M. Equal aliquots of residues 1-39 was added into tubes containing various concentrations of calcium, magnesium, or europium chlorides in TSO buffer. The metal ion concentration ranges investigated were 0.3 to 300 mM for calcium and magnesium and 0.3 to 300 µM for europium ion. The tubes were incubated overnight at 25 °C and then centrifuged for 5 min at 2000 rpm. The supernatant was aspirated and the tubes were counted. The solubility of fragment 1 was investigated as a function of calcium ion concentration in a series of experiments identical with those described above.
Chromatographic Behavior of Fragment 1 and Fragment 1-39—

The behavior of residues 1-39 in the presence of fragment 1 and calcium ions was evaluated by employing gel permeation chromatography. The elution volumes of radiolabeled fragment 1 and residues 1-39 were individually determined on a Sephadex G-100 column (0.9 x 20 cm). Aliquots of each tracer, equal in activity to the sample previously chromatographed, were incubated together in 30 mM calcium ion TSO buffer for 15 min at 37 °C. The mixtures were identically applied to the G-100 column. A second mixture was prepared in a manner similar to that described above with the exception that 0.03 mg of unlabeled fragment 1 was included in the incubation mixture prior to chromatography. The columns were eluted with TSO buffer, and the elution was continued until the previously determined inclusion volume had been exceeded. The fractions were monitored for radioactivity on a Beckman γ-4000 scintillation spectrometer.

Determination of ASCC Titer With Bovine Prothrombin-Phospholipid Complexes—Equal aliquots (0.01 μCi) of radiolabeled prothrombin were added to TSO buffer containing 3 mM calcium ions and allowed to incubate at 37 °C for 15 min. Excess phospholipid (100 μl) was added to each tube and incubated for 15 min at room temperature. Varying dilutions (100 μl) of ASCC were added to the tubes followed by a 3.5-h incubation at 37 °C. Excess S. aureus was added, incubated for 10 min at 25 °C, and sedimented by centrifugation. The pellets were rinsed once prior to counting. Radioactivity was assayed on a Beckman γ-4000 scintillation spectrometer.

RESULTS

1-39 Competition with Fragment 1 for Antibody—The results of experiments designed to reveal competition between fragment 1 and residues 1-39 for ASCC are shown in Fig. 1A. The percentage of labeled fragment 1 bound to ASCC is shown as a function of the molarity of residues 1-39. Consistent with expectation, at low 1-39 molarity, the percentage of fragment 1 bound increases with calcium ion concentration. At calcium ion concentrations of 0.375 and 0.75 mM, the presence of residues 1-39 at 1-39/fragment 1 mole ratios of as high as 10:1 has little effect on the percentage of fragment 1 bound by the antibody. The mole ratio of residues (1-39)/fragment 1 required to yield a 50% inhibition of the fragment 1/antibody interaction is 8/1 at 1.5 mM calcium ions. The results of a similar displacement experiment involving unlabeled fragment 1 rather than residues 1-39 is shown in Fig. 1B for comparison. It is clear that the radiolabeling of fragment 1 alters the antigenicity somewhat, making residues 1-39 appear to be even less antigenic relative to radiolabeled fragment 1. The initial implication of these results is that the residues 1-39 portion of the bovine prothrombin molecule shares antigenic determinants with bovine prothrombin fragment 1, although it is only 3.8% as potent an antigen as the intact fragment 1 molecule. As will be developed below, this conclusion from these data is probably incorrect.

Binding of Radiolabeled 1-39 to Antibody—Direct demonstration of the antigenicity of 1-39 employing the ASCC antibody was accomplished utilizing radiolabeled residues 1-39. The results of the direct binding experiment are presented in Fig. 2 (---). The percentage of 1-39 bound to the antibody represents the fraction of the total protein-bound counts sedimented at any given ASCC dilution. Even at very low ASCC dilutions, only 13% of the protein-bound counts are bound by ASCC at 1 mM calcium ions. At 30 mM calcium ions, no ASCC binding is observed. A similar experiment, performed with magnesium ion rather than calcium, revealed no ASCC binding of the MgCl2/1-39 complex. The results of the competition experiment with fragment 1, described previously, suggests that the ASCC titer with residues 1-39 should be approximately 4% of the fragment 1 titer, corresponding to an antibody dilution of approximately 2000 and we would expect nearly 100% antibody binding of 1-39 at ASCC dilutions less than 500. Failure of this direct experiment to corroborate 1-39 binding to ASCC suggests that a process other than simple competition may be involved when the fragment 1/ASCC binding is observed to decrease in the presence of increasing concentration of 1-39. Arguments will be presented under “Discussion” which support a direct interaction between fragment 1 and 1-39 which blocks the binding of ASCC.

Solubility of (1-39)/Metal I on Complexes—The solubility of tracer amounts of residues 1-39 was investigated as a
function of the calcium, magnesium, and europium ion concentrations. The amount of residues 1–39 precipitated as a function of europium and calcium ion concentration is shown in Fig. 3. The calcium ion dependence curve reaches 60% precipitation at 100 mM metal ion while the europium ion dependence curve appears to peak at 20% precipitation at approximately 20 \( \mu \)M europium ion. The magnesium ion curve (not shown) shows no significant precipitation of residues 1–39 at any magnesium ion concentration investigated. A similar experiment performed with prothrombin fragment 1 showed no significant precipitation across a similar range of calcium ion concentrations as that shown for 1–39 in Fig. 3.

Chromatographic Behavior of Fragment 1/(1-39) Complexes—The results of the gel permeation experiments of fragment 1, 1–39, and various mixtures of the two are shown in Fig. 4. Residues 1–39 are clearly separated from fragment 1 as seen in Fig. 4A. The peak at 16 ml elution volume is attributable to free iodine and provides an internal standard for the comparison of various elution curves. Mixing these tracer amounts of fragment 1 and residues 1–39 together prior to chromatography yields the elution profile in Fig. 4B. A clear decrease in the total counts observed in the 1–39 peak is accompanied by an increase in the number of counts in the position where fragment 1 elutes. The elution position of the fragment 1 peak is slightly retarded to 5.5 ml. The small peak eluting between 9 and 16 ml is unidentified. Addition of a considerable excess of unlabeled bovine fragment 1 to a mixture identical with that employed in Fig. 4B results in the elution profile shown in Fig. 4C. The residues 1–39 peak has nearly disappeared. Counts are concentrated at the elution position of fragment 1 and material eluting ahead of tracer amounts of fragment 1. This latter peak may represent aggregates of 1–39 and fragment 1 which are sufficiently different in molecular weight to be separable from fragment 1 alone.

If, as these gel chromatography studies suggest, fragment 1 and residues 1–39 interact, with the resulting formation of fragment 1/(1–39) complexes, the formation of such complexes would be expected to prevent precipitation of the 1–39 polypeptide, which would normally occur in the presence of 30 mM calcium ions. Such an experiment was performed to verify that this is the case by incubating tracer amounts of fragment 1 and 1–39 with 30 mM calcium ions as above, except that the incubate was centrifuged rather than being applied to the gel column. Compared with results obtained from a control tube containing residues 1–39 and calcium ions alone, 75% of the 1–39 remained in solution when fragment 1 was included in the incubation. This observation is consistent with the results in Fig. 4B.

Prothrombin Antigenicity in The Presence of Phospholipid—Prothrombin has been demonstrated to be immunologically identical with fragment 1 in the antibody system (ASCC) employed in these studies (24). It is a logical inference from a considerable variety of data that prothrombin binds to phospholipid surfaces only after a metal ion-dependent conformational process takes place. As a consequence, the hypothesis that the metal ion-dependent antigenic determinant is also a likely candidate to be the site of phospholipid binding was tested by performing a titer determination of ASCC with prothrombin in the presence of 3 mM calcium ion, in the presence and absence of phospholipid. The effect of phospholipid on prothrombin/ASCC binding is shown in Fig. 5. The titer curve is shifted to lower dilutions of antibody in the

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**Fig. 3. Precipitation of residues 1–39 by calcium and europium ions.** The concentration of 1–39 present in each tube was approximately 2 \( \times \) 10\(^{-8}\) M. \( \bullet \) and \( \circ \), 25 °C; \( \Delta \) and \( \triangle \), 4 °C.

**Fig. 4. Chromatographic evidence for fragment 1/(1–39) complex formation.** Elution volumes of peaks are indicated by arrows. A, elution profiles of separately run (\( \circ \)) radiolabeled bovine prothrombin fragment 1 and (\( \bullet \)) radiolabeled bovine prothrombin residues 1–39; B, samples identical with A but run together; C, sample identical with B except that 0.03 mg of unlabeled fragment 1 was included in the incubation mixture prior to chromatography.
The following conditions were chosen: in all cases \( P + A = PA, K_1 = 10^{10} \text{ M}^{-1} \); \( P + L = PL, K_2 = 10^6 \text{ M}^{-1} \); undiluted antibody concentration = \( 9.4 \times 10^{-4} \text{ M} \), and prothrombin concentration = \( 2 \times 10^{-10} \text{ M} \). (\( \bigcirc \) ) experimental points obtained in the absence of phospholipid; (\( \bullet \) ) experimental points obtained in the presence of phospholipid. Total phospholipid = \( 4.2 \times 10^{-4} \text{ M} \); (---), theoretical titer curve assuming (lipid)_{effective} = \( 4.2 \times 10^{-4} \text{ M} \); (---), theoretical titer curve assuming (lipid)_{effective} = \( 2.1 \times 10^{-4} \text{ M} \).

Fig. 5. The effect of phospholipid on the prothrombin/ASCC titer. Theoretical lines obtained by solving Equation 17 are drawn. The following conditions were chosen: in all cases \( P + A = PA, K_1 = 10^{10} \text{ M}^{-1} \); \( P + L = PL, K_2 = 10^6 \text{ M}^{-1} \); undiluted antibody concentration = \( 9.4 \times 10^{-4} \text{ M} \), and prothrombin concentration = \( 2 \times 10^{-10} \text{ M} \). (\( \bigcirc \) ) experimental points obtained in the absence of phospholipid; (\( \bullet \) ) experimental points obtained in the presence of phospholipid. Total phospholipid = \( 4.2 \times 10^{-4} \text{ M} \); (---), theoretical titer curve assuming (lipid)_{effective} = \( 4.2 \times 10^{-4} \text{ M} \); (---), theoretical titer curve assuming (lipid)_{effective} = \( 2.1 \times 10^{-4} \text{ M} \).

The presence of phospholipid. At an antibody dilution of 10,000, approximately 14% less prothrombin is bound to the antibody in the presence of phospholipid compared to prothrombin binding in the absence of phospholipid.

DISCUSSION

In order to semiquantitatively interpret the results, a model was evoked involving the multiple equilibria depicted in Fig. 6. Included in this model are the metal ion-induced interactions between 1-30 molecules to form dimers, (1-39)/fragment 1 complex formation, and prothrombin-phospholipid interactions. Rigorous quantitative treatment to extract thermodynamic equilibrium constants for the multiple equilibria proposed in the model requires knowledge of the total concentrations of all species involved and the use of a homogeneous antibody population. Thus, extraction of accurate association constants is not completely possible from the data presented here. However, simplified, but reasonable models for quantitatively treating the proposed multiple equilibria are developed and presented in the Appendix for future application to similar experiments performed with homospecific antibodies. The quantitative treatments given in the Appendix have been applied to the data presented here in order to demonstrate that models evoking dimer formation and phospholipid interaction as inhibitors of fragment 1 (or prothrombin) antigenicity can be used to explain the experimental observations when reasonable values of the association constants consistent with the literature for fragment 1/antibody and prothrombin/lipid interactions are used. It is emphasized, however, that this semiquantitative treatment does not define absolute values of the association constants for a nonhomogeneous antibody population. The numbers presented below are used instead as indicators of the relative differences in the orders of magnitude of these constants for the various equilibria required to fit the observed results.

Modeling the Interaction of Fragment 1 with Antibody—

In Fig. 7, the fraction of protein bound to antibody, \( F_{\text{bound}} \), is plotted versus antibody dilution. For each of a series of antibody dilutions, a value of \( K_1 \) was chosen and the fit to the experimental results was evaluated. The best fit within this simplified model was obtained for \( K_1 = 10^9 \text{ M}^{-1} \) (Model 1; 1P: 1A). This value is essentially the same as the \( K_1 \) of \( 1.2 \times 10^{10} \text{ M}^{-1} \) obtained from a Scatchard plot of the fragment 1/anti-

body interaction. The fit to Model 1 is based on an antibody stock concentration of \( 9.4 \times 10^{-6} \text{ M} \). Model 2 (2P:1A), based on the same antibody stock concentration and assuming two noninteracting sites of equal affinity also fits the experimental data with the value of the site affinities, \( k_1 \) and \( k_2 \), equal to \( 0.5 \times 10^9 \). From the site-binding constants, the two thermodynamic equilibrium constants are calculated according to Klotz (31) as \( K_1 = k_1 + k_2 = 1.0 \times 10^{10} \) and \( K_2 = k_1 k_2/(k_1 + k_2) = 2.5 \times 10^{9} \). As expected, Model 2 provides a better fit at high antibody dilution. At high antibody concentrations (low antibody dilutions), the experimental fraction of antibody bound does not reach 1.0 as does the theoretical line. This is probably due to the calcium ion concentration employed. The model assumes that all fragment 1 molecules exist in the calcium-induced conformation, a circumstance unlikely to be fully achieved at 3 mM calcium ion.

For quantitative treatment of the inhibition of fragment 1 and 1-39 binding to antibody by 1-39 or phospholipid, the simplified model of a 1:1 antibody/antigen complex (Model 1) was employed because it was the simplest model which would successfully fit the data. Addition of the second antigen-binding site to the theoretical treatment would be expected to result in higher values of the association constants for equilibria between antigen and inhibitor being necessary to fit the observed decreases in antigen-antibody association.

Modeling the Effect of (1-39) Fragment 1 Complex Forma-

tion on Antibody Binding—The treatment of the fragment 1/(i-39) competition experiment involves the reasonable assumption that, at the antibody dilutions employed in this study, the principal effects of residues 1-39 on fragment 1 antigenicity is a direct result of its interaction with fragment
1. Two equilibria are omitted in this treatment: the interaction of 1-39 with antibody and the dimerization of 1-39. It is assumed that, since the equilibrium association of the (1-39)/antibody interaction \(10^9 \text{M}^{-1}\) is two orders of magnitude lower than the fragment 1/antibody constant \(10^{10} \text{M}^{-1}\) (vide infra), this equilibrium would not significantly affect the fragment 1/antibody and fragment 1/(1-39) interactions. The effect of deleting the 1-39 dimerization reaction would be to result in an apparently lower association constant for the fragment 1/(1-39) interaction. Thus, the value of \(10^9 \text{M}^{-1}\) which gave the best fit for the simplified model (Model 3), which neglected dimerization, could be a lower estimate of the value of this association constant. Fixing the fragment 1/antibody association constant at \(10^{10} \text{M}^{-1}\) and specifying that \((P) = (\text{fragment } 1) = 8.5 \times 10^{-10} \text{M}\) and \((Ab) = 1.08 \times 10^{-9} \text{M}\), estimates of the fragment 1/(1-39) association constant from \(10^9 \text{M}^{-1}\) to \(10^{10} \text{M}^{-1}\) are chosen and the fits to the experimental data are examined (see Table I). The best fit to the experimental data is obtained with a theoretical line calculated assuming that the fragment 1/(1-39) association constant is \(10^{10} \text{M}^{-1}\). Choices of \(10^9\) and \(10^{10}\) result in substantial deviation of the theoretical line from the experimental points.

**Modeling the Effect of 1-39 Dimerization on Antibody Binding** — The effect of self-association of 1-39 must similarly affect estimates of its intrinsic antigenicity *vis-a-vis* the ASCC antibody. Thus, although a calcium ion concentration was chosen for the determination of the (1-39)/ASCC titer curve which did not cause 1-39 precipitation, it is likely that 1-39 self-association is an important determinant of the apparent antigenicity of 1-39. Employing Equation 27 and selecting values of \(K_1\) (characterizing the (1-39)/antibody interaction) and \(K_2\) (characterizing the self-association of 1-39), theoretical (1-39)/antibody titer curves are generated. Details of the choices of parameters are in Fig. 7. The best fit to the experimental data was obtained when \(K_1\) for 1-39 dimerization was chosen to be \(10^{10}\) and \(K_2\) for the (1-39)/antibody interaction was chosen to be \(10^9\). Thus, residues 1-39 are approximately 100-fold less antigenic than fragment 1.

**Modeling the Effect of Phospholipid on Prothrombin/Antibody Interaction** — The results of application of Equation 17 which describes the equilibria in Fig. 6C with the assumption that the association constant for the antibody/lipid-bound protein interaction is zero, is presented in Fig. 5. A 1:1 prothrombin/antibody complex is assumed. The sonicated phospholipid concentration was 4.2 \(\times 10^{-9}\text{M}\). However, only approximately 60% of the total lipid is available for surface binding (25, 26), thus reducing the effective lipid concentration to \(2.5 \times 10^{-9}\text{M}\). We have found further that optimal fits are obtained if it is assumed that each fragment 1 molecule interacts with 60 phospholipid molecules, yielding an effective phospholipid concentration of \(4.2 \times 10^{-8}\text{M}\). The effect of various choices of effective lipid concentration is illustrated in Fig. 5. In addition, the protein/antibody (1:1) association constant was fixed at \(10^{10}\) and the prothrombin/phospholipid association constant was fixed at \(10^{10}\) (10). It is thus apparent that a simple model which assumes that phospholipid-bound prothrombin has a very substantially reduced or no affinity for the antibody adequately fits the observed results. Reducing the lipid concentration by approximately 4 orders of magnitude allows a fit to the data points obtained in the absence of phospholipid. Consistent with an intuitive impression of the equilibria involved, increasing the phospholipid concentration shifts the titer curve to lower dilutions.

The observations reported here which are consistent with the formation of 1-39 complexes with itself and other proteins include: the apparent competition of 1-39 with fragment 1 for sites on the antibody, the precipitation of 1-39 by calcium and europium ions, the solubilization of 1-39 by fragment 1, and the gel filtration behavior of 1-39 and fragment 1. These results, taken individually, do not reveal the mechanism of the association, however. Consideration of the immunologic behavior of the (1-39)/fragment 1 complex has led us to conclude that either the metal ion-dependent antigenic site in fragment 1 is involved at the interface between the two molecules or that this antigenic site is substantially affected by the characteristics of the interface.

The substantial affinity of the 1-39 nonadecapeptide for the fragment 1 region 1 region of prothrombin explains why a simple competition assay for 1-39 antigenicity (Fig. 1) suggests that 1-39 is approximately 1/7 as antigenic as fragment 1, while the simplest interpretation of direct (1-39)/ASCC binding (Fig. 2) suggests that 1-39 is 1/1000 as antigenic as fragment 1. In the first case, 1-39 is bound not to the antibody, but to fragment 1, thus blocking the antigenic site, while in the second, the dimerization of the peptide removes virtually all specific immunologically active sites from exposure to solution, thus eliminating any reaction with antibody. Examination of the equilibria with the thermodynamic models derived in this report indicates that the 1-39 region is actually closer to 1/100 as antigenic as fragment 1.

The precipitation of labeled 1-39, in the presence of calcium and europium ions, from solutions of low 1-39 molarity, suggests that the driving force for this associative process is quite strong. The fact that a number of metal ions are capable of inducing precipitation, albeit with differing efficiencies, and the observation that the rate of the precipitation is considerably greater than that of the slow conformational change, require that both electrostatic and hydrophobic models be entertained as potential explanations of the observed results.

In the investigation of Sarasua et al. (27) of Eu\(^{3+}\) ion complexation with free Gla (I), Z-Gly-DL-Gla-Gly-OEt (II), Phe-L-Leu-L-Gla-L-Gla-L-Leu-OMe (III), Phe-L-Leu-L-Gla-L-Glu-L-Leu-OMe (IV), Z-L-Gla-L-Ser-OMe (V), and Z-D-Gla-D-Gla-OMe (VI), only those peptides which formed complexes consisting of 1 metal/2 peptide stoichiometry precipitated (II and V). Free Gla also formed a 1:M:2P complex, but has 4 charged groups available in the complex to keep it in solution. The precipitation of these peptides as well as residues 1-39 is not apparently related to formation of an amphipathic helix (assuming, reasonably, that Pro-22 isomerization is limiting in 1-39, as it is in bovine prothrombin and fragment 1).

Pletcher et al. (28) have presented convincing evidence that normal fragment 1, in the presence of calcium ions, tends to "associate" to some extent in a manner unrelated to, but in addition to, the formation of an amphipathic helix which we have discussed heretofore. Thus, there is a certain amount of interaction between fragment 1 molecules which is independent of the A \(\rightarrow\) B \(\rightarrow\) C equilibria (6), and hence appears to be a characteristic of segments of the primary structure of the...
metal ion bridges. Thus, precipitation and fast formation of binding involving a series of divalent cations. However, more are clearly different processes. The time dependence of the metal ion complex with protein dependence of fragment 1 self-association, induced by a variety of metal ions (30), suggests that indeed the metal ion coordination behavior of the magnesium ion differs considerably from that of calcium ions. The results presented in those studies are compromised by the high association constants of these peptides for themselves, which are so efficiently induced protein dimerization. The treatment of equilibria involved using the methods derived here demonstrate that the phospholipid-bound material is only weakly antigenic vis-à-vis the ASCC. Further, this treatment clarifies earlier reports (33) that peptides containing Gla residues, including prothrombin residues 12-44, do not interact with phospholipid. The interpretations presented in those studies are compromised by the high association constants of these peptides for themselves, which are likely to be orders of magnitude greater than the association constant of the peptides for phospholipid. In the presence of calcium ions, the intact prothrombin molecule is reported to have an association constant with phospholipid of about 10^6 (10).

The reports of Jackson et al. (14) and Dombrose et al. (10) indicate the existence of a competitive relationship between self-association and phospholipid-binding properties of bovine prothrombin fragment 1. These observations and those presented in this study suggest that the apparent ability of a metal ion to induce prothrombin or fragment 1 to interact with phospholipid involves a composite of self-associative and lipid-binding terms which operate in opposite directions. In this light, calcium ions appear to be optimal metal ions for inducing prothrombin phospholipid binding because they induce relatively weak self-association in the face of functional phospholipid binding. In contrast, an ion such as manganese, which may well induce a phospholipid-binding conformation of prothrombin, will appear not to induce phospholipid binding because it so efficiently induces protein dimerization.

The requirement for a specific metal ion-induced orientation even in the small 1-39 fragment of prothrombin is demonstrated by the lack of Mg^2+-induced precipitation of 1-39 and the failure of the Mg^2+/1-39 complex to bind ASCC. The coordination behavior of the magnesium ion differs considerably from that of calcium and, as a result, fails to direct the 1-39 residues into an antigenically recognizable conformation.

The results presented in this study are consistent with the evolution of a hydrophobic region in an area of bovine prothrombin and fragment 1 containing at least the first 40 NH2-terminal residues. We are led to speculate that, in the presence of metal ion concentration, aggregate size can become quite substantial and lead ultimately to precipitation. Self-association has also been examined in bovine prothrombin and prothrombin fragment 1. The tendency of these materials to self-associate is considerably less than has been observed for small peptides isolated from the Gla-containing NH2-terminal region of the parent molecule. This behavior may be explained by the presence of substantial areas of the fragment 1 region that are unaffected by the calcium ion-induced reorganization of the NH2-terminal tetradecapeptide, thus providing sufficient hydrophilic character to minimize the hydrophobic drive toward aggregation.

This overall behavior constitutes circumstantial support for the conclusion that an essentially hydrophilic region near the prothrombin NH2 terminus in the presence of metal ions becomes significantly hydrophobic as a result of a metal ion-induced conformational change in the protein. Our previous studies with ASCC, fragment 1, and prothrombin in the presence of metal ions and the results reported here further suggest that a specific reorganization in the structure is also involved in the achievement of full metal ion-dependent antigenicity.

We have explored the influence of phospholipid surfaces on the antigenicity of prothrombin in the presence of calcium ions. The data are consistent with a competition between antibody and phospholipid for the same binding site on the protein or binding sites closely related to each other in the protein. The treatment of equilibria involved using the methods derived here demonstrate that the phospholipid-bound material is only weakly antigenic vis-à-vis the ASCC. Further, this treatment clarifies earlier reports (33) that peptides containing Gla residues, including prothrombin residues 12-44, do not interact with phospholipid. The interpretations presented in those studies are compromised by the high association constants of these peptides for themselves, which are likely to be orders of magnitude greater than the association constant of the peptides for phospholipid. In the presence of calcium ions, the intact prothrombin molecule is reported to have an association constant with phospholipid of about 10^6 (10).

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The results presented in this study are consistent with the evolution of a hydrophobic region in an area of bovine prothrombin and fragment 1 containing at least the first 40 NH2-terminal residues. We are led to speculate that, in the presence
of calcium ions, the NH2-terminal 45-50 residues of prothrombin engage themselves in a structure which possesses at least two distinguishable faces. The hydrophobic face presents the hydrocarbon side chains of 15 or so nonpolar residues while the hydrophilic face of the structure contains the 10 Gla residues with their associated calcium ions stabilized by other oxygen ligands and the remaining charged side chains. The antigenicity of this region would logically depend on the proper assembly of each of these faces. As a consequence, we suggest that both the hydrophobic and hydrophilic faces of the metal ion/prothrombin complex are involved in some self-associative processes in vitro, although self-association depends significantly on the size of the fragment containing this region and hence should be minimal in prothrombin and fragment 1. More importantly, we suggest that the hydrophobic face is involved in the interaction of the metal-containing protein with the interior of the phospholipid bilayer. Thus, although electrostatic processes undoubtedly play an important role in the maintenance of a specific protein conformation and in the initial association of prothrombin with the phospholipid surface, a crucial gain in interaction energy may be achieved by hydrophobic interactions between prothrombin structures and the interior of the bilayer. This last point is speculative at this time, since an important alternative hypothesis involving metal ion bridges between prothrombin monomers and the phospholipid surface cannot plausibly be rejected on the basis of available data.

**APPENDIX**

**DERIVATION OF THERMODYNAMIC RELATIONSHIPS CHARACTERIZING PROTEIN-ANTIBODY INTERACTIONS IN THE PRESENCE OF PHOSPHOLIPID**

The following thermodynamic models are derived here because of their importance to the interpretation of the binding data presented in the Results section.

**PROTEIN + ANTIBODY ALONE:**

Model 1: Using a 1:1 antigen:antibody complex characterizes the antigen:antibody interaction:

\( P + A \rightarrow PA \) \hspace{1cm} (1)

where \( P \equiv \text{protein (antigen)}, \ A \equiv \text{antibody} \)

The association constant, \( K_1 \), is given by:

\[ K_1 = \frac{X}{(P_0 - X)(A_0 - X)} \] \hspace{1cm} (2)

where \( X \equiv \text{equilibrium concentration of } PA, \ P_0 \equiv \text{initial protein (antigen) concentration}, \ A_0 \equiv \text{initial antibody concentration} \). Rearrangement of eq. (2) yields the following quadratic equation in \( X \):

\[ X^2 - X(P_0 + A_0) + P_0 A_0 = 0 \] \hspace{1cm} (3)

from which \( F_{BD} \), the fraction of antigen bound to antibody, \( X/P_0 \), can be determined.

In applying model 1 to the data, equation (3) was solved for \( X \) for a series of \( A_0 \) values representing successive dilutions of an antibody stock solution of \( 9.4 \times 10^{-6} \text{M} \). This concentration was estimated as described below. \( P_0 \) (the initial concentration of fragment 1) was \( 2.0 \times 10^{-10} \). The fraction of protein bound to antibody was then calculated for each \( A_0 \) value by \( F_{calc} = X/P_0 \), and compared to the experimental fraction bound (\( F_{exp} \)) over in data points by:

\[ \sum_{i=1}^{n} (F_{exp} - F_{calc})^2 \]

Model 2:

This model is derived for the sake of comparison with Model 1. However, derivation of succeeding models is not feasible employing this model.

Assuming a 2:1 (antigen:antibody) complex characterizes the interaction between antigen and antibody:

\( P + A \rightarrow PA + PA \) \hspace{1cm} (4)

\( P + A \rightarrow 2PA \) \hspace{1cm} (5)

where

\[ K_2 = \frac{X}{(P_0 - X)(A_0 - X)} \] \hspace{1cm} (6)

\[ K_2 = \frac{X}{(P_0 - X)(A_0 - X)} \] \hspace{1cm} (7)

where \( P_0 \equiv \text{initial concentration of protein (antigen)} \) and \( A_0 \equiv \text{initial concentration of antibody} \), \( X \equiv \text{equilibrium concentration of } PA, \ P_0 \equiv \text{initial concentration of } P \). Rearranging gives:

\[ Y = K_2 P_0 X \] \hspace{1cm} (8)

Substitution of (8) into (6) yields:

\[ X^2(K_2^2 + K_1^2) + X^2(K_2 + K_1) - X^2K_2 P_0 A_0 - X^2 K_1 P_0 A_0 = 0 \] \hspace{1cm} (9)

The fraction of antigen bound to antibody is given by:

\[ F_{BD} = \frac{X}{P_0} \] \hspace{1cm} (10)

For examination of the fit of model 2 to the data, it was assumed that the 2 antigen binding sites on the antibody were noninteractive with equal site affinities. Based on a \( K_3 \) value of \( 10^{10} \) (from the Scatchard analysis noted above) and the relation between site affinities \( (K_1) \) and thermodynamic affinities \( (K) \), \( K = K_3 \) \( / \) \( 10^{10} \), a value of \( K \equiv 0.25 \times 10^{10} \) was used.

**PROTEIN-ANTIBODY INTERACTIONS IN THE PRESENCE OF PHOSPHOLIPID:**

Model 3:

Assuming a 1:1 antigen:antibody complex, the following equations apply:

\[ P + A \rightarrow PA \] \hspace{1cm} (11)

\[ P + L \rightarrow PL \] \hspace{1cm} (12)

\[ PL + A \rightarrow PLA \] \hspace{1cm} (13)

where \( P \equiv \text{protein (antigen)}, \ A \equiv \text{antibody,} \) and \( L \equiv \text{lipid} \). The case for which antibody has no affinity for lipid-bound \( P \), i.e. \( K_L = 0 \), yields:

\[ K_4 = \frac{(P_0 - X - Y)(A_0 - X)}{P_0 A_0} \] \hspace{1cm} (14)

\[ K_5 = \frac{(P_0 - X - Y)(L_0 - Y)}{P_0 A_0} \] \hspace{1cm} (15)

where \( X \equiv \text{equilibrium concentration of } PA, \ Y \equiv \text{equilibrium concentration of } L \) and \( P_0 \) is the initial phospholipid concentration. \( P_0 \) and \( A_0 \) are the initial concentrations of protein and antibody, respectively. Solution of (15) for \( X \) yields:

\[ X = \frac{(Y - K_2^2 P_0 A_0 + K_2^2 P_0 X) \pm \sqrt{(Y^2 - 4 Y K_2^2 P_0 A_0)}}{2 Y} \] \hspace{1cm} (16)

Substitution of (16) into (14) yields a cubic equation in \( Y \):

\[ K_2^2 - K_2 K_1 X^3 - (K_2 X^2 - K_1 X + 1) + K_1 P_0 A_0 = 0 \] \hspace{1cm} (17)

\[ K_2^2 - K_2 K_1 X^3 - (K_2 X^2 - K_1 X + 1) + K_1 P_0 A_0 = 0 \] \hspace{1cm} (18)

A more complex model for which \( K_3 \) does not equal 0 yields a quartic equation. Since the data are fit by the simpler model, the derivation is not pursued here.

The model described by equations 11 and 12 is used here to characterize (1) the behavior of the prothrombin:antibody interaction in the presence of phospholipid and (2) the effects of residues 1-39 on the fragment 1:antibody interaction. That the antigen:antibody interactions characterized in the studies reported here fit the 1:1 complex model, eqs. (1) and (2) are demonstrated in the Results section.

Comparison of this model to the data was accomplished by solving equation (17) for \( Y \) over a series of \( X_0 \) values representing successive dilutions of an antibody stock solution of \( 9.4 \times 10^{-6} \text{M} \). This concentration was estimated as described below. \( P_0 \) (the initial concentration of fragment 1) was \( 2.0 \times 10^{-10} \). The fraction of protein bound to antibody was then calculated for each \( A_0 \) value as \( F_{calc} = X/P_0 \), and compared to the experimental value by minimizing the function: \[ \sum_{i=1}^{n} (F_{exp} - F_{calc})^2 \]

1-39-ANTIBODY INTERACTIONS IN THE PRESENCE OF 1-39 DIMERIZATION:

Model 4:

Again assuming a 1:1 antigen:antibody complex:

\[ P + A \rightarrow PA \] \hspace{1cm} (19)

\[ 2P \rightarrow P_2 \] \hspace{1cm} (20)

If \( P_0 \) is the initial 1-39 concentration and \( A_0 \) is the initial concentration of antibody, mass balance yields:

\[ P_0 = (P + P_2) + 2(P_2) \] \hspace{1cm} (21)

\[ A_0 \equiv (A + 1/2) \] \hspace{1cm} (22)

Let \( X \equiv (P + A) \) and \( Y \equiv (P_2) \):

\[ P_0 = X + 2Y \] \hspace{1cm} (23)

\[ A_0 = X \] \hspace{1cm} (24)

Thus:

\[ K_3 \frac{(P_0 - X - 2Y) \equiv X}{Y} \] \hspace{1cm} (25)

\[ K_2 \frac{(P_0 - X - 2Y) \equiv X}{Y} \] \hspace{1cm} (26)
Solution of (25) for \( Y \) in terms of \( X \) and substitution into (26) yields a cubic equation in \( X \):

\[
X^3 + \alpha X^2 + \beta X + \gamma = 0
\]

Examination of the fit of this model to the data was carried out at \( X \) and \( Y \) values ranging from 10 to 10^10. Equation (27) was solved for \( X \) employing a cubic routine adapted from [22] at a series of \( \alpha \) values representing successive dilutions of a stock antibody concentration of 2.5 x 10^12 M. For these calculations \( \beta = 1 - 391 \times 10^{-9} \). The three roots of eq. (27) were tested on the basis of selecting the root that gave the free antibody concentration at any point on the titre curve: \([PA]\) = \([PI - 1/2Po]\) and The total antibody concentration can be expressed in tens of stock antibody, titre curve is given by:

\[
[PA] = \frac{Po}{1 +\frac{Po}{K}}
\]

The total antibody concentration can be expressed in terms of stock antibody, \( \alpha \) and the logarithm (base 10) of the antibody dilution factor \( X \), as:

\[
[PA] = \frac{\alpha \text{ stock}}{10^{10} + [X(\beta)]}
\]

Substitution of this expression for \( \alpha \) into the expression for \([X]\) (eq. 30) yields:

\[
[PA] X = \frac{\alpha \text{ stock}}{10^{10} + [X(\beta)]}
\]

At the inflection point:

\[
\frac{\alpha \text{ stock}}{10^{10} + [X(\beta)]} = \frac{\alpha \text{ stock}}{10^{10}} = 1/X
\]

where \( X \) = log (dilution factor) at the inflection point. Thus:

\[
[PA] = \frac{10^{10} X}{X + 10^{10}}
\]

and for a high affinity antibody/antigen interaction, where \( X >> 10^{10} \):

\[
[PA] = \frac{10^{10}}{X}
\]

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