Ionic Properties of Membrane Association by Vitamin K-Dependent Proteins: The Case for Univalency†

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ABSTRACT: Ionic properties of membrane interaction by prothrombin, protein Z, and other vitamin K-dependent proteins were studied to determine the relevance of a monovalent membrane contact mechanism between one phospholipid headgroup and a calcium-lined pore in the protein [McDonald, J. F., Shah, A. M., Schwalbe, R. A., Kisiel, W., Dahlback, B., and Nelsestuen, G. L. (1997) *Biochemistry 36*, 5120−5127]. For comparison, multivalent ionic interaction was illustrated by peptides of +3 to +5 net charge and by blood clotting factor V. As expected, the peptides were easily dissociated by salt and gave nominal charge−charge interactions (z_az_b values) of −13 to −17. Factor V showed much higher binding affinity despite nominal z_az_b values of about 9. Membrane-bound prothrombin and protein Z showed very low sensitivity to salt as long as calcium was at saturating levels (z_az_b values of approximately −1.3 to −1.4), appropriate for univalent ionic attraction. Prothrombin contains +3 charge groups (Lys-2, Lys-11, Arg-10) that are absent from the GLA domain (residues 1−35) of protein Z, while protein Z contains −4 charge groups (Gla-11, Asp-34, Asp-35) that are absent in prothrombin. Thus, similar z_az_b relationships indicated little role for these surface charges in direct membrane contact. Calcium-saturated protein Z bound to phosphatidylcholine (PC) in a manner which indicated the addition of one calcium ion, bringing the total calcium stoichiometry in the protein−membrane complex to at least 8. Protein Z bound to phosphatidic acid (PA) in a manner suggesting the need for a fully ionized phosphate headgroup, a property expected by ion pairing in an isolated environment. Electrostatic calculations showed that the proposed protein site for phosphate interaction was electropositive. The cluster of hydrophobic amino acids (Phe-5, Leu-6, and Val-9) on the surface of prothrombin was electronegative, suggesting a role in the electrostatic architecture of the GLA domain. Overall, membrane binding by vitamin K-dependent proteins appeared consistent with the formation of an ion pair in an isolated environment.

Vitamin K is required for carboxylation of 9−13 glutamic acids in the amino-terminal 45 residues of the vitamin K-dependent proteins (GLA domain).[1] The resulting γ-carboxyglutamate (Gla) residues are required for calcium and membrane binding. While X-ray (*1, 2*) and NMR structures (*3*) have shown protein folding and calcium binding mechanisms, the ultimate structural goal, creation of a membrane contact site, has remained unclear.

Biophysical studies, designed to determine the membrane binding mechanism, quickly reached an apparent impasse; membrane binding could not be correlated with either hydrophobic or ionic forces. Hydrophobic interactions were inconsistent with properties of protein binding to phospholipid monolayers (*4*) and vesicles (*5*) and with the inability to label prothrombin with membrane-permeable photoaffinity probes (*6*). Especially pertinent was the low surface activity of the proteins, suggesting little hydrophobic area on their surfaces (*4, 7*). While ionic binding mechanisms appeared consistent with the requirement for anionic phospholipid and the involvement of calcium in at least two events [(*8*), eq 1], actual protein−membrane binding (step 3 of eq 1) was

$$\begin{array}{l} P + 7Ca \overset{1}{\rightleftharpoons} P{\bullet}Ca_7 \\ \qquad\quad + \qquad \overset{3}{\rightleftharpoons} P{\bullet}PL{\bullet}Ca_{7+n} \\ PL + nCa \overset{2}{\rightleftharpoons} PL{\bullet}Ca_n \end{array} \qquad (1)$$

not very sensitive to ionic strength (*9*). A common model for membrane contact has suggested the insertion of a small hydrophobic cluster of amino acids (Phe-5, Leu-6, and Val-9) on the surface of the calcium−protein complex (*10*) into the hydrocarbon region of the membrane to a depth that allows simultaneous bridging of protein-bound calcium ions to phospholipid headgroups (*11*−*15*). This binding mechanism appeared to be challenged by several of the earlier results. In addition, chemical modifications such as trinitrophenylation of Lys-3 and Lys-11 (*16*) and removal of the amino-terminal three residues of prothrombin (*17*) had less than a 5-fold impact on membrane affinity (<1 kcal/mol), even though they should disrupt protein structure in the region of the hydrophobic cluster. Site-directed mutation of the hydrophobic residues gave 0−5-fold impacts on membrane affinity at saturating calcium levels (*11, 12, 14*).

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[1] Abbreviations: GLA domain, a somewhat reduced definition, residues 1−35 of vitamin K-dependent proteins; Gla, γ-carboxyglutamic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamne; dansyl-PE, *N*-[(dimethylamino)naphthalenesulfonyl]-phosphatidylethanolamine; PA, phosphatidic acid; BODIPY-PA, 2-(4,4-difluoro-7,7-dimethyl-4-bora-3a,4a-diaza-3-indacenylpentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate.

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The naturally occurring vitamin K-dependent proteins display a 1000-fold range of membrane affinity, with no correlation to differences in hydrophobic amino acids (*18*). Rather, membrane binding properties appeared to correlate with changes at positions 11, 33, and 34. These residues are clustered on the protein surface, near to a calcium-lined pore in the GLA domain. A novel membrane contact was proposed, consisting of membrane attachment through an ion pair that is formed, in an isolated environment, between one phospholipid headgroup and the calcium-lined pore (*18*).

This study tested a number of properties for a correlation between membrane association and a univalent ion pair. Prothrombin and protein Z provided extremes with respect to both hydrophobic and ionic character (6-charge difference) of amino acids in their respective GLA domains. Nevertheless, membrane binding of both proteins showed similar levels of ionic character and other properties, such as a charge−charge parameter ($z_a z_b$) for step 3 (eq 1) of between −1 and −2. Protein Z bound to phosphatidylcholine (PC) in a manner that suggested a calcium stoichiometry for step 2 (eq 1) of 1.0. Electrostatic calculations showed that a prime candidate for ionic interaction, a pore in the GLA domain, was electropositive, while the amino acids of the hydrophobic cluster were very electronegative. Membrane binding may be based on a unique electrostatic arrangement and interaction between the GLA domain and membranes.

MATERIALS AND METHODS

Materials. Bovine brain phosphatidylserine (PS), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (phosphatidic acid, PA), and dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine (dansyl-PE) were purchased from Avanti Polar Lipids, Inc. Egg phosphatidylcholine (PC) was purchased from Sigma Chemical Co. All phospholipids were of high purity (>98%, suppliers estimate). Lissamine rhodamine sulfonyl chloride was from Molecular Probes (Eugene, OR). Other chemicals and reagents were from Sigma Chemical Co. and were of the highest grade available. Polycarbonate filters (0.1 μm diameter) were purchased from Nucleopore Corp. (Costar Co). Highly purified protein kinase C inhibitor peptide (RFARKALRQKNVHEVKN^{5+}) and its noninhibiting analog (RFARKGALEQKNVHEVKN^{3+}) were obtained from GIBCO. Other peptides and their net charge included RKRTLRRL^{5+} and VRKRTLRRL^{5+}, KRTLRR^{4+} (Bachem), and Pyr-KRPSQRSKYL^{3+} (Calbiochem). Bovine protein Z was isolated from barium citrate eluates derived from plasma and purified according to Hashimoto et al. (*19*). Bovine protein Z was highly pure and monodisperse, as previously reported (*18*). Bovine prothrombin was isolated according to Nelsestuen (*20*) and blood clotting factor V according to Pusey et al. (*21*).

Protein Labeling. Prothrombin was labeled with lissamine rhodamine as previously described (*5*). Lissamine rhodamine sulfonyl chloride was dissolved in dimethyl sulfoxide (10 mg/mL) immediately before use and was added dropwise to a solution of prothrombin (2 mg/mL) in 0.1 M $NaCO_3$ buffer to a final level of 25 μL of dye solution/mL of protein. The solution was then stirred at 5 °C for 4 h. Free dye was removed by gel filtration on a column of Sephacryl S-300 (1.3 × 30 cm). Studies were completed within 12 h of protein preparation. An estimate of dye incorporation was obtained from the absorbance of the protein at 280 nm and that of the dye at 550 nm [$E = 13000\ M^{-1}\ cm^{-1}$ (*22*)]. This analysis gave a 4.5 dye/protein ratio.

Vesicle Preparations. Chloroform solutions of phospholipids were mixed in appropriate ratios, and the organic solvent was evaporated by a stream of nitrogen. The sample was then placed under vacuum for 1 h. The dried phospholipids were dispersed in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5) to a final concentration of 2−5 mg/mL. Small unilamellar vesicles (SUVs) were prepared as previously described (*23*) by sonication followed by gel filtration chromatography on Sepharose 4B. Large unilamellar vesicles (LUVs) were prepared by multiple freeze−thaw cycles and extrusion through polycarbonate membranes with a pore size of 0.1 μm (*24*). The vesicles were then dialyzed against Tris buffer (50 mM Tris, 0.1 M NaCl) to produce spherical shape. Phospholipid vesicle sizes were determined by dynamic light scattering as described previously (*25*). SUVs gave average diameters of about 40 nm, and LUVs gave diameters of 110−125 nm.

Equilibrium Binding of Peptides and Proteins to Membranes. Light scattering and fluorescence energy transfer were used to quantitate the binding of proteins and peptides to phospholipid vesicles according to the method of Nelsestuen and Lim (*8*). Equation 2 describes the light scattering changes for assembly of protein on phospholipid vesicles whose diameters are small relative to the wavelength of incident light.

$$I_2/I_1 = (M_2/M_1)^2[(\delta n/\delta c_2)/(\delta n/\delta c_1)]^2 \qquad (2)$$

I_2 and I_1 are the light scattering intensities of the protein−vesicle complex and vesicles alone, respectively. The vesicle concentration was the same in both measurements. M_2 and M_1 are the weight-average molecular weights of the protein−vesicle complex and vesicles alone, and $\delta n/\delta c_2$ and $\delta n/\delta c_1$ are the refractive index increments for protein−vesicle complexes and vesicles, respectively. The refractive index increment was assumed to be equal to the weight-averaged composition of each particle with $\delta n/\delta c = 0.19$ for pure protein and 0.17 for phospholipid (*8*). To characterize membrane binding by charged peptides, each was added to membranes of PS/PC/dansyl-PE (47/47/6) to the saturation level. NaCl was added and the new level of bound protein [$peptide_{bound}$] was determined, along with [binding $site_{free}$], which equaled the amount of peptide that dissociated. [$Peptide_{free}$] was estimated from [$peptide_{total}$] minus the remaining amount of bound peptide, [$peptide_{bound}$]. These values were combined to obtain a K_D at each salt concentration using the relationship in eq 3.

$$K_D = [peptide_{free}][binding\ site_{free}]/[peptide_{bound}] \qquad (3)$$

Factor V− and protein Z−membrane binding was estimated by both light scattering and fluorescence energy transfer methods. Light scattering at 90° to incident light used a 400 nm wavelength. Total binding sites on the membrane were estimated from saturation levels of binding. The other terms of the equilibrium expression were estimated by the methods outlined for the peptides. To obtain Hill plots of the results, the fraction of bound protein was obtained from saturation binding and the M_2/M_1 ratio at a specific condition. The fraction of free sites was determined by subtracting this value from unity. At 50 mM Tris buffer at

25 °C, the equilibrium constant for factor V was determined from association (1×10^8 M^{-1} s^{-1}) and dissociation (0.0017 s^{-1}) rate constants (*26*).

Fluorescence intensities were measured with a FluoroMax (JY/Spex Instruments SA, Inc.) fluorescence spectrophotometer. Fluorescence energy transfer from protein amino acids (excitation at 280 nm) to dansyl groups in the membrane (dansyl-PE, emission at 520 nm) was measured by intensity changes at 520 nm. The intensities (F) are expressed relative to that arising from direct excitation of the dansyl group by the incident light (F_0). As for light scattering, the maximum signal was obtained at saturating levels of protein (F_{max}/F_0). Equilibrium constants were estimated in a manner similar to that described for light scattering. [$Protein_{bound}$] was estimated from F/F_0 at various salt concentrations, and [$protein_{free}$] and [binding $site_{free}$] were estimated from F/F_0, [$protein_{total}$], and F_{max}/F_0. Fluorescence changes due to environmental change of the probes were small as evidenced by almost no change in the dansyl fluorescence upon protein−membrane binding when excitation was at 350 nm.

Application of Debye−Huckel Theory. Quantitation of ionic impact on membrane interaction, for purposes of estimating valences of two interacting species (z_a, z_b) and for comparison of different proteins, used the limiting Debye−Huckel relationship, substituting equilibrium constants as shown in eq 4a [I is ionic strength and A is a constant of approximately 0.5 (*27*)]. Equation 4b gives the relationship for ionic strength impact on a dissociation rate constant (k_{dissn}) resulting from ion binding to the complex.

$$\log(K_D) = \log(K_0) - 2Az_az_b[I]^{0.5} \quad (4a)$$

$$\log(k_{dissn}) = \log(K_0k_{assn}) - 2Az_az_b[I]^{0.5} \quad (4b)$$

These relationships can be used to obtain information of the charge products that may be rate limiting for a variety of biological reactions (*28*−*31*). The limiting case is valid at low ionic strength, which can extend to at least 0.2 M for some reactions (*28, 29*). At higher ionic strengths, the nonlimiting case will apply, which presents a nonlinear relationship. The degree of nonlinearity is dependent on the nature of the reaction and is more prevalent at z_az_b values of ≥4 (*32*). In some cases where ionic strength dependence was analyzed by the limiting and nonlimiting relationships, under conditions similar to those used in this study, z_az_b values often deviated by 2-fold or less (*28, 29*). In all cases, linear analysis of data by eq 4 provides a lower limit for the true z_az_b of interaction of species with opposite charge (*32*). The combination of the levels of calcium needed to saturate both vitamin K-dependent proteins and membranes, low values of z_az_b for the reaction, and resulting small changes in the rate or equilibrium constant, provided experimental deviation that prevented accurate evaluation of nonlinearity. The slopes in different regions of the plot gave little variation for prothrombin.

Alternative analysis of ionic impact by the relationship $K_{Dobs} = K_d[Na^+]^n$ (*27*), where n is the valence, gave similar conclusions to those obtained with eq 4a,b. Since eq 4 applies to any competing ion, it was used to present the result. The sensitivity provided by the logarithmic relationship allowed substantial conclusions to be drawn.

Kinetic Measurements. Kinetics of protein binding and dissociation from vesicles were monitored by fluorescence energy transfer and/or light scattering using a 4800C spectrofluorometer (SLM, Aminco) as described in detail elsewhere (*33*). For prothrombin, rapid kinetics was measured by stopped-flow measurements, using lissamine rhodamine-labeled prothrombin (emission at 600 nm) and phospholipid vesicles containing BODIPY-PA (excitation at 500 nm). Briefly, equal volume syringes were filled with appropriate solutions, and about 40 μL from each syringe was used per injection. The dead time of the instrument was about 6 ms as measured by the fluorescence reaction of pyramine with bovine carbonic anhydrase as described by the manufacturer. For dissociation experiments, one syringe contained labeled protein and labeled vesicles and the other contained a concentration of unlabeled SUVs (PS/PC, 25/75) that was greater than 10 times the concentration of the labeled vesicles in the first syringe. This was sufficient to capture virtually all of the free protein and create irreversible dissociation from the labeled vesicles. The final intensity was measured after several minutes when changes had ceased to occur. Data were fit to first-order dissociations with this value as the final intensity. Control experiments involving protein alone, phospholipid alone, and protein and phospholipid without calcium were performed to ensure that signal changes were due to protein−membrane binding or dissociation events. Because of its slow rate, dissociation kinetics of bovine Z−membrane complexes were carried out manually in the Spex FluoroMax fluorometer, following the addition of unlabeled SUVs under similar conditions.

Dissociation rate constants (k_{dissn}) were estimated by fitting fluorescence intensity change to a first-order reaction, and the values reported were the average of three experiments. In the range used, rates of dissociation were independent of the unlabeled phospholipid concentration. In most cases, the results fit a single exponential decay and rate constant. The exception was low ionic strength (0.06−0.09 M) for prothrombin where two rates were needed to obtain close fit to the data. Curve fitting used the computer program Kaleidagraph and the relationship, $F = F_0 - (F_0 - F_{fin})\exp(-k_{dissn}t)$, where F is the fluorescence intensity at time t, F_0 is the fluorescence at zero time, and F_{fin} is the final intensity at long time. Correlation coefficients of the first order fits were $R > 0.98$.

Electrostatic Modeling. The consistent-valence force field was used for minimization (*34*). Gla residues were assigned charges equivalent to −2.0 with the second carboxyl close to that of Glu^-. Calcium ions were assigned a charge of +2.0. All crystallography waters were removed, the structure was tethered to the X-ray structure with a force constant of 100 kcal/mol. Minimization of 200 iterations with steepest descents was done with DISCOVER 95.0 (Biosym Technologies, 1995).

Electrostatic calculations were done with DELPHI (*35, 36*). The linear Poisson−Boltzmann equation was used. The interior dielectric constant for the protein was set at 4.0 and that of the exterior at 80.0. The ionic strength was 0.145 at room temperature. Charges used were the same as were used for minimization. Visual inspection of the molecule at different contour levels (±3 to ±20 kT/e, where k is the Boltzmann constant and T is room temperature in K) used INSIGHT (Biosym Technologies, 1995). The patterns at

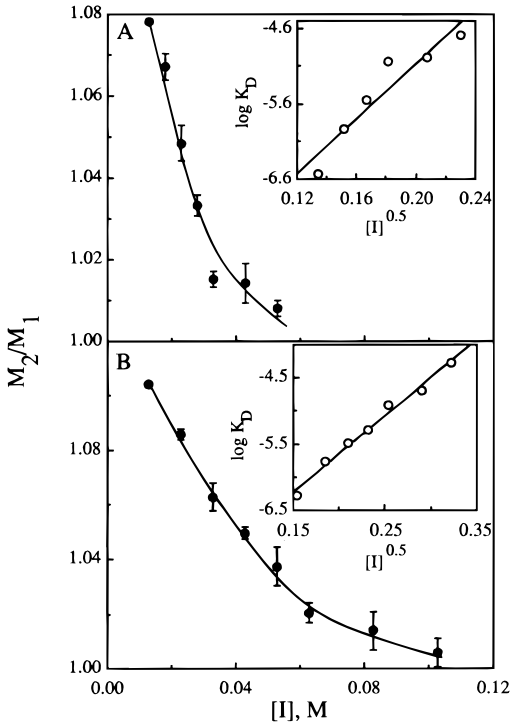

FIGURE 1: Dissociation of peptide−membrane complexes by NaCl. (Panel A). The noninhibitory peptide of protein kinase C (5 μg/mL, net charge = +3) was bound to vesicles (15 μg/mL, PS/PC, 50/50) in 5 mM Tris buffer (pH 7.5) containing NaCl to the ionic strength shown. Binding was detected by light scattering intensity as described in Materials and Methods. M_2/M_1 is the ratio of the molecular weight of the peptide−membrane complex to that of the membrane vesicles alone. Each data point was gathered separately and is the average of three determinations. Saturation of the membrane with peptide gave an M_2/M_1 of 1.14. The results were used to estimate K_D at the various salt concentrations, using eq 3. Plots according to eq 4a (panel A, inset) gave a slope of 17.5, corresponding to a z_az_b value of −17.5. (Panel B). The protein kinase C inhibitor peptide (5 μg/mL, net charge = +5) was bound to the membrane in solutions of various ionic strengths as in panel A. The inset shows a plot of estimated K_D values analyzed by eq 4. The slope of the line drawn is 12.8, corresponding to z_az_b value of −12.8.

different potentials were similar except that the higher electrostatic contours occurred within the lower ones.

Other Methods. Protein concentrations were determined according to ref 37 and phospholipid concentrations according to ref 38 assuming a 25:1 phospholipid to phosphorus weight ratio. Unless described, the buffer system used was 50 mM Tris containing 100 mM NaCl, pH 7.5. The temperature was 25 ± 1 C. Ionic strengths were obtained from total salt concentrations, assuming an activity coefficient of 1.

RESULTS

Membrane Interaction via Multivalent Ionic Interactions. Two examples of ionic membrane binding were used to provide contrast to the vitamin K-dependent proteins. Despite a number of qualifications (Materials and Methods) results with cationic peptides were sufficient to illustrate several properties of a multivalent ionic interaction in aqueous solution. Peptides of +3 charge (e.g., Figure 1A) were dissociated from the membrane by lower salt than peptides of +5 charge (Figure 1B). Equilibrium constants were estimated with eq 3 and were analyzed by eq 4. This relationship did not correct for membrane charge density, which would change as more peptide dissociated. Thus, the z_az_b values were only nominal but served as a general illustration of expected behavior. The low energy per ion pair required multivalency to support an interaction with resulting high values of z_az_b (−13 to −17, Figure 1).

Membrane interaction by blood clotting factor V was also quite sensitive to ionic strength and also illustrated several qualitative properties. First of all, binding was of much higher affinity than that of the peptides, despite lower z_az_b relationships. Consequently, ion pairs exposed to the aqueous environment were insufficient to explain membrane binding by factor V. While higher affinity may arise from nonionic contributions to membrane contact, this z_az_b value did not establish the participation of these forces. The free energy of ion pair formation is described by Coulomb's law (eq 5), where ϵ is the dielectric constant of the medium and r is the distance between the charges in angstroms (*27*).

$$\text{free energy (kcal/mol)} = 331.8 z_a z_b/\epsilon r \qquad (5)$$

By altering the dielectric constant of the medium surrounding an ion pair, a protein can produce high affinity from one or a few ion pairs. A second conclusion was that factor V−membrane contact involved multiple ion pairs with slopes of 9 at low ionic strength (0.04−0.15 M salt, Figure 2B) to 3.5 at moderate ionic strength.

Equation 4b was applied to first-order dissociation rate constants estimated from dissociation half-times (*26*). The result (z_az_b = 5.2, Figure 2B, inset) suggested that the majority of the ionic strength impact on factor V was detected in the dissociation rate constant.

Impact of Ionic Strength on Vitamin K-Dependent Protein−Membrane Complexes. Initial expectation for interaction of the carboxyl-rich GLA domain and multiple calcium ions with anionic lipids was a multivalent ionic mechanism. Early proposals suggested four (*39*) to eight (*40*) calcium bridges between the protein and membrane. The examples in Figures 1 and 2 show that the early proposals for vitamin K proteins would produce interactions that would be exceptionally sensitive to ionic strength. However, the impact of 0.16−0.46 M ionic strength on K_D was only 1.6-fold (*9*), a barely detectable level. Great precision was not applied in the early study but was not needed to conclude that protein−membrane binding was not based on multiple ion pairs in aqueous medium. For example, a divalent interaction (z_az_b = −4.0) would provide a 13-fold change in K_D between 0.16−0.46 M ionic strength. The earlier studies (*9*) did not consider a univalent ion binding mechanism, even though a 1.6-fold change in binding affinity at 0.16 to 0.46 M ionic strength corresponded to a z_az_b value of 0.8.

At equilibrium, factor X showed a small decline in membrane-bound protein between 60 and 400 mM ionic strength (Figure 3A). Nominal estimates of K_D at two data points, 60 and 400 mM ionic strength, gave values of 45 and 310 nM, respectively [maximum M_2/M_1 for factor X of 2.4 (*41*)]. These values corresponded to a z_az_b value of −2.1 (eq 4a). These estimates appeared more reliable than those for the peptides (Figure 1). For example, saturating levels of calcium would maintain a constant, low charge density on the membrane at all protein binding levels. Little plot curvature was apparent, in keeping with a more linear

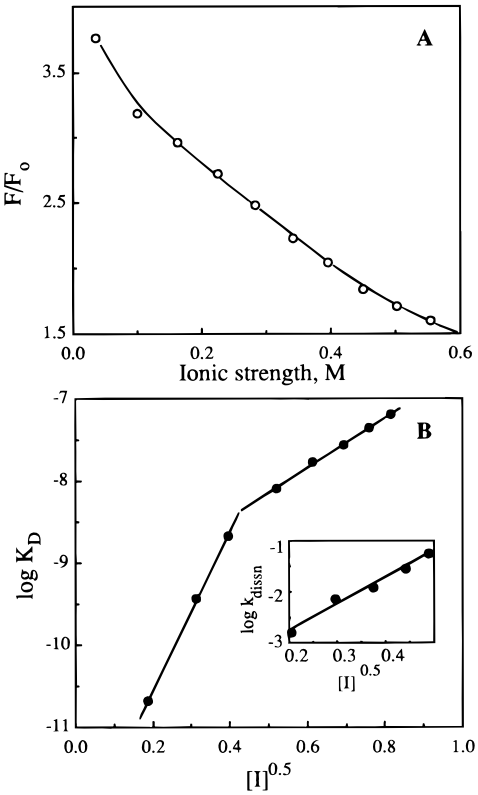

FIGURE 2: Dissociation of factor V from the membrane by NaCl. (Panel A). Factor V (2.6 μg/mL, 8.7 nM) was bound to the membrane vesicles (0.45 μg/mL, 3 nM protein binding capacity, PS/PC/dansyl-PE, 25/70/5) in 50 mM Tris buffer. The amount of bound protein was estimated from the ratio of fluorescence in the presence of protein (F) to that of the vesicles alone (F_0). This titration was typical of seven nonidentical experiments that were conducted at 0.45−23 μg/mL phospholipid, using both light scattering and fluorescence energy transfer to detect protein bound to the membrane. (Panel B) K_D values plotted according to eq 4a were estimated from data in panel A as described in Materials and Methods. The slope of the line drawn at low ionic strength was 8.7, and that at high ionic strength was 3.5. (Inset) Estimation of z_az_b from eq 4b. Rate constants for dissociation of factor V were obtained from the half-time of protein dissociation rate plots presented in (*26*). The slope of the line drawn is 5.2.

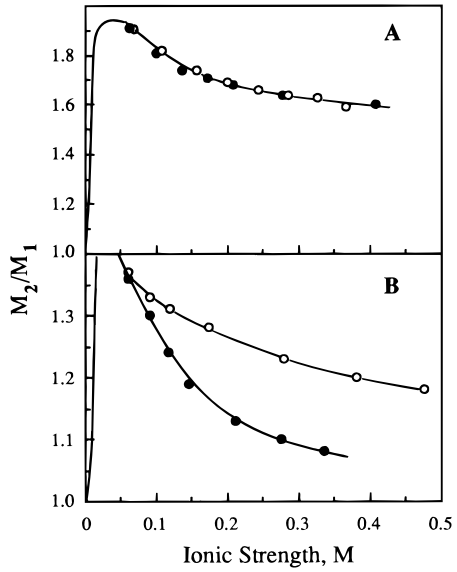

FIGURE 3: Dissociation of vitamin K-dependent proteins from membranes by $CaCl_2$ or NaCl. (Panel A) Factor X (31 μg/mL) and phospholipid (29 μg/mL, PS/PC, 25/75) were titrated with $CaCl_2$ (●) or NaCl (○). The first measurement was taken at 18 mM calcium (60 mM ionic strength) in both titrations. The added ionic strength arose from Tris buffer (pH 7.5) and NaCl. The curve shown below 60 mM ionic strength represents a titration with calcium in a separate experiment. Sequential additions of salt were made, and the amount of protein bound to the membrane was estimated by light scattering as described in Materials and Methods. The range of replicate measurements was less than 2%. Ionic strengh created by one vs seven additions of salt gave a maxium difference of 9%. (Panel B) Solutions of bovine protein S (20 μg/mL) and phospholipid (29 μg/mL, PS/PC, 25/75) were titrated with $CaCl_2$ (●) or NaCl (○) as described in panel A. In both cases, the titrations began in a solution containing 15 mM calcium (60 mM ionic strength from $CaCl_2$, Tris buffer, and NaCl). This was sufficient to begin dissociation of protein S from the membrane. The maximum M_2/M_1 for protein S was 1.49 and occurred at 3 mM calcium.

relationship for reactions with low z_az_b values (*32*). Similar low impact of ionic strength was obtained for $CaCl_2$ titrations of factor IX, protein Z (not shown), and prothrombin (*42*). Ionic strengths provided by NaCl and by $CaCl_2$ were equally effective (Figure 3A), as long as calcium was at saturating levels in the NaCl titration. This property also applied to prothrombin and protein Z (not shown). An exception was protein S, which was more susceptible to dissociation by calcium than by NaCl. For the NaCl titration, the nominal K_D values obtained for protein S at 60 and 400 mM ionic strengths were 200 and 700 nM, respectively [maximum $M_2/M_1 = 1.0$ (*18*)]. These corresponded to a z_az_b of −1.4 (eq 4a). The highest affinity of bovine protein S occurred at 3 mM calcium.

Dynamic measurements were more precise than equilibrium measurements. Dissociation rate constants for prothrombin (Figure 4), plotted according to eq 4b (Figure 4b, inset), gave a slope of 1.4. Association rate constants changed very little over this range (*43*). Thus, interpretation by linear analysis supported univalent binding and that multivalency seemed unlikely. For example, a divalent interaction ($z_az_b = -4$) would give a 35-fold change in K_D over the range of ionic strength tested.

Studies with Protein Z. Bovine protein Z provided several contrasts to prothrombin. It contains several additional hydrophobic residues and five charge differences in the amino-terminal 11 residues. If these amino acids make major contributions to direct membrane contact, prothrombin and protein Z should give different z_az_b relationships. This was not the case.

Figure 5 shows dissociation of protein Z from membranes. This study was limited to two ionic strengths that allowed comparison to results for prothrombin. Error limits prevented more detailed study. Analysis of the data by eq 4b gave a z_az_b value of −1.3 (Figure 5, inset), indistinguishable from that for prothrombin over the same range of ionic strength (Figure 4, inset). Association rates were not studied in detail, but single determinations showed very little change at these ionic strengths (not shown), an observation that also agreed with properties of prothrombin (*43*).

Association of Protein Z with Phosphatidylcholine. While vitamin K-dependent proteins are generally thought to bind to acidic phospholipids, early studies showed that they also bound to PE (*41*). PE also enhanced membrane interaction by annexins (*44*) and increased activity of some blood clotting reactions (*45*). Annexin proteins have been shown to associate with phosphatidylcholine (*44*, *46*). Protein Z

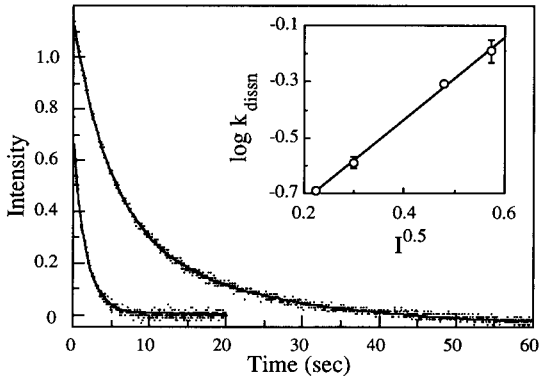

FIGURE 4: Dissociation rates for the prothrombin−membrane complex as a function of ionic strength. Dissociation rates were determined for lissamine rhodamine-labeled prothrombin and vesicles (PS/PC/BODIPY-PA, 25/74/1) as described in Materials and Methods. Intensity is relative, and zero is the value observed after several minutes. Tris buffer (15−25 mM), calcium (10 mM), and NaCl contributed to the ionic strength. The solid lines show curves fit to two exponential decay processes. The ionic strengths used and rate constants (and corresponding amplitudes) that were obtained included 50 mM, 0.205 ± 0.004 s^{-1} (0.756 ± 0.026) and 0.053 ± 0.003 s^{-1} (0.41 ± 0.03); 90 mM, 0.257 ± 0.013 s^{-1} (0.835 ± 0.004) and 0.014 ± 0.003 s^{-1} (0.283 ± 0.036); 230 mM, 0.49 ± 0.004 s^{-1} (0.826 ± 0.014) and 0.024 ± 0.005 s^{-1} (0.066 ± 0.032); and 330 mM, 0.60 ± 0.03 s^{-1} (0.656 ± 0.052, a single rate constant). (Inset) The rate constants for the largest portion of each reaction are plotted according to eq 4b. The slope of the line drawn is 1.45 for a $z_a z_b$ of −1.4.

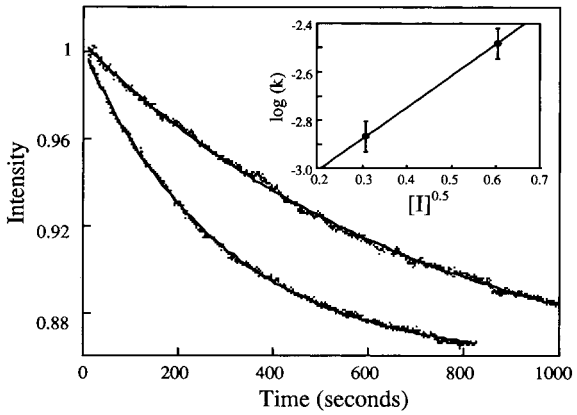

FIGURE 5: Dissociation rates for the protein Z−membrane complex as a function of ionic strength. Dissociation rates were determined for bovine Z as described in Materials and Methods. Intensity is relative to the value measured immediately following addition of unlabeled SUVs. Buffer (50 mM), calcium (10 mM), and NaCl contributed to the ionic strength. The solid lines show curves fit to single exponential decays. The ionic strengths used and rate constants that were obtained were 95 mM, 0.00136 s^{-1}; and 362 mM, 0.0030 s^{-1}. (Inset) The rate constants are plotted according to eq 4b. Error bars are based on standard deviations from three measurements. The slope of the line drawn is 1.3.

also bound to phosphatidylcholine at high calcium and protein concentrations.

Binding was detected by both light scattering and fluorescence energy transfer (Figure 6A). Binding kinetics were very slow ($t_{1/2}$ = 5−20 min at 25 °C). This was expected for protein Z (*18*) and membranes that expressed sites at low frequency. While 5% dansyl-PE was included in the vesicles used in Figure 6, other experiments with pure PC showed the same level of bovine Z binding (data not shown). A distinguishing feature of binding to PC was the lack of

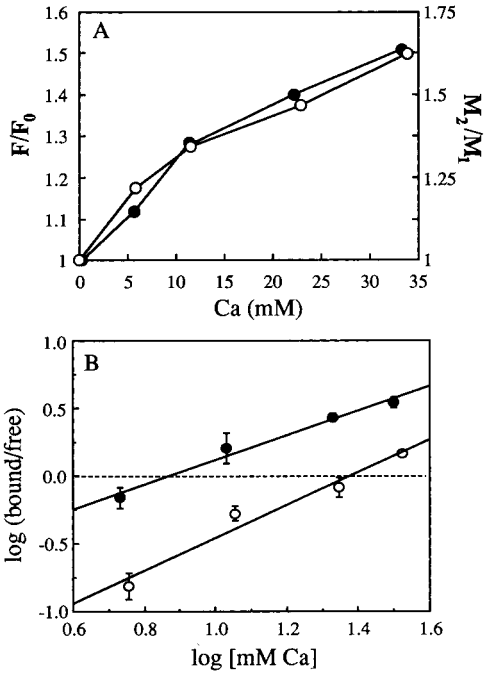

FIGURE 6: Calcium titration of protein Z binding to phosphatidylcholine membranes. (Panel A) Addition of calcium to a mixture of LUVs (9.5 μg/mL, dansyl-PE/PC, 5:95) and bovine Z (95 μg/mL). Protein binding was measured by light scattering (○, M_2/M_1) and fluorescence energy transfer (●, F/F_0). The amount of bound protein was estimated from the ratio of fluorescence in the presence of protein (F) to that of the vesicles alone (F_0). (Panel B) Hill plot of calcium titration. Ratios of bound/free sites were determined from light scattering measurements as described in Materials and Methods. Data sets contained protein:phospholipid ratios of 10:1 (95 μg/mL of bovine Z, ○) and 20:1 (190 μg/mL of bovine Z, ●). The dotted line depicts the x-axis origin at which the membrane sites are half-filled. Error bars are based on standard deviations from three measurements. The line fit to the data at a protein:lipid ratio of 10:1 gave a slope of 1.21 and an intercept of 1.38 (24 mM calcium). The line fit to the data at a protein:lipid ratio of 20:1 gave a slope of 0.91 and an intercept of 0.88 (7.6 mM calcium).

saturation by calcium, suggesting that eq 6 was applicable.

$$Z + n\mathrm{Ca} + \mathrm{PL_{site}} = Z{\cdot}\mathrm{Ca}_n{\cdot}\mathrm{PL_{site}}$$

The slope of the Hill plots (Figure 6B) were approximately

$$K_D = [Z_{free}][Ca]^n[Pl_{site\ free}]/[Z{\cdot}\mathrm{Ca}_n{\cdot}\mathrm{PL_{site}}] \qquad (6)$$

unity. The difference in the x-intercept at two protein concentrations was also consistent with univalent stoichiometry. Binding constants obtained from the data and eq 6 were 23 and 38 nM for the high and low protein titrations (Figure 6B).

Attempts to study prothrombin interaction with phosphatidylcholine were not successful (not shown). Equation 6 shows that a lower membrane affinity requires either higher protein or calcium to detect binding. For prothrombin, these levels may be outside of the feasible range.

Association of Protein Z with Phosphatidic Acid. Isolation of an ion pair in nonaqueous medium requires a fully ionized phosphate. This is provided by phosphodiesters so that binding to PS was relatively constant between pH 7 and pH 9 (Figure 7), a result that was shown in detail for prothrombin (*9*). Phosphomonoesters such as phosphatidic acid (PA) provide a second ionization at about pH 7. Free energy of protein−PA binding at a given pH (ΔG_{pH}) should be

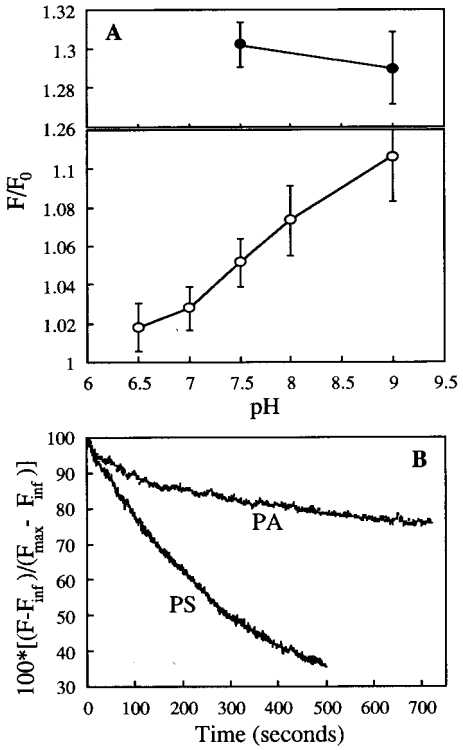

FIGURE 7: Effect of pH on binding and dissociation of protein Z with phosphatidic acid membranes. (Panel A) Binding of bovine Z (38 μg/mL) to LUVs (25 μg/mL) upon addition of calcium (3 mM) as measured by fluorescence energy transfer in buffer containing 25 mM Tris-HCl, 25 mM Bistris-HCl, and 0.1 M NaCl at pH 6.5−9. Results are shown for the following vesicle compositions: PA/dansyl-PE/PC, 20:5:75 (○) and PS/dansyl-PE/PC, 25:5:70 (●). Error bars are based on standard deviations from three measurements. (Panel B) Dissociation of the protein Z−membrane complex at pH 9. Protein−membrane dissociation was carried out as described in Materials and Methods and under the same solution conditions as described for binding in panel A. Curves are labeled to indicate the inclusion of PA versus PS in the vesicle composition.

described by eq 7, where ΔG_{PA-2} is the free energy of binding to fully ionized PA and $\Delta G_{ionization}$ is the free energy change for complete proton release at the pH studied.

$$\Delta G_{pH} = \Delta G_{PA-2} - \Delta G_{ionization} \quad (7)$$

The results with protein Z were qualitatively consistent with expectations. At pH 9.0, binding to PA was of very high affinity and dissociation rates were slower than from PS (Figure 7B). The K_D of the protein Z−PA complex at pH 9 was $\leq 10^{-8}$ M and $\Delta G \leq -11$ kcal/mol. At pH 7.5, the amount of bound protein Z was reduced by about half (Figure 7A, K_D of 5×10^{-7} M, $\Delta G = -8.7$ kcal/mol). The difference ($\Delta G_{ionization}$) of 2.3 kcal/mol correlated with complete proton release from a group with a pK of 7.0. Equation 7 predicted a $\Delta G_{ionization}$ that should diminish ΔG_{pH} to a point where binding should not be detected at pH 7.0. Thus, residual binding at this pH (Figure 7) suggested some nonideal behavior, which may be expected for this complex interaction. While it may seem counterintuitive that phosphodiesters such as PS and phosphatidylglycerol, with fully ionized headgroups but a valence of −1.0, are more effective at pH 7.5 than is a phosphomonoester (PA) with a charge of about −1.7, a critical factor in forming the membrane contact is clearly the absence of proton shielding, a property expected for binding by an isolated ion pair.

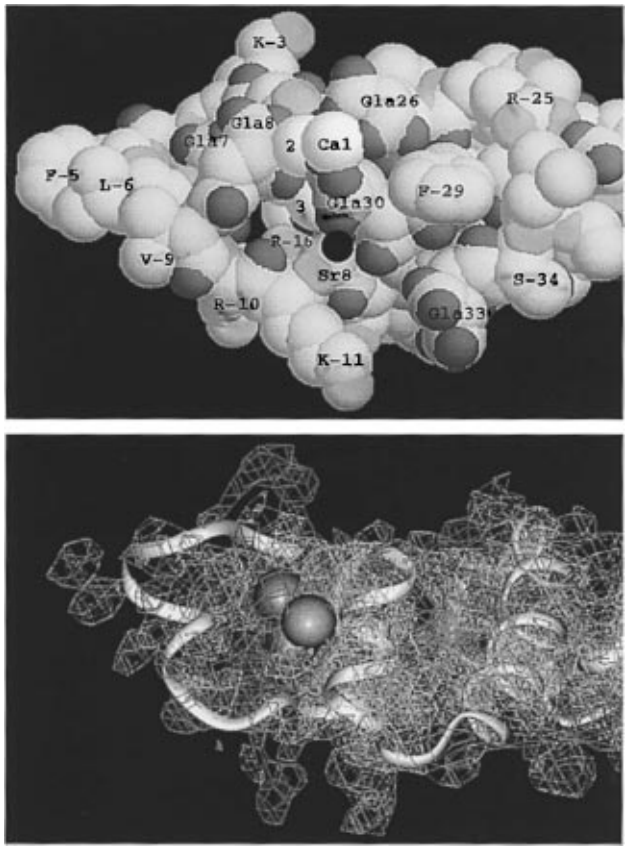

FIGURE 8: Electrostatic and structural comparisons of prothrombin. The upper structure (A) shows the amino-terminal 45 residues of prothrombin in RASMOL (Protein Data Bank reference 2pf2), CPK colors, and space-filled models. Important amino acids are labeled for reference. The approximate position of Sr-8 in the strontium−Fragment 1 structure (*1*) is shown by the black dot. Membrane binding is suggested to occur between a single phospholipid headgroup and the calcium-lined pore that is shown. The lower structure (B) shows the electrostatic potential of the same region of fragment 1, calculated by the program DELPHI at 10 and 20 *kT*/e. That shown in rust is electronegative at 10 *kT*/e and in red is electronegative at 20 *kT*/e. That shown in dark blue is electropositive at 20*kT*/e and in light blue is electropositive at 10 *kT*/e. The profile is approximately the same as that shown in structure A. The amino-terminal 35 residues are prothrombin (*58*), ANKGFLXXVR$_{10}$-KGNLXRXCLX$_{20}$XPCSRXXAFX$_{30}$ALXSL$_{35}$, and protein Z (*59*), AGSYLLXXLF$_{10}$XGHLXKXCWX$_{20}$XICVYXXARX$_{30}$-VFXDD$_{35}$.

DISCUSSION

The primary objective of this study was examination of step 3 of eq 1, the interaction of calcium-saturated protein and calcium-saturated membrane. This condition should isolate step 3 so that properties of the actual membrane contact are observed. Membrane contact by an ion pair in an isolated environment appeared consistent with the properties observed. Although not elaborated in detail, many properties were not easily explained by other contact mechanisms. The primary candidate for the protein component is shown in Figure 8A and consists of a calcium-lined pore. This may interact with a phosphate from the membrane (*18*) to create a univalent contact. The necessary features of this interaction will depend on physical properties described by eq 5, including the distance between the charged groups and the dielectric constant of the medium. Absolute univalency may be altered by participation of several groups with partial charges on both the protein and membrane, resulting in fractional values for $z_a z_b$.

Electrostatic modeling indicated that the region of the pore is electropositive (Figure 8B) and is surrounded by regions of lower electropositivity. An electrostatic potential of 10 *kT*/e corresponds to 250 mV at 300 K, giving a counterion at the plane of this potential an interaction energy of 6 kcal/mol. The pattern of electropositive potential surrounded by electronegative potential has similarity to some forms of phospholipase A2 (*47*) and colicin A (*48*). In some cases, membrane association is highly dependent on an electropositive region, despite a greater number of hydrophobic residues.

The electronegativity (*10*) of the surface of Phe-5, Leu-6, and Val-9 (Figure 8B) probably arises from Gla-7 and Gla-8, whose excess negative charge will be expressed on the surrounding surfaces of the protein. This feature may help explain the very low surface activity of vitamin K-dependent proteins (*4, 7*), despite the presence of this group of amino acids on their surfaces. High electronegativity should diminish the likelihood of penetration into a hydrocarbon, especially that of an electronegative membrane. Of added interest is that proteins containing the greatest electronegativity at other sites on this surface (Gla-33, Asp-34, and Gla-11) also correlated with highest affinity for the membrane (*18*). Thus, it is possible that this hydrophobic cluster serves as a part of the electrostatic architecture of the GLA domain, rather than as a hydrophobic probe. Further studies are needed to fully understand the function of these novel structural and electrostatic features.

Sensitivity of the protein−membrane complex to ionic strength, described by the parameter $z_a z_b$, should represent an aggregate of all forces involved. A similar charge product, despite a 1000-fold difference in dissociation rate constant, suggested a common contact mechanism for prothrombin and protein Z, but of different energy. Protein Z (sequence in the legend to Figure 8) contains several additional hydrophobic amino acids such as Tyr-4, Phe-10, and others (Figure 8). Prothrombin contains three additional cations (Lys-3, Asp-11, Arg-10) that are not found in protein Z, while protein Z contains four anions (Gla-11, Asp-34, Asp-35) that are not found in prothrombin. Another cation, Arg-25 of prothrombin, is replaced in protein Z by tyrosine, but its charge is offset by Arg-29 in protein Z (Phe-29 in prothrombin, Figure 8A). In fact, the only ionic group, other than Gla, that is common to prothrombin and protein Z is amino acid 16. Arg-16 of prothrombin is associated with the cationic pore (Figure 8A). Modifications of this site in human protein C lowered membrane affinity by 3−5-fold (*49*). In any event, similar $z_a z_b$ values for protein Z and prothrombin suggested that the surface groups were not very important in direct membrane contact. Their influence on binding may therefore be indirect.

Similarity of $z_a z_b$ for prothrombin and protein Z placed restrictions on the areas of the protein surface that could be involved in membrane contact. For most surfaces, charged residues would have to contact the surface or penetrate into the interior of the membrane during close approach. For example, the model of protein insertion into the membrane to the depth of the calcium ions (*11*−*15*), would place five charge differences between prothrombin and protein Z (Lys-3, Lys-11, Arg-10, Gla-11) either at or below the membrane surface. This would seem difficult to reconcile with similar ionic properties of binding.

Like annexins (*44*, *46*), protein Z bound to PC at high calcium. Although the K_D was low, binding was not easily detected due to lack of saturability with calcium. Addition of one calcium ion, which may reflect the calcium stoichiometry of step 2 (eq 1), appeared consistent with the binding mechanism proposed. Although the site of an additional calcium ion is not known, the suggested binding site (Figure 8A) may favor Sr-8 (*1*). Sr-1, -3, and -8 might bind a single phospholipid headgroup (*18*).

That protein−membrane dissociation kinetics were closely fit by a single exponential decay and that equilibrium binding suggested uniform affinity, except at the lowest ionic strengths (Figure 5) and highest protein packing densities (*5*, *43*), also supported a univalent binding mechanism. In effect, vitamin K-dependent proteins showed little competition for available PS molecules. For comparison, annexin proteins bind to a number of acidic phospholipids simultaneously and show extreme competition for PS in the membrane. A different equilibrium constant must be used for each annexin that is bound to a vesicle (*50*).

Proposed binding by an isolated ion pair appeared consistent with the inability of high calcium to specifically inhibit membrane contact (Figure 3) and the inability to incorporate phospholipid headgroups into the protein crystals (*10*). In effect, the protein−membrane interface would be needed to alter the environment and create a high energy ion pair. Binding of excess calcium ions and/or the phosphate headgroup may not occur in the water-accessible environment of free protein and phospholipid.

Protein S was an exception that was selectively dissociated by high calcium (Figure 3B). Even the basis for this unique behavior may implicate the proposed binding site. That is, in its amino-terminal 35 residues, protein S contains only two amino acids that are not found in homologous positions of other vitamin K-dependent proteins, Thr-8 and Asn-23 (sequences given in ref 18). Asn-23 occurs at a site that shows many variations in other proteins. However, Thr-8 occurs where all other proteins contain an aliphatic side chain (homologous to Val-9 of prothrombin, Figure 8A). Loss of hydrophobicity did not reduce membrane affinity of protein S (*18*), and a hydrophobic function of this residue is not suggested. However, Thr-8 occurs on the pore of the suggested membrane contact site. It may assist in creating a low-affinity calcium binding site that blocks or modifies the pore in a manner that abolishes membrane contact. While further work is needed, it appeared that the residue most likely to be responsible for the unusual behavior of protein S was associated with the suggested binding site (Figure 8A).

Interactions of protein Z with PC and fully ionized PA were consistent with the proposed binding model. If actual contact is largely based on an isolated ion pair, these structures should function as long as full ionization is realized and/or that K_D is adequate to observe binding by eq 6. In contrast, annexin proteins bound to PA at neutral as well as alkaline pH (*51*). A simpler model of calcium bridging, with less dependence on isolation of ion pairs, may function for annexins (*52*).

The nature of PL−Ca in step 2 of eq 1 is not fully defined and was not the principal focus of this study. Nevertheless, results obtained with protein Z and PC suggested a stoichiometry of 1 per bound protein. It did not appear that Ca−

PL (eq 1) was easily equated with PS_1Ca_1. For example, protein Z binding to membranes of 2% PS did not differ very much from binding to membranes of pure PC (not shown), even though the number of PS molecules was nearly equal to the number of proteins bound at surface saturation. This is evidence of cooperativity between binding affinity and PS density in the membrane. Other recent evidence of cooperativity is the 20-fold difference in binding constant between 20% and 50% PS (*5*). Although protein contact may rely primarily on univalent charge−charge interaction, the PL−Ca structure that is recognized by the protein may involve surface-bound calcium, the formation of which is very complex. For example, in a membrane, the electrostatic potential generates a double layer of calcium so that the surface experiences concentrations that are far higher than those in free solution (*40*, *53*). This phenomenon may counteract the low affinity of calcium association with single PS molecules in aqueous solution. Surface potential is highly cooperative with respect to PS density (*53*). Calcium appears to bind to two PS molecules (*8*, *54*) in the membrane, it causes phase separation in membranes of >50% PS (*55*), and may cause lower levels of clustering in membranes with less PS (*56*, *57*). As a result, increasing the free calcium levels for membranes of 2% PS may never produce the same structures formed in membranes of 20% PS. Definition of the calcium-saturated membrane structure recognized by the protein, PL−Ca in eq 1, will require much additional study.

Overall, many features demonstrated by vitamin K-dependent proteins are consistent with binding through ion pairing that is isolated from the aqueous environment. Identification of the correct membrane contact site should allow design of protein mutations that alter membrane affinity in predictable manners. For example, low affinity of bovine protein C and factor VII for membranes was suggested to arise, in part, from proline-10 (homologous to position 11 of prothrombin), which may disrupt the binding pore [Figure 8 (*18*)]. Indeed, replacement of proline-10 of bovine protein C by His has resulted in a protein with enhanced membrane binding affinity (*60*). This appears to be the first example of enhancing membrane contact by a purposeful design. While further studies are needed to fully understand the binding mechanism, the univalent ionic interaction and the specific site suggested in Figure 8 provide a promising model for defining the roles of specific residues in the membrane binding of vitamin K-dependent proteins.

REFERENCES

1. Seshadri, T., Skrzypckak-Jankum, E., Yin, M., and Tulinsky, A. (1994) *Biochemistry 33,* 1087−1092.
2. Banner, D. W., D'Arcy, A., Chene, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) *Nature 380,* 41−46.
3. Freedman, S. J., Furie, B. C., Furie, B., and Baleja, J. D. (1995) *Biochemistry 34,* 12126−12137.
4. Mayer, L. D., Nelsestuen, G. L., and Brockman, H. L. (1983) *Biochemistry 22,* 316−321.
5. Lu, Y., and Nelsestuen, G. L. (1996) *Biochemistry 35*, 8193−8200.
6. Mayer, L. D. (1983) Ph.D. Thesis, University of Minnesota, St. Paul, MN.
7. Mayer, L. D., Pusey, M. L., Griep, M. A., and Nelsestuen, G. L. (1983) *Biochemistry 22,* 6226−6232.
8. Nelsestuen, G. L., and Lim, T. K. (1977) *Biochemistry 16,* 4164−4171.
9. Resnick, R. M., and Nelsestuen, G. L. (1980) *Biochemistry 19,* 3028−3033.
10. Soriano-Garcia, M., Padmanabhan, K., deVos, A. M., and Tulinsky, A. (1992) *Biochemistry 31,* 2554−2566.
11. Zhang, L., and Castellino, F. J. (1994) *J. Biol. Chem. 269,* 3590−3595.
12. Jalbert, L. R., Chan, J. C., Christiansen, W. T., and Castellino, F. J. (1996) *Biochemistry 35,* 7093−7099.
13. Sunnerhagen, M., Forsen, S., Hoffren, A.-M., Drakenberh, T., Teleman, O., and Stenflo, J. (1995) *Nat. Struct. Biol. 6,* 504−509.
14. Christiansen, W. T., Jalbert, L. R., Robertson, R. M., Jhingan, A., Prorok, M., and Castellino, F. J. (1995) *Biochemistry 34,* 10376−10382.
15. Freedman, S. J., Blostein, M. D., Baleja, J. D., Jacobs, M., Furie, B. C., and Furie, B. (1996) *J. Biol. Chem. 271,* 16227−16236.
16. Welsch, D. J., Pletcher, C. H., and Nelsestuen, G. L. (1988) *Biochemistry 27,* 4933−4938.
17. Weber, D. J., Berkowitz, P., Panek, M. G., Huh, N. W., Pedersen, L. G., and Hiskey, R. G. (1992) *J. Biol. Chem. 267,* 4564−4569.
18. McDonald, J. F., Shah, A. M., Schwalbe, R. A., Kisiel, W., Dahlbäck, B., and Nelsestuen, G. L. (1997) *Biochemistry 36,* 5120−5127.
19. Hashimoto, N., Morita, T., and Iwanaga, S. (1985) *J. Biochem.* (*Tokyo*) *97,* 1347−1355.
20. Nelsestuen, G. L. (1984) *Methods Enzymol. 107,* 507−516.
21. Pusey, M. L., Mayer, L. D., Wei, G. J., Bloomfield, V. A., and Nelsestuen, G. L. (1982) *Biochemistry 21,* 5262−5269.
22. Taylor, D. L., Wang, Y. L., and Heiple, J. M. (1980) *J. Cell Biol. 86,* 590−598.
23. Huang, C. (1969) *Biochemistry 8,* 344−352.
24. Hope, M. J., Bally, M. G., Webb, G., and Cullis, P. R. (1985) *Biochim. Biohys. Acta 812,* 55−65.
25. Bloomfield, V. A., and Lim, T. K. (1978) *Methods Enzymol. 48,* 415.
26. Pusey, M. L., and Nelsetuen, G. L. (1984) *Biochemistry 23,* 6202−6210.
27. Tinoco, I., Jr., Sauer, K., and Wang, J. C. (1995) *Physical Biochemistry. Principles and Applications in Biological Sciences*, pp 1−761, Prentice Hall, Englewood Cliffs, NJ.
28. Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., and Field, D. (1981) *Biochemistry 20,* 6509−6519.
29. Pederson, S. M. (1985) *Biochem. Pharmacol. 34,* 4319−4323.
30. Pocker, Y., and Miao, C. H. (1987) *Biochemistry 26,* 8481−8486.
31. Aviram, I., Myer, Y. P., and Schejter, A. (1981) *J. Biol. Chem. 256,* 5540−5544.
32. Alberty, R. A., and Silbey, R. J. (1992) *Physical Chemistry*, Chapter 8, p 246, John Wiley and Sons, Inc., New York, NY.
33. Lu, Y., Bazzi, M. D., and Nelsestuen, G. L. (1995) *Biochemistry 34,* 10777−10785.
34. Dauber-Osguthorpe, P., Roberts, V. A., Osguthorpe, D. J., Wolff, J., Genest, M., and Hagler, A. T. (1988) *Proteins: Struct., Funct., Genet. 4,* 31−47.
35. Nicholls, A., and Honig, B. (1991) *J. Comput. Chem. 12,* 435−445.
36. Klapper, I., Hagstrom, R., Fine, R., Sharp, K., and Honig, B. (1986) *Proteins 1,* 47−59.
37. Bradford, M. M. (1976) *Anal. Biochem. 72,* 248−254.
38. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem. 28,* 1756−1759.
39. Nelsestuen, G. L. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Siegel, F. L., Eds.) pp 323−332, Elsevier North-Holland, New York.
40. Dombrose, F. A., Gitel, S. N., Zawalich, K., and Jackson, C. M. (1979) *J. of Biol. Chem. 254,* 5027−5040.

41. Nelsestuen, G. L., and Broderius, M. (1977) *Biochemsitry 16*, 4172−4177.
42. Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlback, B., and Nelsestuen, G. L. (1989) *J. Biol. Chem. 264*, 20288−20296.
43. Wei, G. J., Bloomfield, V. A., Resnick, R. M., and Nelsestuen, G. L. (1982) *Biochemistry 21*, 1949−1959.
44. Bazzi, M. D., Youakim, M. A., and Nelsestuen, G. L. (1992) *Biochemistry 31*, 1125−1134.
45. Smirnov, M. D., and Esmon, C. T. (1994) *J. Biol. Chem. 269*, 816−819.
46. Andree, H. A. M., Stuart, M. C. A., Hermens, W. T., Reutelingsperger, C. P. M., Hemker, H. C., Frederik, P. M., and Willems, G. M. (1992) *J. Biol. Chem. 267*, 17907−17912.
47. Scott, D. L., Mandel, A. M., Sigler, P. B., and Honig, B. (1994) *Biophys. J. 67*, 493−504.
48. Lakey, J. K., Parker, M. W., Gonzalez-Manas, J. M., Duche, D., Vriend, G., Baty, D., and Pattus, F. (1994) *Eur. J. Biochem. 220*, 155−163.
49. Thariath, A., and Castellino, F. J. (1997) *Biochem. J. 322*, 309−315.
50. Bazzi, M. D., and Nelsestuen, G. L. (1991) *Biochemistry 30*, 7970−7977.
51. Bazzi, M. D., and Nelsestuen, G. L. (1991) *Biochemistry 30*, 7961−7969.
52. Swairjo, M. A., Concha, N. O., Kaetzel, M. A., Dedman, J. R., and Seaton, B. A. (1995) *Nat. Struct. Biol. 2*, 968−974.
53. McLaughlin, S. G., Szabo, G., and Eisenman, G. (1971) *J. Gen. Physiol. 58*, 667−687.
54. Feigenson, G. W. (1989) *Biochemistry 28*, 1270−1278.
55. Onishi, S., and Ito, T. (1974) *Biochemistry 13*, 881−887.
56. Dibble, A. R., and Feigenson, G. W. (1994) *Biochemistry 33*, 12945−12953.
57. Huang, J., Swanson, J. E., Dibble, A. R., Hinderliter, A. K., and Feigenson, G. W. (1993) *Biophys. J. 64*, 413−425.
58. Magusson, S., Peterson, T. E., Sottrop-Jensen, L., and Claeys, H. (1975) *Proteases and Biological Control* (Reich, E., Rifkin, D. B., and Shaw, E., Eds.) p 123, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
59. Hojrup, P., Jensen, M. S., and Petersen, L. E. (1985) *FEBS Lett. 184*, 333−338.
60. Shen, L., Shah, A., Dahlback, B., and Nelsestuen, G. L. (1997) *Biochemistry* (in press).

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