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Evaluation of Membrane Phase Behavior as a Tool To Detect Extrinsic Protein-Induced Domain Formation: Binding of Prothrombin to Phosphatidylserine/Phosphatidylcholine Vesicles†

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ABSTRACT: The temperature–composition phase diagram of mixed dimyristoylphosphatidylserine (DMPS) and dimyristoylphosphatidylcholine (DMPC) small unilammelar vesicles was determined in the presence and absence of bound bovine prothrombin by monitoring the phospholipid order–disorder phase separation using diphenylhexatriene (DPH) fluorescence anisotropy. The shape of the membrane temperature–composition diagram was essentially unaltered by the binding of prothrombin in the presence of Ca^{2+} although the two-phase (gel/fluid) region was slightly narrowed and shifted by 1–10 °C to higher temperatures. This result does not support the popular idea that extensive domains rich in negatively charged phospholipid are induced in response to prothrombin binding. Instead of implying domain formation, our results demonstrate that the observed increase in melting temperature associated with binding of prothrombin to acidic phospholipid membranes can be accounted for by the observed altered membrane order both in the fluid and in the solid lamellar phases. The membrane order in the liquid-crystalline phase increased with increased acidic lipid content, and much more so for DMPS than for dipentadecanoylphosphatidylglycerol ($DC_{15}PG$). These results demonstrate that simple shifts in membrane phase behavior cannot be properly interpreted to prove the existence of charged lipid domains. In addition, we report the unexpected observation that prothrombin increased the anisotropy of DPH in DMPS/DMPC vesicles in the liquid-crystalline phase in the absence of Ca^{2+} as well as in its presence. This effect was seen to a lesser extent and only at a much higher charged-lipid content for $DC_{15}PG$/DMPC vesicles. Prothrombin fragment 1 with or without Ca^{2+} did not alter the packing in $DC_{15}PG$/DMPC or DMPS/DMPC membranes in this manner. These observations are discussed in terms of the possibility that phosphatidylserine interacts in a specific and Ca^{2+}-independent manner with at least one site on prothrombin.

Crucial proteolytic steps in the blood coagulation cascade are catalyzed by multiprotein complexes that assemble on platelet-derived membranes (Nelsestuen, 1978; Mann, 1987). The protein components of these proteolytic complexes are thought to bind to platelet membranes via electrostatic interactions with negatively charged phospholipids. One such protein complex, the prothrombinase complex, catalyzes the proteolytic conversion of prothrombin to thrombin. The enzyme, factor X_a, associates with its cofactor, factor V_a, on the surface of a platelet or phospholipid vesicle to accelerate conversion.

Factor X_a, and prothrombin are vitamin K dependent proteins containing doubly negatively charged γ-carboxyglutamic acid (GLA)[1] residues. Binding of these proteins to membranes containing negatively charged phospholipids requires Ca^{2+} and has been proposed to occur via "calcium bridging" of GLA residues to negatively charged phospholipids in the membrane (Lim et al., 1977; Dombrose et al., 1979; Wei et al., 1982), although there is no direct evidence in support of this bridging hypothesis. Irrespective of whether "bridging" is involved, the binding site for these proteins is currently envisioned by some as a local pool or "domain" of negatively charged phospholipid that condenses under the surface-bound protein (Lim et al., 1977; Dombrose et al., 1979; Mayer & Nelsestuen, 1981). This model for the formation of domains of negatively charged phospholipids has also been suggested for the binding of other extrinsic membrane proteins (Birrell & Griffith, 1976; Hartmann et al., 1977; Wiener et al., 1985).

We have questioned the concept of negative lipid domain formation in response to binding of extrinsic membrane proteins, in particular the binding of GLA-containing proteins

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[1] Abbreviations: GLA, γ-carboxyglutamic acid; DMPS, 1,2-dimyristoyl-3-*sn*-phosphatidylserine; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; POPA, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidic acid; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; SUV, small unilamellar vesicle(s); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, (diethylaminoethyl)cellulose; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; K_d, equilibrium dissociation constant; T_m, melting temperature.

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such as prothrombin and factor X. On the basis of the shape of membrane phase diagrams, we have failed to find any evidence to support the formation of extensive compositionally distinct domains in dipentadecanoylphosphatidylglycerol ($DC_{15}PG$)[1]/dimyristoylphosphatidylcholine (DMPC)[1] membranes saturated with the GLA-containing fragment 1 region of bovine prothrombin (Lentz et al., 1985). Additionally, studies with pyrene-labeled phosphatidylglycerol and phosphatidylcholine have shown that if domains form in response to prothrombin fragment 1 binding then they must contain less than 15–20 lipid molecules (Jones & Lentz, 1986). This paper extends these conclusions to the physiologically relevant acidic lipid, phosphatidylserine (PS),[1] and to the whole prothrombin molecule. Most significantly, our results demonstrate that shifts in mixed-membrane phase behavior cannot be interpreted in terms of a domain model without the additional information provided by a phase diagram.

A crucial feature of the assembly of the prothrombinase complex is its apparent preference for certain negatively charged phospholipids. We have shown that PS- and phosphatidic acid (PA)[1]-containing vesicles are many times more effective in supporting thrombin production than are phosphatidylglycerol (PG)[1]- or phosphatidylinositol-containing vesicles (Jones et al., 1985). In the current paper, we show that bovine prothrombin alters the membrane order and phase behavior of PS-containing membranes more dramatically than it does PG-containing membranes. The fact that these effects were independent of Ca^{2+} suggests the possibility of a Ca^{2+}-independent, PS-specific, membrane–prothrombin interaction.

Experimental Procedures

Materials. DMPC and the disodium salts of dimyristoylphosphatidylserine (DMPS)[1], $DC_{15}PG$, bovine PS, and palmitoyloleoylphosphatidic acid (POPA)[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), and shown to be greater than 98% pure by thin-layer chromatography (Lentz et al., 1982b). DMPC was stocked in argon-bubbled chloroform while DMPS, $DC_{15}PG$, and POPA were stored as their sodium salts in argon-bubbled chloroform. Solvents were low-residue, HPLC grade. Concentrations of the stock solutions were established by inorganic phosphate determination (Chen et al., 1956). Calcium ion contamination of the acidic lipids was shown to be less than 2 mol % (Lentz et al., 1982b). [^{14}C]DMPC was purchased from Amersham (Arlington Heights, IL). 1,6-Diphenyl-1,3,5-hexatriene (DPH)[1] was purchased from Molecular Probes (Junction City, OR). All other chemicals were reagent grade or better.

Phospholipid Vesicles. Phospholipid mixtures for the preparation of small unilamellar vesicles (SUV)[1] were made from mixtures of lipid stock solutions. To the stock of one component (usually DMPC) was added [^{14}C]DMPC at less than 0.1 mol %. The specific activity of [^{14}C]DMPC in this stock was established by scintillation counting and phosphate analysis. The composition of vesicles prepared from mixtures of two lipids was then obtained by scintillation counting to obtain the mole fraction of one component and phosphate analysis to obtain the total phospholipid concentration. To prepare SUV, the lipid was lyophilized from a frozen benzene solution overnight after evaporation of the chloroform. The resulting dry white powder was then dispersed in prewarmed buffer (100 mM NaCl, 10 mM TES,[1] and 0.02% NaN_3, with or without 0.1 mM Na_2EDTA,[1] depending on the experiment). The mixture was vortexed thoroughly and sonicated at a temperature above the lipid liquid-crystalline phase transition in a Heat Systems W350 sonicator equipped with a Heat Systems cup horn as described by Lentz et al. (1982b). Large vesicles were removed by ultracentrifugation (Beckman TL-100, 25 min, 72000 rpm) (Lentz et al., 1980). Vesicles were maintained above their phase transition temperature throughout the ultracentrifugation and at all times prior to use in an experiment.

Prothrombin Purification and Handling. Bovine prothrombin was isolated by barium citrate precipitation from freshly collected bovine plasma (Mann, 1976). The barium citrate precipitate (a gift from the laboratory of Dr. Richard Hiskey, Department of Chemistry, UNC) was dissolved in 120 mL of 200 mM Na_2EDTA and 120 mL of 20 mM sodium citrate and 0.9% NaCl per liter of starting plasma. Contaminating protein was removed by additions of ammonium sulfate to 10% and then to 35%. After each ammonium sulfate addition, the solution was centrifuged, and the supernatant was retained for the next ammonium sulfate cut. Ammonium sulfate was added to the supernatant from the 35% cut to bring the ammonium sulfate concentration to 65%. Precipitate obtained following centrifugation was dissolved in 100 mL of 25 mM sodium citrate at pH 6.0 and then dialyzed into this citrate buffer to remove ammonium sulfate and to prepare the protein for purification by ion-exchange chromatography on Whatman DEAE-cellulose[1] (Mann, 1976). Prothrombin obtained from the ion-exchange column was purified further on a Bio-Gel polyacrylamide gel filtration column eluted with buffer containing 20 mM Tris,[1] 300 mM NaCl, 0.02% NaN_3, and 1 mM benzamidine hydrochloride. Prothrombin was stored at −70 °C in the presence of protease inhibitors [1 mM benzamidine hydrochloride and either 1 mM diisopropyl fluorophosphate (DFP) or 1 mM phenylmethanesulfonyl fluoride (PMSF)] and high-salt buffer (20 mM Tris, 300 mM NaCl, and 0.02% NaN_3). When prothrombin obtained in this manner was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), staining by Kodavue (Eastman Kodak, Rochester, NY) revealed only one major band and no more than two very minor bands. A final purification step was performed 1 day prior to an experiment by HPLC on a Perkin-Elmer Isopure LC system using a MonoQ HR 5/5 ion-exchange column (Pharmacia, Norwalk, CT). This step removed small quantities (<10%) of proteolysis products that occasionally formed during storage.

Prothrombin fragment 1 was obtained from Dr. Richard Hiskey's laboratory (Department of Chemistry, UNC–Chapel Hill) after its formation by cleavage of prothrombin with Ecarin (prothrombin activator isolated from *Echis carinatus* snake venom) purchased from Sigma (Morita et al., 1976; Deerfield et al., 1986).

Protein concentrations were determined by absorbance measurements using extinction coefficients at 280 nm of 1.44 mL mg^{-1} cm^{-1} for prothrombin and 1.05 mL mg^{-1} cm^{-1} for fragment 1 (Mann, 1976) and light-scattering corrections from the absorbance measured at 320 nm (Donovan, 1969). Prothrombin concentrations were also determined by using the synthetic chromogenic substrate S2238 (Helena Laboratories, Beaumont, TX) to assay thrombin formation from activated prothrombin (Rosing et al., 1980).

Prior to use in an experiment, both prothrombin and its fragment 1 portion were concentrated by using a stirred ultrafiltration cell (Amicon, Danvers, MA) or an RCF-Confilt centrifugal concentrator (Bio-Molecular Dynamics, Beaverton, OR) and preincubated at 40 °C for 15 min in buffer containing 0.1 mM Na_2EDTA or 5 mM $CaCl_2$ to allow the protein to assume its ion-dependent binding conformation (Nelsestuen, 1976).

Fluorescence Anisotropy Measurements. A small volume (~1 μL) of a stock solution of DPH (0.3 mM) in acetone was added to SUV suspensions to achieve a final dye:lipid ratio of 1:300. The vesicles were then vortexed thoroughly and incubated for 1 h before use to achieve maximal partitioning of DPH into the bilayer. DPH-containing vesicles and buffer (100 mM NaCl, 10 mM TES, 0.02% NaN_3, and either 0.1 mM Na_2EDTA or 5 mM Ca^{2+}) or vesicles, buffer, and protein were added to a stirred microcuvette (Helma Cells, Jamaica, NY) to a final volume of 1.4 mL and final concentrations of 0.075 mM phospholipid and 34 μM protein. This phospholipid concentration was required by the experimental conditions to obtain sufficient fluorescence intensity.

The mixture in the cuvette was allowed to equilibrate at 48 °C for 10–20 min before beginning the fluorescence measurements during a cooling scan. The cuvette temperature was maintained with a programmable, external water bath (RTE-8 bath equipped with an ETP-3 programer, Neslab Instruments, Portsmouth NH) and continuously monitored with a Model 5810 Digitec digital thermometer using a Model 702A Yellow Springs Instrument probe (Yellow Springs, OH) inserted directly into the cuvette.

Measurements of DPH fluorescence anisotropy were made with an SLM 48000 spectrofluorometer (SLM Aminco, Urbana, IL). Anisotropy measurements were taken every 0.2 °C at scan rates of 30 °C/h. Cooling scans were performed prior to heating scans. Details of the measurements and of the calculation of microviscosity and microviscosity activation energy are given in the literature (Lentz et al., 1976, 1978). These parameters, as previously validated by comparison with calorimetric data (Lentz et al., 1976, 1978, 1982a), were used for detecting phospholipid phase transitions and thereby used to construct temperature–composition diagrams showing the phospholipid phase transition as a function of the acidic lipid content of vesicles. For the approach and arguments used here to be valid, sufficient prothrombin or prothrombin fragment 1 had to be present in a vesicle suspension to saturate surface binding sites to better than 95% at every composition. The large ratio of protein concentration to lipid concentration used in these experiments was chosen on the basis of previously determined protein binding constants (Nelsestuen & Broderius, 1977; Cutsforth et al., 1989) in order to achieve this level of binding site saturation.

Light Scattering. Light-scattering measurements (Nelsestuen & Lim, 1977) were performed at a 90° angle on an SLM 48000 spectrofluorometer to verify the binding of prothrombin or prothrombin fragment 1 to DMPS/DMPC vesicles or to try to detect prothrombin-induced DMPC SUV fusion. Experiments were carried out with both emission and excitation monochromators at 313 nm and with slits at 1 and 4 nm, respectively. Protein was preincubated with 5 mM Ca^{2+} to achieve its calcium-induced binding conformation and then added sequentially with stirring to a 1-mL cuvette containing 0.05–0.1 mM SUV and assay buffer (100 mM NaCl, 10 mM TES, and 5 mM Ca^{2+} filtered through a 0.22-μm filter). Scattering intensities were analyzed essentially by the method of Nelsestuen and Lim (1977). Following completion of a binding isotherm, Na_2EDTA was added to a final concentration of 50 mM to determine reversibility. Reversible increases in scattering intensity were interpreted to indicate binding in the absence of fusion.

Results

Effects of Prothrombin, Ca^{2+}, and Prothrombin and Ca^{2+} on the Phase Behavior of DMPS/DMPC SUV. The phase behavior of mixed DMPS/DMPC SUV in the presence or absence of Ca^{2+} and/or prothrombin was determined by monitoring temperature-dependent changes in the anisotropy of DPH to detect lipid phase transitions. Representative plots of the temperature dependence of the DPH-derived microviscosity activation energy are shown in Figure 1 for cooling scans of (A) 33–42 mol % and (B) 64–71 mol % DMPS vesicles with EDTA alone (top plots), with protein plus EDTA (second from top), with protein plus Ca^{2+} (second from bottom), and with Ca^{2+} alone (bottom plots).

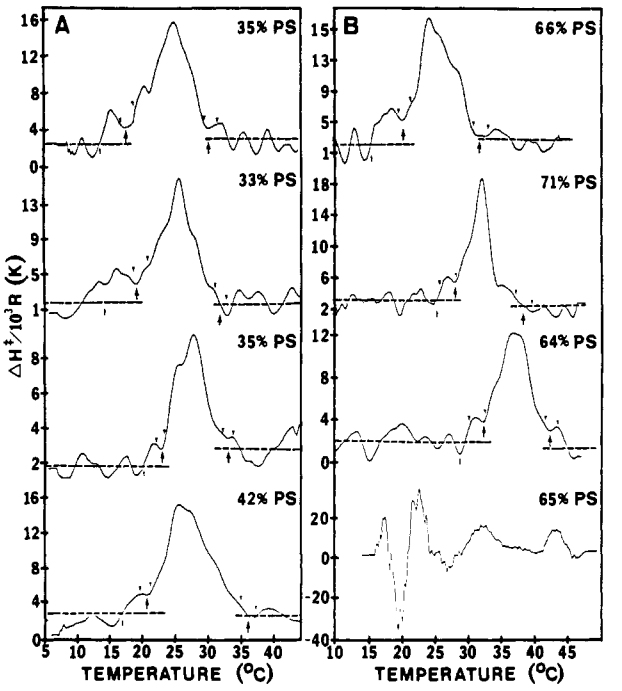

FIGURE 1: Temperature dependence of the DPH-derived microviscosity activation energy (ΔH^*) obtained from representative cooling scans of small unilamellar vesicles composed of mixtures of DMPS and DMPC. Panel A shows data for vesicles containing 33–42 mol % DMPS, and panel B shows results for vesicles containing 64–71 mol % DMPS. For both panels, the plots from top to bottom are for lipid alone, for vesicles in the presence of bovine prothrombin, for vesicles in the presence of prothrombin and Ca^{2+}, and for vesicles in the presence of Ca^{2+} alone. The molar ratio of protein to lipid was equivalent for each lipid composition and was chosen to ensure ≥95% surface saturation of charged membranes with bound prothrombin. Heavy arrows define the upper and lower limits of the main transition, and arrowheads mark the errors in the assignment of these limits. Small arrows mark what we interpret to be the lower limit of the pretransition. The data for 65 mol % PS vesicles in the presence of Ca^{2+} but in the absence of prothrombin are not marked with arrows, as it was not possible to delimit a clear phase transition in this sample. The peaks shown for this sample below 25 °C are due to noise which resulted from the extensive aggregation and possibly fusion of these vesicles in the presence of Ca^{2+}.

As previously reported (Lentz et al., 1976, 1981, 1987), the order/disorder phase transition in SUV is broad and complex due to packing inhomogeneities caused by the high curvature of these vesicles. In addition, SUV display a gel-to-gel phase transition (the "pretransition"; Lentz et al., 1978) that is very difficult to detect with DPH fluorescence anisotropy (Lentz et al., 1987). In Figure 1, we have indicated by heavy arrows the temperatures that define the upper and lower limits of the main phase separation in DMPS/DMPC SUV. Arrowheads indicate our estimates of the errors in these assignments, while small arrows mark the lower limit of what we interpret to be the pretransition. The focus of the current work, however, is not the pretransition but rather the main transition and, in particular, the liquidus line of the membrane phase diagram. It is this portion of the phase diagram whose shape should contain information about the possible induction by pro-

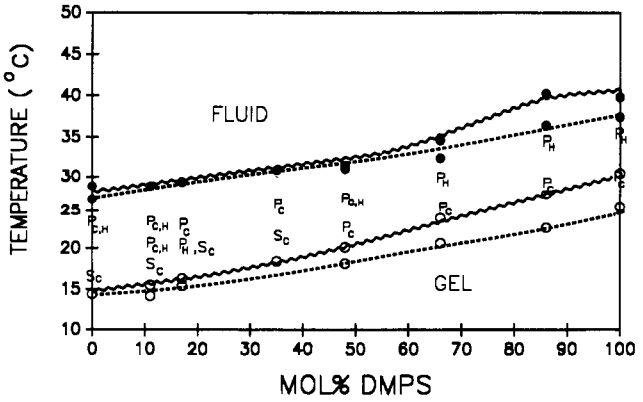

FIGURE 2: Temperature–composition diagram for DMPS/DMPC small unilamellar vesicles. Closed circles indicate the upper temperature boundaries of the phase transition, and the open circles mark the lower boundaries for cooling scans (dashed lines) and for heating scans (wavy lines). The peak temperatures are indicated by P and temperatures of shoulders by S. The subscripts "C", "H", or "C,H" indicate that the feature was observed in a cooling scan, a heating scan, or both cooling and heating scans, respectively.

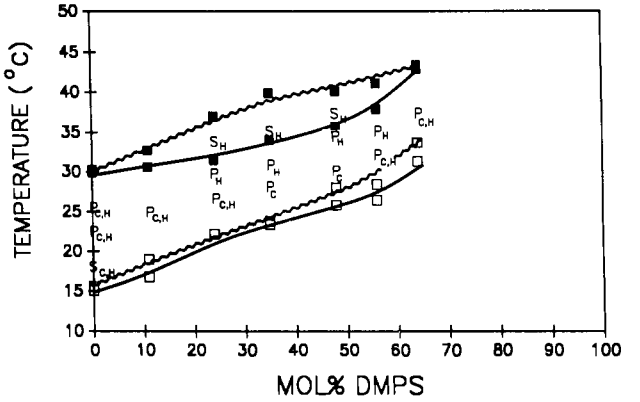

FIGURE 3: Temperature–composition diagram for DMPS/DMPC small unilamellar vesicles in the presence of prothrombin and 5 mM Ca^{2+}. The lower and upper boundary temperatures of the main transition are plotted as open squares and closed squares, respectively, for both cooling (solid lines) and heating (wavy lines) scans. The peak and shoulder temperatures are labeled as in Figure 2.

thrombin of separated DMPS-rich fluid domains.

It is clear from Figure 1 that the phase behavior of DMPS/DMPC SUV was altered by all the components added in our experiments. The effects of Ca^{2+} on the phase behavior and structure of PS or PS/PC[1] vesicles have been widely studied [e.g., see Hauser and Shipley (1984), Silvius and Gagné (1984), and Feigenson (1989)] and will not be reported here in detail. Calcium has long been known to induce in PS-rich membranes formation of a highly ordered PS–Ca^{2+}–PS, dehydrated, interbilayer complex with an extremely high order-to-disorder phase transition (Portis et al., 1979; Hauser & Shipley, 1984; Feigenson, 1989). Our experiments with DMPS/DMPC SUV plus Ca^{2+} were consistent with these earlier reports. For example, a cooling scan with 40 mol % DMPS/DMPC SUV (Figure 1, bottom) showed, as expected, a slight increase in the phase transition temperature [e.g., see Silvius and Gagné (1984)]. Aggregation and precipitation of the vesicles occurred below the transition, probably to produce the highly ordered PS–Ca^{2+}–PS gel phase, so a subsequent heating scan did not reproduce the cooling scan. At higher DMPS content (e.g., 65 mol % in Figure 1), extensive aggregation, and possibly fusion (Düzgünes et al., 1981), occurred even above the transition, and the phase behavior was extremely complex, reflective of formation of dehydrated gel phases (Silvius & Gagné, 1984).

The phase behavior of DMPS/DMPC SUV is summarized in Figure 2 in terms of a temperature–composition diagram. In this figure, the upper (closed circles) and lower (open circles) delimiting temperatures of the main phase transition as well as the temperatures corresponding to peaks (P_C, $P_{C,H}$, P_H) and shoulders (S) are plotted as a function of vesicle composition for both cooling and heating scans. The temperature values of peaks were obtained from plots of microviscosity activation energy versus temperature such as shown in Figure 1. The delimiting temperatures of the transition were determined both from microviscosity activation energy plots and, when noise in these plots caused ambiguity, from Arrhenius plots of microviscosity. Deviations of such Arrhenius plots from linearity have been established to be indicative of a membrane phase change (Suurkuusk et al., 1976; Lentz et al., 1976). Uncertainty in these determinations was generally less than or on the order of ±1 °C. Cooling scans (dashed lines) and heating scans (wavy lines) were essentially identical for low DMPS content. DMPS/DMPC SUV with high DMPS content, however, showed a slighly higher liquidus line during heating scans than in the prior cooling scans. This irreversibility was probably due to instability of SUV due to their size, and the upward shift in the phase transition may indicate size growth of vesicles. This type of irreversibility was also seen with $DC_{15}PG$/DMPC SUV above 50 mol % $DC_{15}PG$, and electron microscopy confirmed the occurrence of size growth (Lentz et al., 1982a).

Delimiting phase separation temperatures from both cooling (solid lines) and heating scans (wavy lines) are summarized in a temperature–composition diagram in Figure 3 for DMPS/DMPC SUV samples containing prothrombin and Ca^{2+}. As in Figure 2, the closed and open symbols indicate the upper and lower delimiting temperatures, respectively. The presence of prothrombin and Ca^{2+} shifted the phase transition of DMPS/DMPC SUV to increasingly higher temperatures the greater the DMPS content of the membrane (compare Figures 2 and 3). A high-temperature shoulder appeared in the heating scans at intermediate DMPS content and resulted in higher temperatures at the liquidus line for heated samples than for cooled samples. This shoulder may be due to the formation of calcium-mediated intermembrane interactions. Due to the strength of the interaction of PS with Ca^{2+}, there may be incomplete blockage of this interaction by prothrombin. We stress that this irreversibility was slight and that no aggregation or precipitation of vesicles, as seen in the presence of Ca^{2+} alone, was observed at any DMPS content in the presence of saturating concentrations of prothrombin. In contrast to these results with PS/PC vesicles, cooling and heating scans were reversible for PG/PC SUV in the presence of prothrombin fragment 1 and Ca^{2+} (Lentz et al., 1985). This would be consistent with a weaker interaction of Ca^{2+} with PG/PC membranes than with PS/PC membranes which is suggested by the smaller upward shift in melting temperature caused by calcium in the case of PG than in the case of PS (van Dijck et al., 1978). Our temperature–composition diagram is not extended beyond 66 mol % DMPS because, at higher DMPS content, the delimiting temperatures of the transition could no longer be determined from anisotropy measurements for vesicles incubated with prothrombin and Ca^{2+} (see Effect of Prothrombin on PS/PC Membrane Order).

DMPS/DMPC SUV in the presence of prothrombin plus EDTA exhibited reversible phase behavior, so only data from cooling scans are summarized in the temperature–composition diagram shown in Figure 4. Also summarized in Figure 4 are the delimiting temperatures (asterisks) for transitions observed in cooling scans performed on DMPS/DMPC SUV

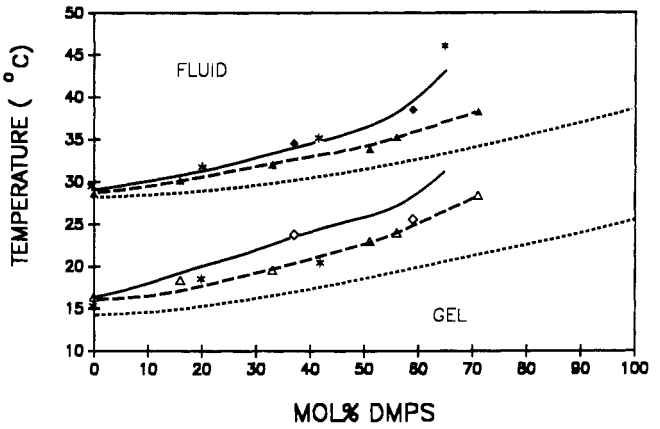

FIGURE 4: Summary of the effect of added components on DMPS/DMPC phase transitions. The upper and lower limiting temperatures of the order/disorder phase transition from cooling scans of mixed DMPS/DMPC SUV are plotted as a function of membrane DMPS content. Closed symbols indicate the upper temperature boundaries of the phase transitions, and open symbols mark the lower boundaries. Results are recorded for measurements made on vesicles alone (from Figure 2; short dashed lines), in the presence of Ca^{2+} (asterisks), in the presence of prothrombin alone (triangles; long dashed lines), and in the presence of prothrombin and Ca^{2+} (from Figure 3; solid lines). The solidus point for 65 mol % DMPS/DMPC SUV in the presence of Ca^{2+} is not shown because of the complex gel phase behavior seen in this sample (Figure 1). The delimiting temperatures of transitions observed for SUV containing 35 and 58 mol % DMPS in the presence of prothrombin fragment 1 plus Ca^{2+} are also shown (diamonds).

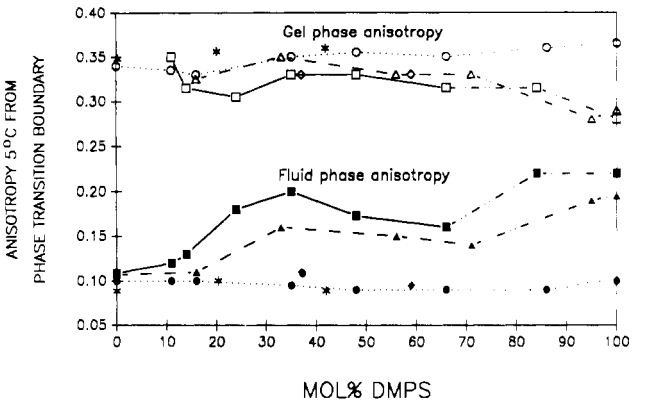

FIGURE 5: Fluorescence anisotropies of DPH incorporated in DMPS/DMPC SUV incubated 5 °C above or below the phase transition are plotted in the lower and upper half of the figure with closed and open symbols, respectively. Anisotropy measurements were made on vesicles alone (circles), in the presence of Ca^{2+} (asterisks), in the presence of prothrombin (triangles), in the presence of prothrombin and Ca^{2+} (squares), and in the presence of prothrombin fragment 1 and Ca^{2+} (diamonds). For vesicles rich in PS that had poorly defined phase transitions upon addition of prothrombin (indicated by mixed dashed and dotted lines), the anisotropy is reported for 5 °C above or below the transition of the most PS-rich vesicle with a measurable transition.

in the presence of Ca^{2+} as well as in the presence of prothrombin fragment 1 plus Ca^{2+} (diamonds at 35 and 58 mol % DMPS), and, for comparison, the liquidus and solidus phase lines drawn through the data presented in Figures 2 and 3.

The phase behavior of DMPS/DMPC SUV in the presence of prothrombin and Ca^{2+} was very different from the phase behavior in the presence of Ca^{2+} alone at 65 mol % PS (Figure 1). The difference was mainly due to the complex gel phase behavior in the presence of Ca^{2+} alone. The presence of prothrombin prevented the aggregation and precipitation of membranes seen in the presence of Ca^{2+} alone. Apparently, prothrombin binding blocked the formation of the PS–Ca^{2+}–PS complex, and the phase behavior of DMPS/DMPC SUV in the presence of prothrombin and Ca^{2+} reflects the binding of the Ca^{2+} form of the protein to the DMPS/DMPC bilayers rather than the formation of the PS–Ca^{2+}–PS complex. At 20 and 42 mol % PS, the transition was broader with a lower peak temperature (both ~3 °C lower) and a lower temperature gel line (~1.5 and 3 °C, respectively) in vesicles plus Ca^{2+} (Figure 1 and Figure 4, asterisks) than in vesicles in the presence of Ca^{2+} and prothrombin. However, the *fluidus* phase line was similar in the presence of Ca^{2+} and prothrombin and Ca^{2+}. Apparently, the structural response of the *fluid* phase of DMPS/DMPC membranes to the binding of prothrombin plus Ca^{2+} was similar to the response to Ca^{2+} alone for membranes composed of ≤42 mol % PS.

The phase transition shift induced by prothrombin was not absolutely dependent on Ca^{2+}. Prothrombin plus EDTA also caused the transition to be shifted to higher temperatures but to a lesser extent than in the presence of Ca^{2+} (Figure 4). As in the presence of Ca^{2+}, the transition was also ill-defined at very high DMPS content in the absence of Ca^{2+}. The total calcium concentration in the experimental samples containing lipid, protein, and EDTA was determined by atomic absorption. The concentration of calcium not chelated to the EDTA was calculated to be less than 7 μM. We used the prothrombin–Ca^{2+} binding constants reported by Deerfield et al. (1987) to calculate the occupancy of Ca^{2+} binding sites on prothrombin at this Ca^{2+} concentration. At a Ca^{2+} concentration of 7 μM, the [metal bound]:[protein total] ratio was less than 0.04, which is well below that (0.5) required for prothrombin to assume the Ca^{2+}-dependent conformation. As a control, 7 μM Ca^{2+} was added to vesicles, and the transition temperature was measured and found to be identical with the transition of vesicles incubated only with EDTA (data not shown). Therefore, the shift in lipid phase behavior seen with prothrombin and EDTA was not due to a trace amount of Ca^{2+}.

Effect of Prothrombin on PS/PC Membrane Order. In order to obtain further insight into the nature of the interaction between prothrombin and acidic lipid membranes, we examined the effect of prothrombin on membrane order in both the fluid and solid lamellar states. Under most conditions, the steady-state fluorescence anisotropy of DPH provides a reasonable estimate of membrane order (Jänig, 1979), assuming that the probe is aligned mainly with the lipid acyl chains (Lentz, 1988).

The DPH anisotropy is recorded in Figure 5 at temperatures 5 °C above (lower curves) and 5 °C below (upper curves) the main phase transition of DMPS/DMPC vesicles in the presence and absence of Ca^{2+} or in the presence and absence of prothrombin with Ca^{2+} or EDTA. At high DMPS content, where the phase transition was undetectable in the presence of protein (see Figure 3), the anisotropy was recorded and plotted 5 °C above or below the transition boundaries of the last measurable transition. We have previously noted that, for mixtures of similar phospholipids, the anisotropy of DPH at a fixed, reduced temperature relative to the bilayer phase transition is independent of bilayer composition (Lentz et al., 1976, 1981, 1982a). Consistent with these earlier reports, 5 °C above or below the phase boundaries, the anisotropy of DPH incorporated into DMPS/DMPC SUV in the presence or in the absence of Ca^{2+} remained constant in the absence of protein (Figure 5, asterisks and circles).

In contrast to the behavior of other membrane proteins such as cytochrome *c* (Van & Griffith, 1975), prothrombin significantly increased membrane order in fluid phase membranes. In the presence of calcium, bound prothrombin caused

Table I: Changes in Peak Transition Temperature Due to Prothrombin or Fragment 1 Binding

peptide[a]	neutral lipid	negative lipid	ΔT_m[b] (°C)	reference
II or f1 (? μM)	DMPC (lower melting)	DPPA (30%)	−3.5	Mayer & Nelsestuen (1981, 1983)
II (10 μM) or f1 (6 μM)	DPPC (higher melting)	bovine PS (20%)	+3	Mayer & Nelsestuen (1981, 1983)
II (34 μM), II (2 μM)	DMPC (higher melting)	POPA (23%)	+6, +3	this work
II or f1 (34 μM)	DMPC (lower melting)	DMPS (35%)	+3	this work
f1 (36 μM)	DMPC (lower melting)	$DC_{15}PG$ (33%)	+10	Lentz et al. (1985)
II (34 μM), II (7 μM)	DMPC (lower melting)	$DC_{15}PG$ (28%)	+8, +5–6	this work

[a] II, prothrombin; f1, prothrombin fragment 1. [b] Change in transition temperature due to protein binding is reported as ΔT_m which is calculated by subtracting the T_m of mixed lipid vesicles in the absence of protein from the T_m in the presence of prothrombin or prothrombin fragment 1.

an increase in anisotropy for vesicles which contained ≥20 mol % DMPS (Figure 5, closed squares), indicating ordering of the liquid-crystalline phase. At high DMPS content, where the transition disappeared in the presence of prothrombin, the fluid phase became very ordered. This membrane ordering effect of prothrombin was calcium independent (Figure 5, closed triangles), although membrane ordering was not quite as great in the presence of EDTA as in the presence of 5 mM Ca^{2+}. The ordering effect seen with the intact prothrombin molecule was not seen with the fragment 1 region of prothrombin (Figure 5, closed diamonds). This is consistent with the report that prothrombin fragment 1 did not alter bilayer fluidity as measured by fluorescence polarization (Dombrose et al., 1979).

Below the phase transition, the DPH anisotropy was slightly lower in the presence of prothrombin or fragment 1 than in their absence (Figure 5, upper curves), indicating that the gel phase was slightly disordered by these peptides. This was also a Ca^{2+}-independent effect, but, unlike the effect above the phase transition, the gel phase disorder occurred with fragment 1 as well as with whole prothrombin. This membrane disordering was not very substantial except at high DMPS content where the transition was broad and poorly defined. These data reveal the reason for the loss of the phase transition at high DMPS content in the presence of prothrombin: the fluid phase was ordered by the protein, and the gel phase was disordered, with the ultimate effect, at sufficiently high DMPS content, that the two phases became indistinguishable.

As a control, we showed that the observed protein-induced anisotropy shifts were not due to movement of DPH out of the membrane and partitioning into the protein. As with the parent fluorophore DPH, measurements with the fluorescent phospholipid 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC)[1] incorporated in DMPS/DMPC SUV with high PS content showed that the presence of prothrombin, with or without calcium, raised the anisotropy of probe in fluid phase vesicles by 0.07 and resulted in the loss of the phase transition (data not shown). Since DPHpPC should remain securely anchored in the membrane, changes in DPHpPC anisotropy, which were comparable to the observed changes in DPH anisotropy shown in Figure 5, indicate changes in lipid order.

Effect of Prothrombin on PG/PC Membrane Order. Figure 6 shows the anisotropy of DPH in the gel and fluid phases of $DC_{15}PG$/DMPC SUV. As in Figure 5, the data are plotted 5 °C above the fluidus and below the solidus phase boundaries. In agreement with the results for DMPS (Figure 5), the order in the fluid phase of $DC_{15}PG$/DMPC vesicles was not altered by prothrombin fragment 1 at any composition. Also similar to the results with DMPS, prothrombin in the presence (Figure 6, lower closed squares) or absence (lower closed triangles) of Ca^{2+} increased the fluid phase anisotropy with increasing $DC_{15}PG$ concentration but not nearly as dramatically at low acidic lipid contents as for DMPS (compare Figures 5 and 6). It seems that the Ca^{2+}-independent influence of prothrombin

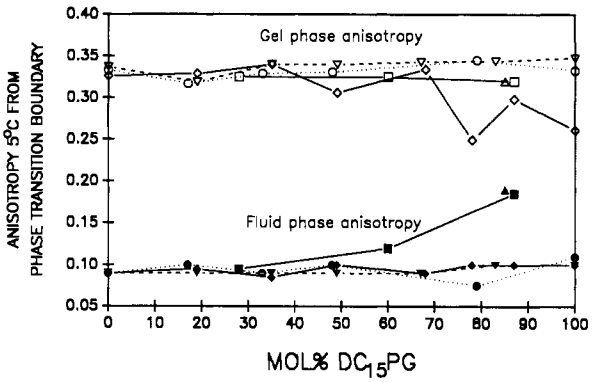

FIGURE 6: Fluorescence anisotropies of DPH incorporated in $DC_{15}PG$/DMPC SUV incubated 5 °C above or below the main order/disorder phase separation are plotted in the lower and upper half of this figure, respectively. Symbols are as defined in Figure 5 with the addition of inverted triangles for measurements made on vesicles in the presence of fragment 1. Vesicles containing ~85 mol % PG had poorly defined phase transitions upon addition of prothrombin. For these vesicles, the anisotropy is reported 5 °C above or below the transition of the most PG-rich vesicle with a measurable transition.

on acidic lipid-containing membranes demonstrates at least a partial specificity for PS- as opposed to PG-containing membranes.

Below the phase transition, the anisotropy of DPH was unaffected by prothrombin fragment 1 alone (Figure 6, open inverted triangles) and was decreased in response to fragment 1 plus Ca^{2+} only at high PG concentrations (78–100 mol % $DC_{15}PG$; Figure 6, upper curve, open diamonds). In the absence of Ca^{2+}, the anisotropy was equivalent to that measured in vesicles alone. It is apparent that prothrombin fragment 1 disordered the gel phase of vesicles with high $DC_{15}PG$ content, but only in the presence of Ca^{2+}. By contrast to the effect of fragment 1, whole prothrombin, in the presence or absence of Ca^{2+}, had little if any effect on the fluorescence anisotropy of DPH in PG/PC gel phase membranes (open squares and triangles in Figure 6). Since the disordering effect was Ca^{2+} dependent and specific to fragment 1, it may be a result of the interaction of the Ca^{2+} conformation of prothrombin fragment 1 with the negatively charged membrane.

Effect of Prothrombin on the Phase Behavior of Various Acidic Lipid Membranes. In Table I, we have summarized our DPH anisotropy measurements of the transition temperature shifts resulting from the interaction of prothrombin or prothrombin fragment 1 plus Ca^{2+} with a variety of lipid surfaces composed of 20–35 mol % acidic lipid. Data reported earlier by Mayer and Nelsestuen (1981, 1983) and by Lentz et al. (1985) are also reproduced in Table I. As we shall show under Discussion, these data, taken together, demonstrate that interpretation of phase transition shifts in terms of lipid domain formation can be misleading.

Effect of Different Prothrombin and Ca^{2+} Concentrations. A reviewer has expressed the concern that the phase behavior shifts and changes in membrane order that we have observed

are due mainly to Ca^{2+} or perhaps to the high concentrations of prothrombin that we have used. Although we believe that our phase behavior measurements (Figures 1, 3, and 4) distinguish between the effects of Ca^{2+} and of prothrombin and Ca^{2+}, we have performed additional control experiments to address the concerns of this reviewer. Our measurements of phase behavior have been made at prothrombin concentrations sufficiently high that surface binding would be essentially (>95%) saturated. This was crucial for the construction and meaningful interpretation of membrane phase diagrams. As summarized in Table I, Mayer and Nelsestuen used prothrombin and prothrombin fragment 1 concentrations (~6–10 μM) that led to somewhat lower surface saturation (~80%, 1981; ~90%, 1983). In order to determine the effect of partial surface saturation, measurements with POPA/DMPC and $DC_{15}PG$/DMPC membranes were made at reduced prothrombin concentrations. As summarized in Table I, the upward shift of T_m was reduced in the membranes with the lower saturation of binding sites. However, the shifts were still in the same direction observed for the fully saturated membrane. This demonstrates that our observation of shifts in a direction opposite to that predicted by the domain model of Mayer and Nelsestuen cannot be explained on the basis of anomalies associated with use of high concentrations of protein. It should be noted that the data sets obtained at low prothrombin concentrations, especially the POPA/DMPC data, were somewhat noisy due to visible aggregation of the sample during the cooling scan. This is in agreement with the expectation that partial saturation of the membrane surface (~60% for both $DC_{15}PG$/DMPC and POPA/DMPC) should leave some areas free to form Ca^{2+}-mediated interbilayer complexes. We avoided such aggregation in the case of our measurements for the temperature–composition diagrams since these were made at protein concentrations sufficient to saturate even 10 mol % PS vesicles.

With regard to the effect of Ca^{2+} concentration, Nelsestuen and Broderius (1977) reported that, in the presence of 0.2 mM Mn^{2+}, tight binding occurred to PS-containing membranes at Ca^{2+} concentrations as low as 1–2 mM. In response to a reviewer's suggestion, we took advantage of this observation to test the effect of Ca^{2+} concentration on our observations. The transition was essentially unchanged when 2 mM Ca^{2+} and 0.2 mM Mn^{2+} were substituted for 5 mM Ca^{2+} (data not shown). This demonstrates that the shift in T_m was dependent on the extent of surface saturation rather than on Ca^{2+} concentration. This is additional evidence that the transition temperature shift observed in the presence of prothrombin and calcium resulted from the binding of the Ca^{2+}-associated prothrombin to the membranes and not from the effect of Ca^{2+} alone on the acidic lipid membranes.

Discussion

Does Prothrombin or Fragment 1 Binding Induce Lateral Acidic Lipid Domains? Mayer and Nelsestuen (1981, 1983) observed different prothrombin-induced shifts in phase transition temperature depending on whether the acidic lipid in the vesicle melted at a temperature lower or higher than the neutral lipid. These authors argued that removal of acidic lipids into protein-induced domains should shift the membrane phase transition toward that of the neutral lipid. Since they observed such a pattern in the case of two lipid mixtures (see Table I), Mayer and Nelsestuen concluded that acidic lipid domains formed in response to the binding of prothrombin (Mayer & Nelsestuen, 1981) or of other coagulation proteins (Mayer & Nelsestuen, 1983).

Mayer and Nelsestuen reported results for a lower melting PC in combination with a higher melting PA and a higher melting PC in combination with a lower melting PS. They did not, however, report results for PS/PC and PA/PC combinations with the reversed relationship of transition temperatures. As recorded in Table I, we have made these measurements. The phase transition of POPA/DMPC vesicles was shifted to a higher temperature in the presence of protein as predicted by the domain formation model for a lipid mixture with a neutral lipid which is higher melting than the acidic lipid (see Table I). However, the transition was shifted upward to a temperature *greater* than that characteristic of pure DMPC SUV (Lentz et al., 1987). Such a large shift in transition temperature clearly cannot be explained solely on the basis of the acidic lipid domain model; other effects must come into play. Other data in Table I argue against the domain model for prothrombin binding. For instance, although DMPC is the lower melting component in DMPS/DMPC and $DC_{15}PG$/DMPC vesicles, prothrombin binding produced an *increase* in the phase transition temperature of these vesicles (Table I). These data demonstrate that protein-induced shifts in the phase behavior of mixed acidic lipid vesicles cannot be used to test the domain model for protein binding.

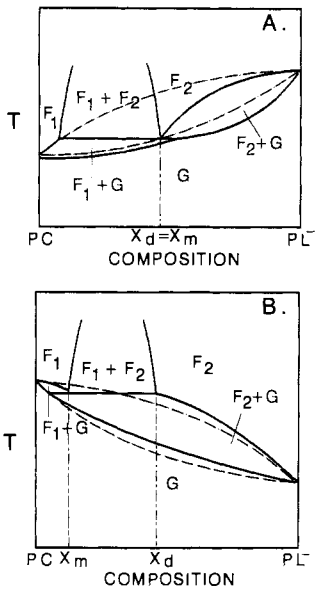

FIGURE 7: Theoretical phase diagrams implied by the lateral domain model for acidic membranes with a lower melting neutral lipid (PC; panel A) or a lower melting acidic lipid component (PL^-; panel B). These diagrams were drawn with the assumption that there was no phase separation in the absence of protein (dashed line) in either the fluid (F) or the gel (G) phase but that there was a protein-induced separation of an acidic lipid-rich, protein-bound, fluid phase (F_2) and a protein-free, acidic lipid-poor fluid phase (F_1). At the composition labeled X_d, the protein-induced domain composition equals the overall composition of the membrane.

A phase diagram can be used to provide a rigorous test of whether thermodynamically defined, compositionally distinct fluid phases form in response to prothrombin binding. To illustrate the importance of constructing complete phase diagrams to test the protein-induced domain model, we present in Figure 7 simple hypothetical phase diagrams drawn according to the thermodynamic principles of phase equilibrium (Levin et al., 1956; Yeh, 1970). These phase diagrams were suggested by the lateral domain model for membranes containing acidic lipid and a lower melting PC (Figure 7A) or a higher melting PC (Figure 7B). In the absence of protein, the dashed curves in both panels are meant to illustrate phase diagrams to be expected for lipids that mix ideally in the plane of the membrane, i.e., no fluid–fluid phase separation. The

solid curves in Figure 7 illustrate the complex phase behavior (lateral fluid phase separations) expected for normally completely miscible lipids under the influence of extrinsically bound protein, according to the protein domain model. These hypothetical phase diagrams assume further that the phase behavior of pure neutral or acidic lipid membranes was not altered by protein binding. Shifts in pure lipid phase behavior would reflect a change in the relative stabilities of the gel and fluid phases, perhaps due to protein-induced changes in lipid packing, in headgroup conformation, in headgroup hydration, etc. Such shifts are observed (Lentz et al., 1985) but cannot reflect domain formation, so they have not been included in our hypothetical diagrams.

Several points are immediately clear from the hypothetical phase diagrams in Figure 7. First, both the existence and direction of a shift in T_m are dependent on the particular composition of the membrane being studied. For example, for Figure 7A,B, no shift in T_m would be seen for membranes containing <10% acidic phospholipid or, for Figure 7B, for membranes containing >80% acidic phospholipid. At other compositions, only negative shifts would be expected for phase diagrams such as Figure 7A for which the monotectic composition (X_m) equals the domain composition (X_d). Systems such as illustrated in Figure 7B would be expected to show a positive shift in the neighborhood of X_d (as predicted by Nelsestuen and colleagues) but a negative shift in the neighborhood of X_m. Second, the situation is even further complicated by the fact, as mentioned above, that *observed* shifts in T_m will also reflect the general influence of protein binding on the relative free energies of the gel and fluid phases, a phenomenon that we have ignored in constructing the phase diagrams in Figure 7. For this reason, the only clear indication of fluid–fluid phase separation (i.e., domain formation) is provided by the different shapes of the *fluidus* phase lines for the protein-containing versus the protein-free membranes. Fluid domain formation is indicated by a horizontal region of the *fluidus* phase line, bounded on the side of the higher melting component by an invariant monotectic point (X_m in Figure 7).

Because of the irreversibility of DMPS/DMPC SUV phase behavior at high DMPS content, our experimental temperature–composition diagram is not a true equilibrium phase diagram. However, this irreversibility likely reflects interbilayer complexes that form in the gel phase. Assuming microscopic reversibility exists on cooling through the liquidus line, the shape of this line should still obey the phase rule and reveal the occurrence of lipid domains in the fluid phase. In the study reported here, there was no significant difference in the *shape* of the diagram when prothrombin was present. On this basis, we conclude that binding of prothrombin in the presence or absence of Ca^{2+} does not cause the formation of extensive lateral domains in DMPS/DMPC model membranes. Similarly, we concluded earlier (Lentz et al., 1985) that binding of prothrombin fragment 1 in the presence of Ca^{2+} did not cause the formation of extensive thermodynamic domains in $DC_{15}PG$/DMPC membranes. Together, all our data indicate that thermodynamically defined domains enriched in acidic lipids do not form in response to binding of prothrombin or prothrombin fragment 1 to mixed acidic/neutral lipid membranes. We have previously pointed out (Lentz et al., 1985) that changes in lipid packing associated with prothrombin fragment 1 binding could contribute to observed changes in phase behavior observed by us and by Mayer and Nelsestuen. The current work indeed demonstrates (see Figures 5 and 6) that prothrombin binding induced changes in packing (i.e., in membrane order) that can account for the observed upward shift in the phase diagram.

There is a caveat related to the approach used here and elsewhere (Mayer & Nelsestuen, 1981, 1983; Wiener et al., 1985) in which lipid lateral organization is inferred from studies of lipid phase behavior. We have pointed out previously (Lentz et al., 1985; Jones & Lentz, 1986) that such studies have an inherent limit of resolution with regard to the size of the domain they can detect, namely, roughly 30 lipid molecules. Protein-induced domains much smaller than this will not melt with sufficient cooperativity to be detected by these thermodynamic measurements. Bound prothrombin has been reported to cover ~50 lipid molecules. Therefore, a prothrombin-induced binding domain should be thermodynamically detectable by studies of lipid phase behavior. We have extended these limits using spectroscopic measurements (Jones & Lentz, 1986) to conclude that domains larger than 10 acidic lipids were not likely to form. Finally, we have shown (Cutsforth et al., 1989) that the observed binding of prothrombin to acidic membranes can be explained by a model in which only four acidic lipids are associated with specific sites on membrane-bound prothrombin.

Do Prothrombin and Prothrombin Fragment 1 Interact in the Same Way with Acidic Lipid Membranes? Prothrombin fragment 1 binding has often been assumed to be a model for the interaction of whole prothrombin with membranes (Dombrose et al., 1979; Lentz et al., 1985; Marsh et al., 1981). However, cautions have been raised regarding equating the response of these two peptides to Ca^{2+} (Prendergast & Mann, 1977; Bloom & Mann, 1978), and there is at least one report that the interaction of fragment 1 with PS/PC monolayers is somewhat different from the corresponding interaction of prothrombin (Lecompte et al., 1980). Although our results support the commonly held notion that the membrane binding of whole prothrombin is dominated by its GLA-containing fragment 1 portion, at least three of our observations indicate that there are differences in the mode of interaction of these two peptides with membranes.

First, the effects of these two peptides on the phase behavior of $DC_{15}PG$/DMPC SUV were different. While the effects of fragment 1 and prothrombin were nearly identical at low PG content, prothrombin did not induce as great an increase as fragment 1 in the phase separation range of 65 mol % $DC_{15}PG$ membranes (data not shown). Although we obtained limited data for the prothrombin–$DC_{15}PG$ system, the data were sufficient to determine that the shape of the PG/PC phase diagram in the presence of prothrombin (data not shown) was different than in the presence of fragment 1 (Lentz et al., 1985). Second, fragment 1 produced neither the Ca^{2+}-dependent nor the Ca^{2+}-independent increase in the fluorescence anisotropy of DPH associated with fluid phase PS/PC (Figure 5) and PG/PC membranes (Figure 6). Finally, neither prothrombin alone nor prothrombin plus Ca^{2+} altered the phase behavior of pure DMPC vesicles (Figure 4). Separate studies showed there to be no irreversible increase in light scattering when pure DMPC SUV were incubated with prothrombin plus Ca^{2+} (data not shown), which indicated that vesicle fusion did not occur under these conditions. Previously, we have reported (Lentz et al., 1985) that DMPC vesicles fused when treated with prothrombin fragment 1. This ability to induce fusion must have been a nonspecific effect due to the peptide fragment properties of prothrombin fragment 1 and not a property of the intact prothrombin molecule.

We conclude that the whole prothrombin molecule interacts with acidic lipid membranes by a somewhat different mech-

anism than does its N-terminal fragment 1 portion. This could be due to somewhat different conformations of isolated fragment 1 and fragment 1 in the parent molecule and/or to a contribution to binding from other parts of the prothrombin molecule.

Is There a Ca^{2+}-Independent Interaction of Prothrombin with PS/PC Membranes? There are several reports in the literature of calcium-independent interactions of prothrombin with monolayers (Lecompte et al., 1980, 1989; Lecompte & Miller, 1980) and bilayers (Bull et al., 1972; Lecompte et al., 1984; Prigent-Dachary et al., 1986). The interaction was reported not to occur with prothrombin fragment 1 (Lecompte et al., 1980) and to be dependent on the percentage of PS in the vesicles or monolayers (Lecompte & Miller, 1980; Lecompte et al., 1984, 1989; Prigent-Dachary et al., 1986). Nelsestuen et al. (1978) commented that prethrombin 1 displayed calcium-independent binding to "very acidic" membranes. "Very acidic" seemed to refer to greater than 20 mol % PS.

Two aspects of our results are suggestive of a PS-specific, Ca^{2+}-independent interaction of prothrombin with acidic lipid membranes. First, as summarized in Figure 4, prothrombin altered the phase behavior of DMPS/DMPC SUV in both a Ca^{2+}-dependent and a Ca^{2+}-independent fashion. The effect of prothrombin increased with increasing DMPS surface concentration. Second, prothrombin produced an acidic lipid-dependent, Ca^{2+}-independent increase in the fluorescence anisotropy of DPH associated with acidic lipid membranes (Figures 5 and 6). This effect was much more pronounced in PS-containing than in PG-containing membranes, although an effect could be seen in membranes containing a sufficiently high PG content (Figure 6). Ca^{2+} alone produced neither the alteration in phase behavior nor the change in membrane order observed in the presence of prothrombin. In the absence of Ca^{2+}, fragment 1 had no acidic lipid-dependent effect on either the phase behavior or the acyl chain order of either PS- or PG-containing membranes (Lentz et al., 1985; Figures 5 and 6). Preliminary experiments with equivalent molar concentrations of the prethrombin 1 portion of prothrombin (prothrombin minus fragment 1) have demonstrated the same effects seen with whole prothrombin (Tendian and Lentz, unpublished results). These data seem consistent with the existence of an acidic lipid-specific, Ca^{2+}-independent binding site on prothrombin, probably outside of the fragment 1 region. This binding site appears to show preference for PS as compared to PG, consistent with other data from our laboratory that suggest the existence of acidic lipid binding sites on prothrombin that bind PS in preference to PG (Cutsforth et al., 1989). This possibility is consistent with other observations in our laboratory that demonstrate a protein conformational shift in the prethrombin 1 portion of bovine prothrombin that occurs upon binding specifically to PS-containing vesicles (Lentz et al., 1990; Wu & Lentz, 1990).

It should be pointed out that there are other possible, and more trivial, explanations for our observations: first, that the observed Ca^{2+}-independent effects result from nonspecific peptide adsorption at high peptide concentration; second, that impurities in prothrombin preparations (ours and others) account for the Ca^{2+}-independent interaction observed. We are currently attempting direct binding measurements with prothrombin and prothrombin fragments and both PS- and PG-containing membranes to address these possibilities.

Conclusions

Three principle conclusions result from this work. *First*, prothrombin does not induce extensive, thermodynamically defined domains of negatively charged lipid in DMPS/DMPC or $DC_{15}PG$/DMPC membranes. Mayer and Nelsestuen (1981, 1983) misinterpreted protein-induced shifts in the phase behavior of mixed neutral/negatively charged lipid vesicles to support the domain model originally proposed by Lim and Nelsestuen (1977) and Dombrose et al. (1979). *Second*, the detailed molecular mechanisms of interaction of prothrombin and prothrombin fragment 1 with acidic lipid membranes share significant common features, but also clearly have some differences. *Third*, a Ca^{2+}-independent prothrombin–lipid interaction appears to increase the acyl chain order in the liquid-crystalline phase of acidic lipid membranes. This ordering effect has some phosphatidylserine specificity and has not been detected previously. Our results are consistent with the existence of a Ca^{2+}-independent binding site(s) in the prethrombin 1 portion of prothrombin, although additional direct binding experiments will be needed to completely eliminate possible nonspecific artifacts.

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