Equilibria Involved in Prothrombin- and Blood-Clotting Factor X-Membrane Binding†

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ABSTRACT: The study of prothrombin- and factor X-membrane interaction by light-scattering intensity measurements at 90° is reported. This technique, which uses a fluorometer as a light-scattering photometer, can be applied to measurement of free and membrane-bound protein concentrations, from which equilibrium constants can be obtained. The following equilibria adequately describe the observed properties of prothrombin-membrane interaction

$$\left.\begin{array}{l} \mathrm{P} + i\mathrm{Ca} \overset{1}{\rightleftarrows} \mathrm{P}_{i\mathrm{Ca}} \overset{2}{\rightleftarrows} \mathrm{P'}_{i\mathrm{Ca}} \\ \qquad\qquad + \\ \mathrm{PL} + j\mathrm{Ca} \overset{3}{\rightleftarrows} \mathrm{PL}_{j\mathrm{Ca}} \end{array}\right\} + m\mathrm{Ca} \overset{4}{\rightleftarrows} \mathrm{P'} \cdot \mathrm{PL}_{i+j+m\mathrm{Ca}}$$

where P and PL are protein and phospholipid, P_{iCa} and PL_{jCa} are the calcium complexes, P'_{iCa} is the protein after undergoing a calcium dependent transition, and $P'\cdot PL_{i+j+mCa}$ is the protein-membrane complex. Several lines of evidence indicate that i, j, and m are interrelated and m decreases to 0 when i and j are saturated. In agreement with this, direct calcium binding measurements indicate m values of 3.2 ± 1.5 and 1.1 ± 1.5 at 0.5 and 1.2 mM calcium, respectively. The total number of functional calcium ions in the complex ($i + j + m$) is 6 to 9 based on Hill coefficients for the reactions and direct calcium binding measurements. In reaction 3, the maximum stoichiometry of calcium per acidic phospholipid is 1:2. While the details of factor X-membrane binding were not determined in quite as great detail, the equilibria (identified) appear the same but a major difference is the calcium concentration needed to initiate protein-membrane binding.

Blood coagulation consists of a cascade of zymogen to enzyme conversions involving a group of very specific proteases. At least two of these reactions, the conversion of factor X to Xa and the conversion of prothrombin to thrombin, are not simple proteolytic reactions but require membrane-bound complexes of protease, substrate, and an additional protein (factor VIII for X to Xa conversion and factor V for prothrombin to thrombin conversion) (see Davie and Fujikawa, 1975, for a review). The factor X hydrolyzing proteins, factors VII and IX, together with factor X and its substrate, prothrombin, comprise the vitamin K dependent proteins in blood coagulation. These proteins contain γ-carboxyglutamic acid residues (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974; Howard and Nelsestuen, 1975; Bucher et al., 1976) and bind to membranes containing acidic phospholipids in the presence of calcium ions (Papahadjopoulos and Hanahan, 1964; Bull et al., 1972). Current understanding of how these proteins bind to the membrane is limited to the knowledge that, along with calcium or other cations and γ-carboxyglutamic acid residues, intact protein structure is essential for formation of a protein-membrane complex (Nelsestuen, 1976; Nelsestuen et al., 1976). Focusing on prothrombin, the most thoroughly studied of these proteins, some of the important questions for which no data are available include: does prothrombin penetrate the membrane or is it bound only to the membrane surface? Is the protein-membrane interaction entirely ionic or do hydrophobic contacts occur as well? How many ionic sites are there? Since prothrombin is an elliptical molecule (Lamy and Waugh, 1953), does it lie flat on the membrane surface or does it bind at one end and protrude from the surface? What are the dissociation constants for binding prothrombin and the other proteins to membranes of different compositions? Eventual reconstruction of the blood-coagulation process requires careful determination of these and other parameters.

A major difficulty in seeking answers to these questions and others has been that the methods employed for studying these protein-membrane interactions, primarily gel filtration, produce very qualitative results (cf. Bull et al., 1972). The recent application of fluorescence energy transfer has advanced the protein-membrane binding studies by allowing determination of the cation requirement and cation specificity of protein-membrane binding (Nelsestuen et al., 1976). That technique, however, is limited by the requirement for major chemical modification of the phospholipid and the necessary assumption that only a single form of protein-membrane complex is formed. Clearly, if the binding of proteins to phospholipid membranes whose compositions approximate physiological composition is to be studied, other methods must be employed.

The investigations reported here answer many of the points brought up including the development of methods which can be used to obtain dissociation constants for the protein-membrane complexes, the number of cations required, and more detailed identification of the equilibria involved in prothrombin- and factor X-membrane binding.

Materials and Methods

Bovine prothrombin and its thrombin cleavage product, prothrombin fragment 1,[1] and bovine factor X were prepared

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[1] The nomenclature of the prothrombin breakdown products is as suggested by the Task Force on the Nomenclature of Blood-Clotting Zymogens and Zymogen intermediates. Prothrombin fragment 1 is the 23 000-dalton amino-terminal region containing the membrane-binding site, prethrombin 1 is the 52 000 dalton carboxyl-terminal region which is composed of prothrombin fragment 2 (12 000 daltons) and prethrombin 2 (40 000 daltons and the direct precursor of thrombin).

as described previously (Nelsestuen et al., 1976). These proteins were quantitated using $\epsilon^{1\%}_{280nm}$ values of 14.1 (Cox and Hanahan, 1970), 10.0 (Heldebrant and Mann, 1973), and 12.4 (Jackson, 1972), respectively. Phospholipids purchased from the Sigma Chemical Co. including supplier's purity estimates are: phosphatidylserine from bovine brain (98%), phosphatidylcholine from egg yolk (95%), phosphatidylethanolamine from egg yolk (98%), and Folch fraction III from bovine brain (reported to contain 80–90% phosphatidylserine).

Membrane Preparation. Phospholipid vesicles were prepared by mixing the appropriate amounts of the phospholipids (10–100 mg total) in organic solvent, drying under vacuum to remove the solvent, and dispersing in buffer (usually 5 mL) by direct probe sonication for three 1.5-min intervals with a Bransen sonifier. The sample was maintained in an ice bath during and between sonications. The product was chromatographed on a 2.5 × 20 cm column of Sepharose 4B. The elution pattern consists of an excluded peak of large vesicles and a second peak which penetrates the gel matrix (Huang, 1969). The vesicles eluting in the last half of the included peak were pooled for use in these studies. This procedure yields only about 10 to 30% of the total phospholipid as these single bilayer vesicles but this is quite sufficient for the studies reported. The phospholipid concentration of the stock solutions and vesicle preparations were based on organic phosphate determined by the method of Chen et al. (1956) using a (w/w) conversion factor of 25 (phospholipid/P) for phosphatidylserine, phosphatidylcholine, and phosphatidylglycerol, a factor of 22 for phosphatidic acid, and a factor of 23.5 for phosphatidylethanolamine. In all cases phospholipid composition is expressed as the molar ratio of phospholipids present before sonication and chromatography. Where a membrane composition is given as a percentage or mole fraction of acidic phospholipid, the remainder of the phospholipid is phosphatidylcholine.

It was observed that phospholipids containing crude phosphatidylserine (Folch fraction III) produced a much higher yield of the single bilayer vesicles under these conditions than the pure phospholipid and the product vesicles were more stable. One preparation showed no detectable change in average vesicle diameter over a period of several months. A major contaminant of this crude phosphatidylserine preparation is cerebrosides. Therefore, 1% (w/w) cerebrosides (Sigma Chemical Co.) were routinely included in the pure phospholipid membrane preparations. This appeared to enhance the yield of small vesicles, although no comprehensive study of yield or stability of the vesicles produced was performed. Changes in the average diameter of the vesicles occurred with variable rates. In those cases where single bilayer vesicles were essential for the correct analysis, studies were usually completed within 8–12 h of membrane preparation and corrections for membrane changes were included where necessary. By pooling the same fractions from the Sepharose 4B column, the average vesicle radius measured according to Lim et al. (1977) was 180 ± 30 Å for very different phospholipid compositions.

Calcium Binding Measurements. Calcium binding to protein and membranes was measured by determination of the average partition coefficient for calcium in Sephadex G-25. Columns of Sephadex G-25 were packed to a volume of 0.2 mL in 1-mL serological pipets. The columns were equilibrated with the appropriate solution ($^{45}Ca^{2+}$, protein, phospholipid) by loading 0.3 mL onto the column and allowing the solution to drain to the surface of the gel. The tip of the pipet was wiped, the column chamber above the gel was rinsed with buffer, and the contents of the column were eluted with 0.5 mL of buffer. The quantity of material (protein, phospholipid, and/or ^{45}Ca) eluted from the gel was measured. Protein was quantitated by its 280-nm absorbance, phospholipid by organic phosphorus according to Chen et al. (1956), and $^{45}CaCl_2$ by scintillation counting. Assigning $^{45}CaCl_2$ a partition coefficient of 1.0 since it penetrates all regions of the gel, the total volume of the column could be determined from the known specific activity and concentration of $^{45}CaCl_2$ in the solution applied to the column. The penetration volumes for protein and phospholipid were measured separately by equilibrating the columns with known concentrations and measuring the total quantity eluted. The penetration volume for macromolecules (which is the exclusion volume for molecules of molecular weight > 5000 daltons) was determined to be 0.53 relative to the penetration volume for calcium.

If a solution of $^{45}Ca^{2+}$ with protein and/or phospholipid is applied to the column, the bound calcium will display a partition coefficient of 0.53 and the average partition coefficient for calcium will be less than 1.0. The fraction of unbound calcium is equal to $(x - 0.53y)/0.47y$, where x is the ^{45}Ca eluted from the column equilibrated with protein and/or phospholipid and y is the ^{45}Ca eluted from the same column equilibrated with the same total $^{45}CaCl_2$ concentration but without protein or phospholipid. From the known calcium and protein and/or phospholipid concentrations in the solutions applied to the column, and the fraction of calcium which is bound, the calcium bound per unit of protein or phospholipid is calculated. The quantity of protein and/or phospholipid added was manipulated so the fraction of calcium bound was 0.2 to 0.8. It was found that the Sephadex gel bound 75 nmol of calcium per mL of bed resin with a dissociation constant of 1.6×10^{-4}. A correction was made for this bound calcium in all determinations.

Relative Molecular Weight Determination by Light Scattering. Relative 90° light-scattering measurements were made in a Perkin-Elmer Hitachi Model MPF-2A fluorescence spectrophotometer equipped with a Hitachi Model QPD33 recorder and a thermostated cell holder. The excitation and analysis monochromators were set at the same wavelength (usually 320 nm) and the recorder was zeroed with buffer in the cuvette. Samples were added to the cuvette and the light scattering from the protein–phospholipid complex was compared as described below to the light scattering from the phospholipid alone. The volume of sample was 1.5 mL and the quantity of the single bilayer phospholipid vesicles used was usually 30–100 μg, although much smaller quantities could be measured. Under these conditions, precautions were taken to eliminate dust particles in the reagents. Noise from dust particles appeared as peaks on the recorder which were easily identified and, when not severe, were excluded in data evaluation. Excessive contamination by dust produced very unstable readings which were corrected by centrifugation for 15 min at 10 000g.

Under conditions where the light-scattering particles are much smaller than the wavelength of light, such as the case in our experiments, the equation describing light scattering for a particle in solution is:

$$\frac{Kc}{I_s/I_{s_0}} = \frac{1}{M} + 2Bc \qquad (1)$$

where I_s/I_{s_0} is the ratio of scattered to incident light intensities, K is a constant of the instrument and solution, c is the concentration of the scattering component in g per mL, M is the molecular weight of the scattering component, and B is the second virial coefficient which corrects for deviation from ideality due to particle interaction (Doty and Edsall, 1951).

The evidence obtained indicates that, at the concentrations of protein and phospholipid used in these studies, the term $2Bc$

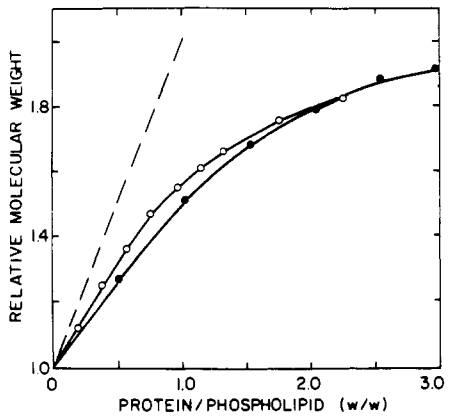

FIGURE 1: Relative molecular weight determination by light scattering. The light scattering from 120 μg of 25% phosphatidylserine vesicles in 1.5 mL of 1 mM calcium (I_{s_1}) was assigned the value 1.0 and the light scattering (I_{s_2}) was measured after addition of protein. The values plotted for prothrombin (●) and factor X (○) have been corrected for light scattering from unbound protein. The dotted line gives the value of M_2/M_1 if all of the protein bound to the phospholipid.

in eq 1 can be neglected. Within experimental error, the light-scattering intensity from both protein and single-bilayer phospholipid vesicles was directly proportional to concentration up to at least 1.0 mg/mL which is several fold higher than the concentrations generally used. Based on one estimate, the error introduced by neglecting the $2Bc$ term in eq 1 for measurements of single bilayer vesicles at 100 μg per mL was less than 2%.

Assuming that the term $2Bc$ is also negligible for the protein–membrane complex produced under the conditions used in these studies, the ratio of light-scattering intensities for the protein–membrane complex (I_{s_2}) to that of the membrane vesicles alone (I_{s_1}) measured under the same conditions becomes:

$$\frac{I_{s_2}}{I_{s_1}} = \frac{\left(\frac{\partial n_2}{\partial c_2}\right)^2 M_2 c_2}{\left(\frac{\partial n_1}{\partial c_1}\right)^2 M_1 c_1} \qquad (2)$$

where $\partial n_2/\partial c_2$ and $\partial n_1/\partial c_1$ are the change in refractive indexes with concentrations for the protein–membrane complex and the membrane vesicles alone, respectively.

Refractive index measurements were made in a Model B-S differential refractometer (Phoenix Precision Instruments, Co., Philadelphia, Pa.). The value of $\partial n/\partial c$ for prothrombin and phospholipid (50% phosphatidylserine vesicles) were 0.192 and 0.172, respectively. The value of $\partial n/\partial c$ was not dependent on membrane composition; between 10 and 50% phosphatidylserine, the value of $\partial n/\partial c$ was 0.172 ± 0.01 and the value of 0.172 was used for all membrane compositions. A $\partial n/\partial c$ value of 0.192 was used for factor X. Within a range of ±3%, it was found that $\partial n/\partial c$ for a solution of equal concentrations of prothrombin or factor X and phospholipid was unaffected by binding the protein to the membrane by the addition of 1 mM calcium. Therefore, the value of $\partial n_2/\partial c_2$ in eq 3 can be estimated from the ratio of protein to phospholipid in the complex and the values of $\partial n/\partial c$ for the free proteins and for phospholipid. For example, a 1:1 complex of protein to phospholipid will have a $(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)$ value of 1.06. In determination of M_2/M_1 from eq 3 below, the overall error due to the $\partial n/\partial c$ terms could be about 15% since this term is squared and contains uncertainties in both refractive index and concentration determination. Since a substantial portion of this potential error is systemic, the results presented have less error when used for comparative purposes.

In the present case, where the term $2Bc$ in eq 1 is negligible and the number concentration of light-scattering particles is unchanged (i.e., $C_1/M_1 = C_2/M_2$), the ratio of light-scattering intensities in eq 2 will be equal to:

$$\frac{I_{s_2}}{I_{s_1}} = \left(\frac{\partial n_2/\partial c_2}{\partial n_1/\partial c_1}\right)^2 \left(\frac{M_2}{M_1}\right)^2 \qquad (3)$$

In application of eq 3 to the procedures given below, the value of I_{s_2} must be corrected for light scattering due to unbound protein. This was accomplished by assigning $(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)$ a value of 1 and estimating the percentage of free and bound protein from the value of I_{s_2}/I_{s_1} and the known ratio of added protein and phospholipid. Since the light-scattering intensity of free protein never exceeded 10% of the total light-scattering intensity, this approximate correction which assumes $\partial n_2/\partial c_2 = \partial n_1/\partial c_1$ introduces no more than a 1% error in the calculated values.

Finally, the ratio of M_2/M_1 in eq 3 was estimated as follows: The ratio of M_2/M_1 was estimated assuming $(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1) = 1$. From the resulting ratio of protein to phospholipid in the complex, a second value of $(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)$ was calculated as outlined above and used to obtain a second value of M_2/M_1. This calculation was recycled until a constant value for M_2/M_1 resulted.

A problem which can arise in the study of calcium-mediated protein–membrane interaction by relative light scattering is the aggregation of the membranes by calcium ions, a process which has been studied extensively by other workers including Papahadjopoulos et al. (1976). In the studies reported here and in accompanying communications we found that the membranes containing only phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine were not affected in this way by any of the calcium concentrations used. Membranes of low phosphatidylserine content (<30%) were also not seriously affected. Membranes of high (>50%) phosphatidylserine content, however, showed increased light scattering as the calcium concentration was increased.

The measurement of protein–membrane binding by light scattering is dependent upon a system which does not undergo secondary aggregation of the protein–membrane complexes. Such a secondary aggregation will cause an overestimate of the amount of protein bound and the estimated dissociation constants will be low. We have carefully avoided conditions of calcium concentration and membrane composition which cause the vesicle fusion described above. The reversibility of the protein–membrane interaction (implying that the protein is not catalyzing vesicle fusion) was shown by adding EDTA[2] to prothrombin–protein complexes. The result was a return of light scattering intensity to that of the free protein plus free vesicles. While this was not determined for every experimental point, it was tested in a number of cases including membranes of high phosphatidylserine content (80% phosphatidylserine) which are the most prone to vesicle fusion.

The results of a typical experiment measuring prothrombin and factor X binding to phospholipid vesicles is shown in Figure 1. From comparison of the observed molecular weight to the theoretical molecular weight if all the protein were bound (dotted line, Figure 1) the concentration of free and bound protein was estimated. Protein additions were made from a concentrated solution of prothrombin containing calcium or manganous ions so the protein was in its phospholipid binding

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

conformation (Nelsestuen et al., 1976). The added cations did not significantly alter the cation concentration in the cuvette. The conditions employed ensured that all experiments in these communications were conducted at equilibrium and were not affected by the slow rate of the prothrombin transition (Nelsestuen, 1976).

Unless specified, all experiments were carried out in the standard buffer (0.05 M Tris (pH 7.5)–0.1 M NaCl) at 25 ± 1 °C.

Results

Calcium Titration of the Protein–Phospholipid Complex. Previous studies have identified two distinct calcium dependent equilibria in prothrombin- and factor X–phospholipid binding (Nelsestuen, 1976; Nelsestuen et al., 1976). The first reaction is a calcium-dependent protein transition involving calcium-binding sites on the protein only. The second calcium-dependent reaction involves calcium binding either to the phospholipid or to the protein–phospholipid complex (Nelsestuen et al., 1976). In order to investigate this second reaction further, we determined a set of calcium titration curves measured by light scattering as shown in Figure 2. In order to eliminate the effects of the calcium-dependent protein transition, 0.2 mM manganous ion was included. Binding of manganous ion to the protein brings about the protein transition but manganese will not bind the protein to the phospholipid (Nelsestuen et al., 1976). The properties of the protein–membrane interaction are similar to the results obtained previously (Nelsestuen et al., 1976) by fluorescence energy transfer. If the value at 1 mM calcium is assigned 100% reaction and a Hill plot is made of the data in Figure 2 at a protein/phospholipid ratio of 5/1, the reaction is half maximal at 0.09 mM calcium and the Hill coefficient is 1.68. The results from fluorescence energy transfer treated in a similar manner gave a half-maximal reaction at 0.085 mM calcium and a Hill coefficient of 2.2 (Nelsestuen et al., 1976). The differences in these values may be due to the different phospholipid compositions and to the different protein to phospholipid ratios used since both of these factors affect the result (Nelsestuen and Broderius, 1977). In other comparisons of these techniques, the rate constants for protein–membrane binding measured by either light scattering (not shown) or fluorescence energy transfer (Nelsestuen et al., 1976) were identical and both in turn are identical with the rate constants for the protein transition (Nelsestuen, 1976). The agreement of these very different methods serves to confirm the validity of each technique.

The results from the light-scattering experiment shown in Figure 2 illustrate some features of protein–phospholipid binding which were not detected by fluorescence energy transfer. First of all, it is apparent that not all of the protein binds to the phospholipid regardless of the calcium concentration. When the protein/phospholipid ratio is 0.36, the increase in relative molecular weight of the membrane vesicles is only 0.19 and about 50% of the prothrombin must remain free in solution. This lack of quantitative binding becomes more pronounced at higher protein/phospholipid ratios and implies the existence of a calcium-independent equilibrium. The studies using fluorescence energy transfer did not detect this nonquantitative binding. The conditions selected for those studies (low protein/membrane ratios and high phosphatidylserine content) would result in a very low percentage of unbound protein at 1 mM calcium and the results presented by Nelsestuen et al. (1976) characterize the calcium-dependent reaction only.

Another feature of the protein–phospholipid interaction which is evident in Figure 2 at higher protein to phospholipid

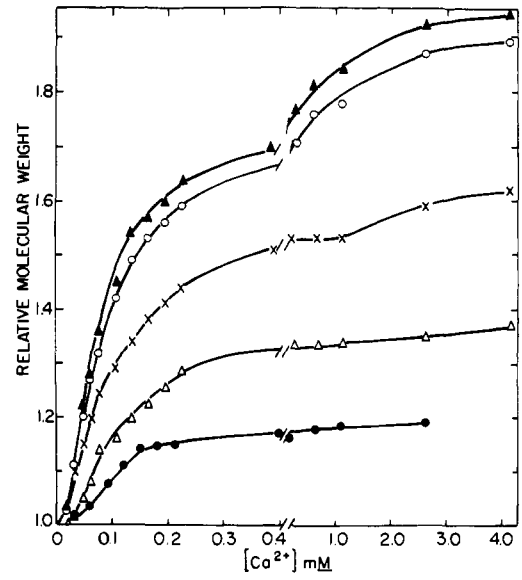

FIGURE 2: Calcium titration of prothrombin–membrane binding measured by light scattering. Phospholipid (214 μg of 30% Folch fraction III) and varying amounts of prothrombin were mixed in 1.5 mL of buffer containing 0.2 mM manganous ion. Calcium was added to the concentrations indicated and the relative molecular weights of the vesicles or protein–vesicle complexes were determined by relative light scattering after subtraction of light scattering by unbound protein. The amounts of prothrombin added are: 78 μg (●), 156 μg (○), 260 μg (X), 650 μg (△), and 1040 μg (▲).

ratios is the existence of at least two types of calcium-dependent protein–membrane binding reactions. There is a cooperative process occurring at low calcium concentrations followed by a slow increase in binding as calcium is increased from 0.4 to 4 mM.

These results suggest no fewer than three separable equilibria. Two are calcium dependent and a third is calcium independent. These will be documented more thoroughly below. Although the calcium titration curve for factor X–phospholipid shows that binding occurs at higher calcium concentrations (Nelsestuen et al., 1976), experiments such as those given for prothrombin in Figure 2 indicated that the general characteristics are similar and the types of equilibria involved would also appear to be similar.

Studies at Varying Protein Concentrations. In order to study the complex equilibria implied by the data in Figure 2, the membrane-bound protein and free protein concentrations were determined at varying total protein and calcium concentrations by the method shown in Figure 1. Double-reciprocal plots of these data are shown in Figure 3. At low calcium concentrations (<0.2 mM), these plots show considerable upward curvature and have different intercepts indicating a complex reaction. At intermediate calcium concentrations (>0.3 mM < 5 mM), these plots are linear giving the same intercept on the ordinate (indicating an identical number of total binding sites on the membrane) but with different slopes (indicating different dissociation constants at the different calcium concentrations). At very high calcium (>10 mM, Figure 3B) the plots are identical indicating that the binding is saturated with respect to calcium and the dissociation constant contains no calcium term. The linearity of these double-reciprocal plots at intermediate and high calcium concentrations implies a single class of independent binding sites.

The Effect of Varying Phospholipid Concentration and Vesicle Size. Since the individual sites are independent (from above), it would be expected that, in the equilibrium constants describing the protein–membrane binding, the phospholipid

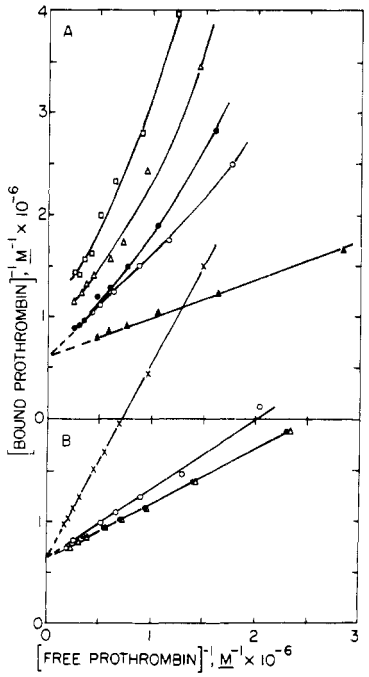

FIGURE 3: Double-reciprocal plots of prothrombin-membrane binding under conditions of varying calcium concentrations. A gives the results obtained as described in Materials and Methods for prothrombin binding to 120 μg of 25% phosphatidylserine at 0.08 mM (□), 0.12 mM (△), 0.16 mM (●), 0.30 mM (○), and 1.0 mM (▲) calcium. B gives the results for prothrombin binding to 160 μg of 30% Folch fraction III at 1 mM (X), 5 mM (○), 10 mM (●), and 30 mM (△) calcium.

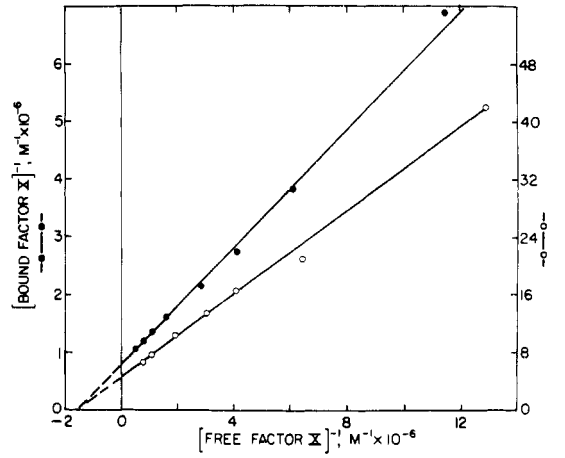

FIGURE 4: Double-reciprocal plots of factor X binding to vesicles of varying size and concentration. The results of factor X binding to vesicles of 14% phosphatidylserine are given using 90 μg of single bilayer vesicles, (●) and 36 μg of multibilayer vesicles (the exclusion peak from Sepharose 4B) (○).

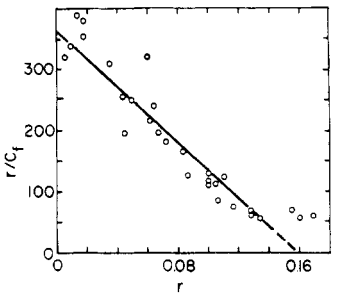

FIGURE 5: Scatchard plot of calcium binding to membrane vesicles of 50% phosphatidylserine. C_f is the free calcium concentration and r is the μmol of calcium bound per mg of phospholipid. The line drawn gives a saturation binding of 0.50 calcium ion per exposed phosphatidylserine (see text for details) with a dissociation constant of 4.4×10^{-4}.

term should be expressed as the concentration of protein binding sites and should be independent of vesicle size or concentration. That this is true is shown by Figure 4 where factor X binding to vesicles of similar composition but of different size and at different vesicle concentration give similar dissociation constants. Other studies using prothrombin and other membrane compositions substantiate this observation.

Calcium Binding to the Phospholipid and to the Prothrombin-Phospholipid Complex. As pointed out previously (Nelsestuen et al., 1976), the cooperative binding reaction seen in Figure 2 appears to be a function of calcium binding either to the phospholipid or to the prothrombin-phospholipid complex. Therefore, calcium binding to phosphatidylserine-containing membranes was determined with the results shown in Figure 5.

The ratio of calcium ions bound per phosphatidylserine residue was estimated as follows: The maximum capacity of single bilayer vesicles of 50% phosphatidylserine with a radius of 180 Å is 1.2 g of prothrombin/g of phospholipid (Nelsestuen and Broderius, 1977). Assuming a membrane thickness of 50 Å, the outer surface of the membrane represents 64% of the total membrane surface and it is assumed that 64% of the total phosphatidylserine is present on the outer membrane surface of these vesicles. The vesicles used in Figure 5 were not chromatographed to obtain single bilayer vesicles and the maximum capacity of these membranes was determined separately to be 0.9 g of prothrombin per g of phospholipid. From this, it is estimated that 47% (from (0.9/1.2) × 64) of the phosphatidylserine is exposed in the vesicles used in Figure 5. The line drawn in the Scatchard plot (Figure 5) is the best fit straight line which gives an intercept with a value of exactly 0.50 Ca ion per exposed phosphatidylserine residue. The data agree well with this stoichiometry and give a dissociation constant of 4.4 × 10^{-4}. Phosphatidylserine does not distribute itself entirely randomly between the inner and outer membrane surface of small vesicles but prefers the inner surface. The amount of exposed phosphatidylserine estimated could be too high by about 15% according to the distribution reported by Berden et al. (1975). An intercept at 15% lower bound calcium may actually fit most of the experimental results better than the theoretical line drawn for the case of random distribution (Figure 5). This stoichiometry and binding affinity is in agreement with some (Bangham and Papahadjopoulos, 1966; Blaustein, 1967) but differs considerably from other (Barton, 1968) studies on calcium binding to phosphatidylserine. Hauser et al. (1976) have demonstrated that ionic strength has a major impact on calcium binding to phosphatidylserine monolayers. It is therefore important to measure calcium binding by the membrane under the desired conditions. The results given in Figure 5 would apply to the conditions consistently used in our studies.

The results of calcium binding to the prothrombin-membrane complex are shown in Table I. Calcium bound to prothrombin alone, to the phospholipid alone, and to the prothrombin-phospholipid complex were determined at two free calcium concentrations. The complex binds 3.2 ± 1.5 calcium ions more than the sum of the components at 0.5 mM calcium and 1.0 ± 1.5 calcium ions more at 1.2 mM calcium. These values should correspond to the value of m in eq 5 (below). Despite the large standard deviation associated with these values, the trend of the mean values which indicates a large difference in calcium bound to the complex vs. the individual components at low calcium with decreasing differences at high

TABLE I: Calcium Binding to the Complex.

	[Ca] (mM)	Bound calcium[a]
Components		
Phospholipid (50% PS)[b]	1.2	4.9 ± 0.6 μmol/60.0 mg
Prothrombin	1.2	6.0 ± 0.5 μmol/μmol
		Sum: 10.9 ± 0.8 μmol/μmol of PT + 60 mg of P·L
Complex		
Prothrombin + phospholipid	1.2	11.8 ± 1.1 μmol/μmol of PT + 60 mg of P·L
Extra calcium bound to the complex		+0.9 ± 1.4 μmol/μmol[d]
Components		
Phospholipid (25% PS)[c]	0.5	1.94 ± 0.3 μmol/72 mg of P·L
Prothrombin	0.5	3.8 ± 0.4 μmol/μmol
		Sum: 5.7 ± 0.5 μmol/μmol of PT + 72 mg of P·L
Complex		
Prothrombin + phospholipid	0.5	7.7 ± 0.8 μmol/μmol of PT + 72 mg of P·L
Extra calcium bound to the complex		+2.0 ± 0.9 μmol/μmol[e]

[a] Determined by the procedure outlined in Materials and Methods. Each value is the average of 12 determinations. [b] Capacity is 1.1 mg of prothrombin/mg of phospholipid. [c] Capacity is 0.72 mg of prothrombin/mg of phospholipid. [d] Since 81% of the protein is estimated to be phospholipid bound from the dissociation constants for these conditions (Nelsestuen and Broderius, 1977), this value is +1.1 ± 1.5 calcium ions per membrane-bound prothrombin molecule and the total calcium bound per membrane-bound prothrombin molecule is 12.9 ± 1.2. [e] Since 62% of the protein is estimated to be phospholipid bound from the dissociation constant for these conditions (Nelsestuen and Broderius, 1977) this value is +3.2 ± 1.5 calcium ions per membrane-bound prothrombin molecule and the total calcium bound per membrane-bound prothrombin molecule is 8.9 ± 1.2.

calcium is consistent with the observations in Figures 2 and 3 above. It follows that the complex binds no more calcium than the individual components at very high calcium concentrations (>10 mM).

Discussion

The results presented here and previously (Nelsestuen, 1976; Nelsestuen et al., 1976) are consistent with the following model for prothrombin- and factor X-phospholipid binding at very high calcium concentrations:

$$\left.\begin{array}{l} P + xCa \overset{K_1}{\rightleftarrows} P_{xCa} \overset{K_2}{\rightleftarrows} P'_{xCa} \\ \qquad\qquad + \\ PL + yCa \overset{K_3}{\rightleftarrows} PL_{yCa} \end{array}\right\} + \overset{K_4}{\rightleftarrows} P' \cdot PL_{x+yCa} \qquad (4)$$

In this equation, P is factor X or prothrombin, P′ represents these proteins after undergoing the calcium-dependent transitions, and PL is a phospholipid membrane containing acidic phospholipids and is expressed as the concentration of protein-binding sites. P_{xCa} and PL_{yCa} represent calcium complexes with the protein and phospholipid and $P'PL_{x+yCa}$ represents the protein·phospholipid complexes.

Reaction 1 of eq 4 consists of calcium binding to the protein which has been studied extensively. Most literature reports indicate about 10 or 11 calcium binding sites on prothrombin which are half-saturated at about 0.6 mM calcium. There are some differences, however, in the reported degree of cooperativity displayed by these sites and the location of these sites in the prothrombin molecule. Bajaj et al. (1975) and Prendergast and Mann (1977) have reported that there are 6 sites on the fragment 1 region of prothrombin and 4 sites on the fragment 2 region. They reported cooperative calcium binding for the sites on the fragment 1 region. Although not as extensive, the results of Nelsestuen et al. (1975) appear to support these conclusions. Stenflo and Ganrot (1973) reported very similar calcium binding properties to intact prothrombin but did not study the prothrombin breakdown products. Henriksen and Jackson (1975) reported that calcium binding to prothrombin and fragment 1 shows cooperativity but reported 10 calcium binding sites on the fragment 1 region. Benson and Hanahan (1975) reported that all of the calcium binding sites of prothrombin are found in the fragment 1 region of the molecule and also reported that calcium binding showed no cooperativity. Independent evidence supports the model presented by Bajaj et al. That calcium binding to prothrombin is cooperative is required by the observation that reaction 2 of eq 4 has a Hill coefficient of 2.6 when titrated with calcium (Nelsestuen, 1976). The Hill coefficient can be taken as the number of ligands which bind simultaneously (Monod et al., 1965). Results of recent experiments which determined terbium(III) binding to these proteins indicated 11 binding sites on prothrombin, 7 of which are found on fragment 1 (Brittain et al., 1976). Since terbium(III) often binds to calcium sites very tightly, this latter evidence lends considerable support to the results of Bajaj et al. (1975). The reported discrepancies in the calcium-binding properties of fragment 1 could be due to protein denaturation. We have observed that fragment 1 loses its membrane-binding ability under prolonged storage at 4 °C without loss of molecular weight (Nelsestuen, 1976). These modified molecules may have altered calcium-binding characteristics.

The total number of calcium-binding sites on factor X has been reported to be 20 which are half-saturated at about 0.6 mM calcium (Henriksen and Jackson, 1975).

Reaction 2 of eq 4 is the calcium-dependent protein transition which requires 3 or 4 calcium ions in the case where P is prothrombin and is accompanied by some further calcium binding (Nelsestuen, 1976). Prendergast and Mann (1977) have reported a calcium-dependent aggregation of fragment 1 molecules. This association is not related to the protein transition since it is strongly dependent on protein concentration (the transition is not) and displays a different calcium concentration requirement. The relationship of this association reaction to these studies is not known and would not significantly affect the light-scattering measurements. This self-association might have considerable effect on calcium-binding measurements (reaction 1).

Reaction 3 of eq 4 is calcium binding to the membrane. The

existence of this as an independent equilibrium is implied by the observations that (i) the calcium-dependent step in prothrombin–membrane complex formation involves calcium-binding sites of greater affinity than those found on prothrombin and therefore were concluded to be formed by the membrane or by the protein–membrane complex (Nelsestuen et al., 1976) and (ii) at very high calcium concentrations these sites can be saturated such that the equilibrium constant describing protein–phospholipid complex formation (K_4) contains no calcium term (from Figure 3B). Recent studies by Prendergast and Mann (1977) demonstrate that magnesium ions (included to catalyze the protein transition (reaction 2)) result in appearance of cooperative calcium-binding sites on prothrombin fragment 1 of higher affinity than those observed without magnesium. This could affect the validity of the first argument above since it is based on observations made in the presence of manganous ions (e.g., Figure 2). Other lines of evidence, however, also indicate that reaction 3 is a necessary component. Nelsestuen and Broderius (1977) observe that the calcium dependence of protein–membrane binding in the presence of manganese (as shown in Figure 2) is dependent on membrane composition. Therefore, membrane–calcium interaction is an important aspect of these results and we feel that eq 4 represents the correct equilibria.

Finally, reaction 4 in eq 4 represents the formation of the protein–phospholipid complexes.

At calcium concentrations below 5 mM, the following set of equilibria appear to adequately describe prothrombin and factor X–phospholipid binding:

$$\begin{aligned} P + i\mathrm{Ca} &\overset{K_1}{\rightleftarrows} P_{i\mathrm{Ca}} \overset{K_2}{\rightleftarrows} P'_{i\mathrm{Ca}} \\ &\quad + m\mathrm{Ca} \overset{K_4}{\rightleftarrows} P' \cdot PL_{i+j+m\mathrm{Ca}} \\ PL + j\mathrm{Ca} &\overset{K_3}{\rightleftarrows} PL_{j\mathrm{Ca}} \end{aligned} \tag{5}$$

The symbols in eq 5 are the same as in eq 4 but the number values for the calcium ions (i,j,m) are interdependent and vary with calcium concentration. Expressing the equilibria in this manner rather than as in eq 4 serves to emphasize that the protein–membrane interaction (reaction 4 of eq 5) is calcium dependent. By direct measurement (Table I) m is 3.2 ± 1.5 at 0.5 mM calcium and 1.1 ± 1.5 at 1.2 mM calcium. At calcium concentrations where protein is entirely in the $P'_{i\mathrm{Ca}}$ state the intercept on the x axis of the double-reciprocal plots (cf. Figure 3) will be equal to $-[\mathrm{Ca}]^m/K_4$ for reaction 4 of eq 5. The dissociation constant obtained in this way at a given calcium concentration will be assigned the value, K_4^{Ca}, and, according to this model, would consist of:

$$K_4^{\mathrm{Ca}} = \frac{K_4}{[\mathrm{Ca}]^m} = \frac{[P'_{i\mathrm{Ca}}][PL_{j\mathrm{Ca}}]}{[P' \cdot PL_{i+j+m\mathrm{Ca}}]} \tag{6}$$

At calcium concentrations where reaction 2 is not saturated (below 1 and 2 mM calcium for prothrombin and factor X, respectively), the free protein will consist of both $P_{i\mathrm{Ca}}$ and $P'_{i\mathrm{Ca}}$ and a further term is identified:

$$K_4^{\mathrm{Ca}^*} = \frac{[P'_{i\mathrm{Ca}} + P_{i\mathrm{Ca}}][PL_{j\mathrm{Ca}}]}{[P' \cdot PL_{i+j+m\mathrm{Ca}}]} \tag{7}$$

The values of K_4^{Ca} can be obtained by multiplying $K_4^{\mathrm{Ca}^*}$ by the value, $[P'_{i\mathrm{Ca}}]/[P'_{i\mathrm{Ca}}] + [P_{i\mathrm{Ca}}]$. This can be determined at each calcium concentration from data presented in previous studies on the protein transition (reaction 2 of eq 5) (Nelsestuen, 1976; Nelsestuen et al., 1976). For purposes of determining the amount of membrane-bound protein at a given calcium concentration, the most useful value is $K_4^{\mathrm{Ca}^*}$. If this value is used, the free protein term is the total free protein, a value which is more readily obtained from the data (see Figures 1 and 2) and which is more easily applied. The data indicate that eq 7 can be applied at all calcium concentrations above the cooperative binding reaction (>0.3 mM calcium for prothrombin in Figure 2).

Prothrombin has been reported to undergo partial dimerization (Cox and Hanahan, 1970). Recent studies on human prothrombin indicate that this is a partial end-to-end dimerization (Agarwal et al., 1977) with interaction between the prethrombin 2 regions. The extent of this dimerization at the low concentrations of protein used in these studies is not known but should be less than 50% (Agarwal et al., 1977). This could have some effect on the observed values of $K_4^{\mathrm{Ca}^*}$ if the dimerized molecules have altered affinity for the phospholipid. The evidence indicates that this type of protein–protein interaction is not occurring on the vesicle surface as evidenced by no secondary binding to the membrane as the protein concentration is increased (see Figure 3) and also by the changes in radius of the membrane vesicles as prothrombin binds to the surface (Lim et al., 1977). The physiological significance of this partial dimerization is not known and the concentrations where it has been examined are tenfold or more above plasma prothrombin concentrations.

By including manganous ion in the reaction, the calcium dependence of reaction 2 is eliminated and the calcium requirements of reactions 3 and 4 of eq 5 are observed. Under these conditions, the calcium titration curve given in Figure 2 is obtained. A possible relationship to eq 5 is as follows: At low calcium concentrations (<0.3 mM) the binding reaction is due to both reaction 4 and a minimum reaction 3. A minimum reaction 3 is proposed to be the formation of a "binding site nucleus" which the protein can recognize. This initial complex produces high affinity calcium binding sites (m) which are filled and the protein–membrane complex is then completed. This would account for the cooperative nature of the calcium titration curve for prothrombin- and factor X–membrane binding seen in Figure 2 and reported previously (Nelsestuen, 1976; Nelsestuen et al., 1976). At intermediate calcium concentrations (from 0.3 to 5 mM), this model predicts that all of the binding sites are nucleated and the maximum number of binding sites remains constant. In this calcium range, the values of j and i increase to x and y (eq 4) and the value of m decreases from that needed to complete the protein–membrane complex to zero, whereupon eq 5 becomes eq 4. This trend in the value of m is seen in the direct calcium binding measurements given in Table I. At 0.5 mM calcium the value of m is 3.2 ± 1.5 and at 1.2 mM calcium m is 1.1 ± 1.5. Based on the titration curve for factor X–membrane interaction which requires about 0.2 mM calcium for half-reaction (Nelsestuen et al., 1976), the model presented would predict that factor X requires a greater number of membrane-bound calcium ions for nucleation of its binding site. Due to the data of Prendergast and Mann (1977) showing higher calcium affinity in the presence of magnesium, we cannot eliminate a variation of this model in which the cooperative titration results of Figure 2 are a function of calcium binding to both the membrane and the protein.

The total number of calcium ions required to form the complete prothrombin–membrane complex is 8.9 ± 1.2 or less from the data given in Table I at 0.5 mM calcium. The minimum number of calcium ions cannot be determined from these data since we do not know if unessential calcium ions are included in this value. Based on arguments using the Hill coefficients for the protein transition and phospholipid binding (Nelsestuen et al., 1976), the minimum number of essential

calcium ions for protein-membrane binding is 6. The total calcium bound per mole of membrane-bound prothrombin increases at higher calcium concentrations (13 ± 1.2 at 1.2 mM Ca^{2+}, Table I). This increase probably represents nonfunctional calcium ions but the possibility that the number of functional calcium ions is variable and exceeds 8.9 ± 1.2 at higher calcium concentrations cannot be ruled out. This increase would have to occur without change in the number of acidic phospholipid molecules involved in the interaction.

It has been shown previously that, while manganous ion will catalyze the protein transition (reaction 2 of eq 5), it is not capable of forming the protein-membrane complex (Nelsestuen et al., 1976). Manganous ion does bind to phospholipid (Barton, 1968), however, and we have reported that high manganous ion concentrations actually inhibit prothrombin-membrane interaction (Nelsestuen et al., 1976). In this latter case, the manganous ion may be preventing calcium from binding to the membrane thereby inhibiting protein-membrane complex formation. This may also explain the observation that high manganous ion concentrations inhibit thrombin generation by factor Xa·Ca·phospholipid complexes (Bajaj et al., 1976). The cation specificity studies for prothrombin-membrane interaction reported previously (Nelsestuen et al., 1976) may represent a study of the ability of these cations to form a "nucleation site" for protein binding. The cation specificity and the known coordination properties of these cations should assist in eventual reconstruction of the protein-membrane interaction site.

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