Calcium- and Prothrombin-Induced Lateral Phase Separation in Membranes†

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ABSTRACT: Calcium and prothrombin changed the observed phase transition temperature (T_m) of appropriately constituted phospholipid vesicles. The results are consistent with clustering of the acidic phospholipid molecules to form a prothrombin binding site. Transition temperatures were obtained by monitoring the fluorescence intensity and fluorescence depolarization of the lipid-soluble probes 8-anilino-1-naphthalenesulfonic acid and diphenylhexatriene, respectively. In all cases, calcium and prothrombin caused the observed T_m to increase or decrease toward the value of the pure neutral phospholipid. For example, calcium caused a 2 °C increase in the observed T_m of vesicles containing 20% bovine brain phosphatidylserine (PS) and 80% dipalmitoylphosphatidylcholine (DPPC). Prothrombin binding to these vesicles caused a further 2 °C increase in the T_m. Conversely, calcium or calcium plus prothrombin caused 2 and 3.5 °C decreases, respectively, in the observed T_m of membranes of 30% dipalmitoylphosphatidic acid and 70% dimyristoylphosphatidylcholine. The changes are due to clustering of the acidic phospholipid molecules, which enriches the bulk membrane in the neutral phospholipid. The observed T_m therefore increases or decreases, depending on whether the acidic phospholipid component had a T_m higher or lower than the neutral phospholipid. In agreement with this interpretation and with previous studies, manganese caused negligible changes in the T_m and inhibited the effect of calcium. In addition, the concentration dependence of the calcium-induced increase in the T_m (for PS–DPPC vesicles) indicated a dissociation constant of about 0.5 mM for calcium–phosphatidylserine binding, which agrees with direct calcium binding measurements. The inhibitory effect of manganese on the calcium-induced changes was overcome by prothrombin; in the presence of manganese and calcium, prothrombin still induced the full 4 °C increase. Light-scattering studies demonstrated that, in both cases, prothrombin-induced changes in the T_m correlate closely with actual prothrombin binding to the phospholipid vesicles. Lateral phase separation appears to be an integral part of prothrombin–membrane binding.

The proteolytic conversion of prothrombin to thrombin by blood-clotting factor X_a is a membrane-assisted reaction [see Davie & Fujikawa (1975), Stenflo & Suttie (1977), and Jackson & Nemerson (1980) for a review]. The importance of acidic phospholipids in this reaction has also been demonstrated (Papahadjopoulos & Hanahan, 1964; Bull et al., 1972). Prothrombin and factor X bind to the surface of membranes containing acidic phospholipids, forming protein–acidic phospholipid complexes (Lim et al., 1977). These complexes are apparently mediated by a calcium bridge between protein and phospholipid.

Two cation-dependent phenomena involved in prothrombin–membrane binding were detected by Nelsestuen (1976) and the very different cation specificities of these processes established (Nelsestuen et al., 1976). The first process is a calcium-dependent protein conformational change. Nelsestuen & Lim (1977) proposed that the second process involved a calcium–phospholipid interaction which may entail lateral phase separation of the acidic phospholipids. The vitamin K dependent proteins may then bind to areas enriched in acidic phospholipid.

Changes in the transition temperature (T_m)[1] of membranes containing acidic phospholipids have been used to monitor metal-induced (Papahadjopoulos et al., 1974; Jacobsen & Papahadjopoulos, 1975) and protein-induced (Boggs et al., 1977a,b) lateral phase separation. In comparison to most other metal ions, calcium displays unique characteristics in its interaction with the polar head groups of acidic phospholipids (Papahadjopoulos, 1968; Hauser et al., 1976, 1977; Kurland et al., 1979; Portis et al., 1979). Calcium also initiates lateral phase separation (Ito & Ohnishi, 1974; Ohnishi & Ito, 1974; Papahadjopoulos et al., 1974; Galla & Sackmann, 1975; Ito et al., 1975; Jacobson & Papahadjopoulos, 1975). Certain proteins induce structural reorganization of membranes. Intrinsic (Trauble & Overath, 1973; Birrell & Griffith, 1976; Boggs et al., 1977b; Mombers et al., 1977) and extrinsic (Boggs et al., 1977; Kimelman et al., 1979) proteins have been shown to cause the formation of lipid domains. Most of these studies, however, were conducted with vesicle preparations containing ≥50% acidic phospholipid. For example, when vesicles containing less than 50% acidic phospholipid were used, it was concluded that calcium-induced lateral phase separation was absent (Papahadjopoulos et al., 1974) or was small and slow in forming (Ito et al., 1975). Most biomembranes contain less than 20% acidic phospholipid. Therefore, if proteins of the prothrombinase complex cause structural reorganization in membranes with nearly physiological acidic phospholipid content, very sensitive techniques must be employed to observe these changes.

The fluorescence intensity (Brand & Gohlke, 1972; Yguerabide & Foster, 1979) and fluorescence depolarization (Cogen et al., 1973) of probes embedded in a membrane undergo characteristic changes during phase transition. Both techniques were used in the studies below to monitor changes in the T_m induced by calcium and prothrombin. The latter

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[1] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; PS, bovine brain phosphatidylserine; ANS, 8-anilino-1-naphthalenesulfonic acid; DPH, diphenylhexatriene; EDTA, ethylenediaminetetraacetic acid. Transition temperature and T_m are synonymous and refer to the temperature at the midpoint of the phase transition.

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components caused changes in membrane structure which appear to be lateral phase separation.

Materials and Methods

Bovine prothrombin was purified and quantitated as previously described (Resnick & Nelsestuen, 1980). Phospholipids (bovine brain phosphatidylserine, dipalmitoylphosphatidic acid, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylcholine) were purchased from Sigma Chemical Co. and are reported to be greater than 95% pure. ANS and DPH were purchased from Sigma Chemical Co. and Molecular Probes, Inc., respectively.

Single bilayer vesicles containing phospholipid mixtures were prepared according to Huang (1969) with the modifications of Nelsestuen & Lim (1977). Phospholipid concentrations were estimated by organic phosphate measurement (Chen et al., 1956) by using a phospholipid/phosphorus weight ratio of 25. ANS was incorporated into phospholipid vesicle preparations by injecting the appropriate amount of an ethanol solution of ANS (1 mg/mL) into a vortexing vesicle solution. DPH was incorporated by the same procedure by using 1 mg of DPH per mL of tetrahydrofuran. The final molar ratio of fluorescent probe to phospholipid was 1:100. These solutions were equilibrated at room temperature for 30 min. Nitrogen was then lightly bubbled through the solution at 4 °C as previously described (Jonas et al., 1977).

All fluorescence and light-scattering measurements were obtained by using a Hitachi Perkin-Elmer Model 44A fluorescence spectrophotometer. ANS fluorescence measurements were taken with excitation and emission wavelengths set at 360 and 470 nm, respectively. For DPH measurements, an excitation wavelength of 350 nm and an emission wavelength of 430 nm were used. Scans of fluorescence intensity as a function of temperature were obtained while decreasing the temperature at a rate of 1 °C/min unless otherwise noted. The cuvette temperature was continuously monitored with a thermistor temperature probe. In agreement with previous results (Papahadjopoulos et al., 1973), transition temperatures obtained from such cooling scans were about 1 °C lower than those obtained from heating scans. Nevertheless, both procedures gave the same relative changes in the presence of metal and protein. Replicate values for transition temperatures were within ±0.25 °C.

Fluorescence depolarization measurements were made with the addition of a Perkin-Elmer polarization accessory. Polarization intensities were obtained and evaluated according to the fluorescence polarization relationship $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ (Weber, 1952) where P is polarization, $I_{\parallel}$ is the fluorescence intensity with the polarizers in a parallel arrangement, and $I_{\perp}$ is the fluorescence intensity with the polarizers in a perpendicular arrangement. In practice, fluorescence intensities were measured for all combinations of excitation and emission polarizer arrangements:

		excitation	
		90°	0°
emission:	90°	L_H	L_V
	0°	V_H	V_V

The resulting equation $P = V_V - L_V(V_H/L_H)/[V_V + L_V(V_H/L_H)]$ was used to evaluate the data. The V_H/L_H ratio is commonly referred to as the G value and is used to correct for the depolarization effect of grating monochromators. Alternatively, the transition temperature for the membrane was measured by continuous scanning of the fluorescence emission with polarizers in the L_H arrangement. This latter technique does not allow calculation of the polarization value but is much faster and actually yields a more precise transition temperature.

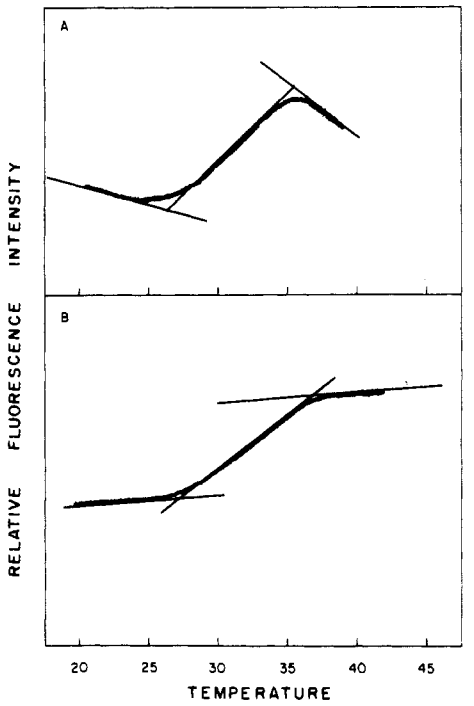

FIGURE 1: Fluorescence intensity scans of phospholipid vesicles as a function of temperature. (A) Fluorescence intensity of ANS dissolved in phospholipid vesicles. (B) Continuous scan of polarized fluorescence intensity of DPH dissolved in phospholipid vesicles. Both polarizers were set at 90°.

The amount of protein bound to phospholipid vesicles was determined by 90° light scattering according to the relationship

$$\frac{I_{s2}}{I_{s1}} = \left(\frac{dn_2/dc_2}{dn_1/dc_1}\right)^2\left(\frac{M_{r2}}{M_{r1}}\right)^2$$

where I_{s2} is the scattering intensity from the protein–phospholipid vesicle complex measured at 320 nm, I_{s1} is the scattering intensity of the initial vesicles, dn_2/dc_2 is the refractive index increment of the protein–phospholipid complex, dn_1/dc_1 is the refractive index change for phospholipid alone, M_{r2} is the molecular weight of the complex, and M_{r1} is the molecular weight of the vesicles alone. The reader should refer to previous works (Nelsestuen & Lim, 1977) where derivation of this equation and a more detailed description of its implications are given.

Results

Determination of Transition Temperatures. The first method used to measure the T_m of phospholipid vesicles involved the fluorescence intensity of ANS, which shows a characteristic inflection concomitant with phase transition (Levine, 1972; Overath & Trauble, 1973; Trauble & Overath, 1973). The T_m was defined as the midpoint of the straight line drawn through the inflection (Figure 1A).

Alternatively, the T_m was determined by monitoring the fluorescence depolarization of a membrane-soluble probe (DPH). Molecular rotation and therefore fluorescence depolarization are sensitive to membrane fluidity and changes during phase transition. Figure 1B demonstrates a temperature scan of DPH (dissolved in 20% PS and 80% DPPC vesicles) fluorescence intensity with both polarizers set at 90°. The T_m was obtained at the midpoint of the inflection as shown. Figure 2 demonstrates the dependence of the actual fluorescence depolarization values on membrane structure.

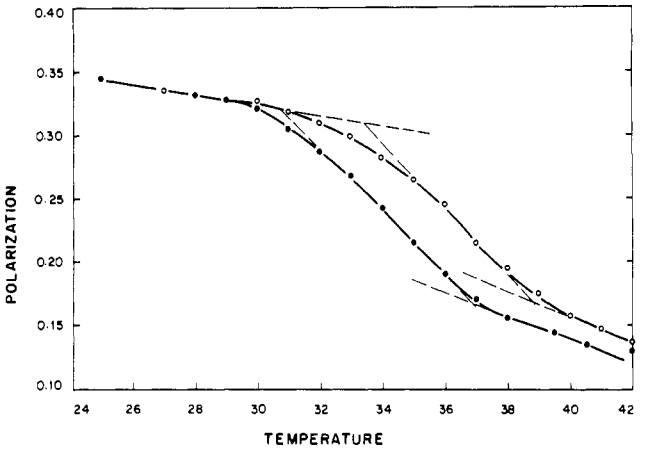

FIGURE 2: Phase transitions of 20% PS and 80% DPPC vesicles as monitored by DPH fluorescence polarization. Polarization points were obtained as described under Materials and Methods while decreasing the temperature at a rate of 1 °C per 15 min. vesicles in the absence of Ca^{2+} and prothrombin (●) and vesicles in the presence of 0.2 mM Mn^{2+}, 2.0 mM Ca^{2+}, and prothrombin [prothrombin/phospholipid (w/w) = 2.0] (○) are shown. The midpoint of the inflections in the two scans occurs at 33.5 and 36.5 °C, respectively.

Transition temperatures obtained at the midpoint of the inflection (Figure 2) compare very well with values obtained from the continuous scan shown in Figure 1B. Determination of a T_m from fluorescence polarization values (Figure 2) requires that several measurements be taken at each temperature. The instrumental manipulations and the necessity of maintaining a constant temperature during the measurements contribute to potential experimental error in a single polarization point. Several points are also required to obtain an accurate T_m. Consequently, continuous monitoring of one polarized fluorescence intensity as a function of temperature provides a more precise and convenient measure of the T_m. The continuous scanning technique illustrated in Figure 1B was used for most subsequent measurements involving DPH.

The approach described here provides a very sensitive method of monitoring lateral phase separation based on the following rationale. Upon undergoing lateral phase separation, the fluorescent probe molecules contained within the phosphatidylserine "clusters" will not contribute to the observed T_m because membrane segments high in bovine brain phosphatidylserine do not undergo phase transition in the temperature range monitored (Figure 1). The T_m measured will therefore reflect the bulk lipids which are enriched in DPPC. According to this rationale, the observed T_m should increase with lateral phase separation, and the upper limit would be 41 °C (the T_m of pure DPPC vesicles as determined by the above method). However, phospholipids on the interior of the vesicle, generally inaccessible to the reagent, as well as boundary phospholipids (those in contact with the PS cluster and the bulk phospholipids) will probably decrease this value so that maximum phase separation on the exterior of the vesicle will give an observed T_m somewhat less than 41 °C.

An example of these results is shown in Figure 2 where the binding of prothrombin to 20% PS and 80% DPPC vesicles caused changes in the temperature dependence of DPH depolarization, indicating a shift in the T_m from 33.5 to 36.5 °C. This change monitors alterations in membrane structure which are consistent with lateral phase separation.

Transition temperatures of different preparations of 20% PS and 80% DPPC vesicles obtained by the ANS and DPH methods ranged from 31.5 to 33.5 °C. This range agrees with transition temperatures of PS–DPPC vesicles measured by differential scanning calorimetry (Boggs et al., 1977b). Since the T_m of any given preparation was reproducible within ±0.25 °C, the larger range is probably attributable to inaccuracies in phospholipid mixing or the preparation procedure. In experiments where comparisons were necesary, phospholipid vesicles were all taken from the same preparation on the same day.

Transition temperatures obtained by DPH-polarized fluorescence intensity scans typically were 0.5–1.0 °C higher than the transition temperatures obtained by ANS scans. Because ANS is located at the membrane surface and DPH is located within the hydrocarbon chain region, this may reflect a real difference in the environment which the two probes experience. Alternatively, the probes may monitor different stages of the phase transition. In either case, relative changes in the observed transition temperatures are measured (below), and the results show that both probes monitor similar changes.

Calcium has been shown to increase the affinity of ANS for membranes, thereby causing an increase in the total fluorescence intensity (Kasai et al., 1969; Rubalcava et al., 1969; Rubalcava et al., 1969; Vanderkooi & Martonosi, 1969). Many proteins also bind ANS, resulting in an increase in the fluorescence intensity (Stryer, 1965; Daniel & Weber, 1966; McClure & Edelman, 1966). While both of these properties were observed in our studies, controls indicated that they did not affect measurements of transition temperatures or our interpretation of the data. For example, pure DPPC vesicles containing ANS were prepared. While actual ANS fluorescence intensities varied considerably, the T_m values obtained in the presence and absence of 2.0 mM Ca^{2+}, 0.2 mM Mn^{2+} plus 2.0 mM Ca^{2+}, and prothrombin (prothrombin/phospholipid, w/w, ratio of 2.5) were constant. Similarly, T_m values of 20% PS and 80% DPPC vesicles were invariant in the presence or absence of prothrombin when calcium was omitted. No binding of protein or metal ion to these membranes should occur under these conditions. These results therefore indicate that actual binding of metal or protein to the phospholipid must occur in order to elicit a change in the observed T_m.

Effects of Metal and Prothrombin Binding on Membrane Structure As Determined by ANS. Small changes in the T_m induced by calcium binding to 20% PS and 80% DPPC vesicles indicate that calcium does cause lateral phase separation in membranes containing nearly physiological amounts of acidic phospholipid (Figure 3). This T_m change is half-maximal at 5×10^{-4} M calcium. Apparent dissociation contstants obtained by direct measurement of calcium binding to 100% and 50% phosphatidylserine vesicles under similar conditions were 2.5×10^{-4} M (Bangham & Papahadjopoulos, 1966; Portis et al., 1979) and 4.4×10^{-4} M (Nelsestuen & Lim, 1977), respectively. Calcium titration of the T_m change (Figure 3) agrees very well with these values and suggests that the changes observed in the T_m are approximately proportional to the amount of calcium bound.

Previous reports have indicated that, although calcium and manganese have very similar binding constants for phosphatidylserine, manganese does not induce lateral phase separation (Hauser et al., 1976; Puskin, 1977). In agreement with this observation and our general interpretation of the data, manganese caused virtually no change in the observed T_m (Figure 3). Furthermore, 0.2 mM Mn^{2+} depressed the effects of calcium, perhaps by direct competitive inhibition of calcium binding to the phospholipids (Figure 3).

The binding of prothrombin to 20% PS and 80% DPPC vesicles caused a more pronounced structural reorganization of the phospholipids than did metal binding, as demonstrated by larger changes in the T_m (Figure 3). Prothrombin actually

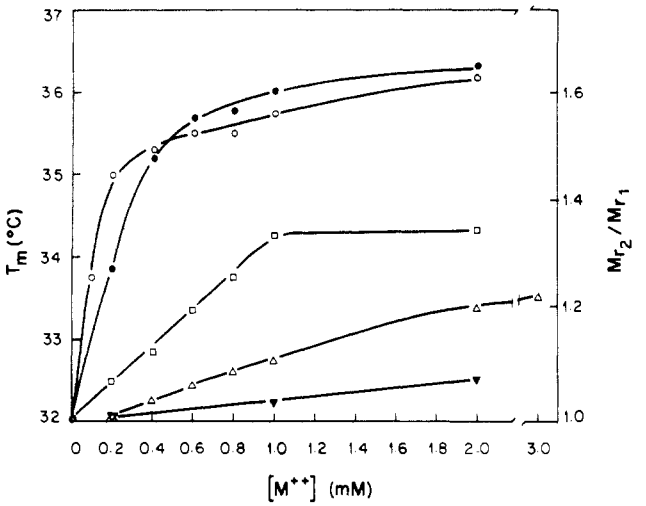

FIGURE 3: Effects of divalent cations and prothrombin binding on the T_m of 20% PS and 80% DPPC vesicles as determined by ANS fluorescence intensity. The phospholipid concentration was 0.2 mg/mL. Titrations were conducted with Mn^{2+} (▼), Ca^{2+} (□), Ca^{2+} in the presence of 0.2 mM Mn^{2+} (△), and Ca^{2+} in the presence of 0.2 mM Mn^{2+} plus prothrombin [prothrombin/phospholipid (w/w) = 2.5] (○). Prothrombin binding to phospholipid vesicles as monitored by relative light scattering (●) is also shown. M_{r2} is the molecular weight of the prothrombin–phospholipid complex, and M_{r1} is the molecular weight of the phospholipid vesicle alone. The conditions for the binding determinations were 0.2 mM Mn^{2+}, variable Ca^{2+}, and 0.2 mg of phospholipid per mL.

overcame manganese inhibition of calcium-induced changes [e.g., compare (△) with (○), Figure 3] so that the maximum change in the T_m (about 4.25 °C) was the same in the presence or absence of manganese. This observation indicates that the full calcium-induced lateral phase separation is not a prerequisite to prothrombin–membrane binding. On the other hand, protein-induced lateral phase separation appears to be an integral and constant part of the prothrombin–membrane interaction.

Prothrombin-induced changes in the T_m compare closely to actual prothrombin binding to the membrane. The latter was monitored by relative light scattering [Figure 3, compare (○) and (●)]. The binding data indicate a maximum change in M_{r2}/M_{r1} of 0.62 which is half-maximum at 0.24 mM Ca^{2+} (Figure 3). These values agree with previous results for similar systems (Nelsestuen & Lim, 1977). The T_m change is half-maximal at about 0.12 mM Ca^{2+}. This difference in calcium titration of T_m changes vs. protein–membrane binding was always observed when manganese was present (also see below) and suggests that the first protein molecules cause a disproportionate change in phospholipid structure.

Figure 4 shows the relationship between T_m changes and prothrombin binding to PS/DPPC vesicles incubated with calcium plus manganese (A) or calcium alone (B). Both vesicle samples had a T_m of 31.75 °C prior to the addition of divalent cations, and the increases caused by the metal ions alone (at zero protein, Figure 4) are similar to those shown in Figure 3. Prothrombin binding to PS–DPPC vesicles corresponds exactly to the elevation of the transition temperature when calcium is the only divalent cation (Figure 4B). In the presence of manganese, the T_m change precedes protein–membrane binding (Figure 4A). This is similar to the results in Figure 3. The midpoint for protein–membrane binding occurs at a prothrombin/phospholipid ratio of 0.75 in both systems (Figure 4A,B).

Because ANS interacts with prothrombin, further control studies were run. For the ascertainment of whether ANS alters protein–membrane binding, characteristics of pro-

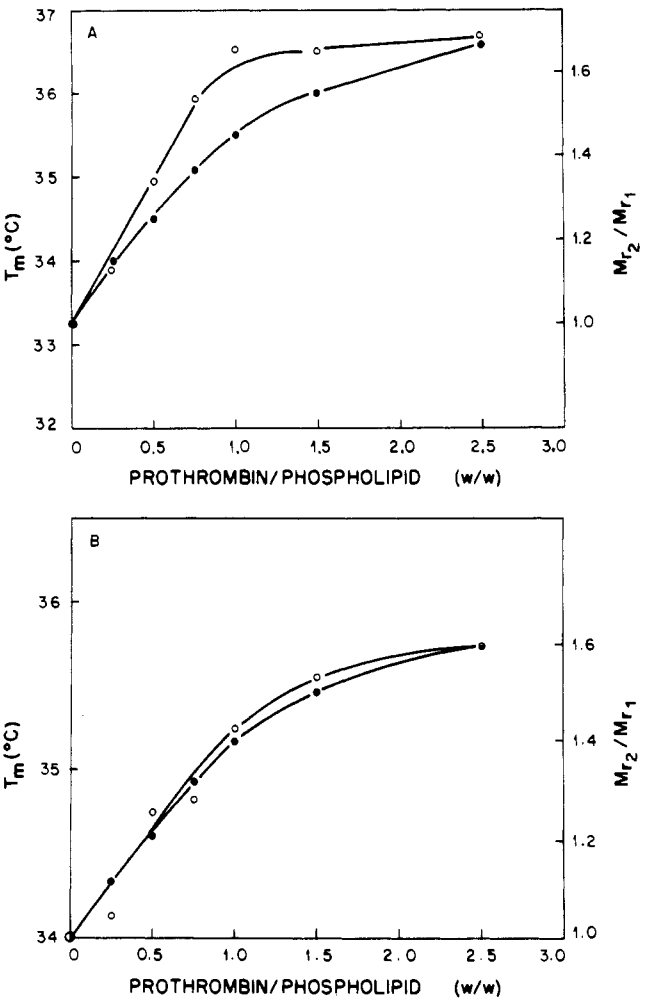

FIGURE 4: Effects of prothrombin binding on transition temperatures of 20% PS and 80% DPPC vesicles. The phospholipid concentration was 0.2 mg/mL. In (A), the phospholipid was equilibrated with 0.2 mM Mn^{2+} and 2.0 mM Ca^{2+} at 45 °C for 30 min prior to protein additions. In (B), the phospholipid was equilibrated with 2.0 mM Ca^{2+} at 45 °C for 30 min prior to protein additions. The prothrombin was also equilibrated with 2.0 mM Ca^{2+} prior to addition. Transition temperatures obtained by ANS fluorescence intensity scans (○) as well as prothrombin–membrane binding results (●) are shown.

thrombin binding to 20% PS and 80% DPPC vesicles were determined by methods described previously (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). The dissociation constant for prothrombin–membrane binding was 0.8 μM in the presence or absence of ANS. This value compares favorably with a value of 0.7 μM prothrombin obtained under similar conditions by Nelsestuen & Lim (1977) and indicates that the probe does not interfere with protein–membrane interactions. Light-scattering intensity as a function of temperature was also monitored. Neither calcium nor calcium plus prothrombin induced fusion or aggregation of the phospholipid vesicles (Lim et al., 1977). Light-scattering intensity changes observed between 40 and 30 °C were of a magnitude which could be attributed to changes in the refractive index increment which occur during phase transition (Yi & MacDonald, 1973). Major alteration in the amount of the protein–membrane complex, therefore, does not occur in the presence of ANS or as a result of the phase transition.

Suurkuusk et al. (1976) have shown that DPPC vesicles undergo aggregation over a period of hours while stored at low temperatures. They also demonstrated that the T_m increases as one cycles through the transition temperature. The present study utilized low phospholipid concentrations and few cycles through the phase transition. No significant aggregation of

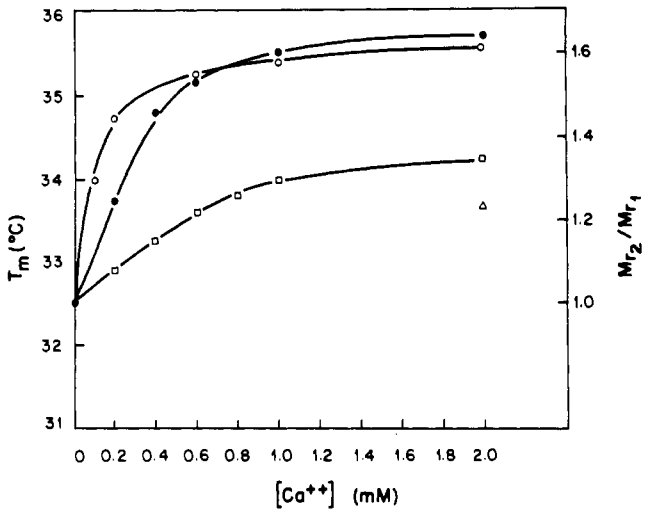

FIGURE 5: Transition temperatures of phospholipid vesicles upon binding of metals and prothrombin as monitored by DPH polarized fluorescence intensity. The phospholipid concentration was 0.2 mg/mL. Experiments include vesicles in buffer only (□), vesicles in the presence of 0.2 mM Mn^{2+} (△), vesicles in 0.2 mM Mn^{2+} plus prothrombin at a prothrombin/phospholipid (w/w) ratio of 2.5 (○), and prothrombin binding to vesicles as monitored by relative light scattering (●).

the vesicles was observed in the time course of the experiment. In addition, no changes in the T_m were observed after several T_m determinations over a 2-h period.

Effects of Metal and Prothrombin Binding on Membrane Structure As Monitored by DPH Polarized Fluorescence Intensity. Due to possible complications (outlined above) in interpreting the results of the ANS fluorescence intensity studies, we repeated critical experiments with the lipid-soluble fluorescent probe DPH. Unlike ANS, DPH does not directly bind calcium or prothrombin as evidenced by the insensitivity of DPH fluorescence intensity to both cation and protein concentrations. In addition, fluorescence depolarization provided a theoretically different method of monitoring phase transition. Transition temperatures were obtained as described above with the results shown in Figure 5. The maximum T_m change in the presence of 2.0 mM Ca^{2+} was 1.75 °C. This compares to a maximum T_m change of 2.25 °C detected with ANS. The total T_m shift when prothrombin was bound at saturating conditions was 3.00 °C, which compares to a total monitored by ANS of 4.25 °C. These small differences may be due to the use of different lipid preparations or to the different environments in which the probes exist.

The calcium and prothrombin dependences of these T_m changes were identical for both ANS and DPH: (a) the calcium concentration at half-maximal change was 0.5 mM; (b) the effect of calcium was depressed in the presence of 0.2 mM Mn^{2+}; (c) the calcium concentration at which the elevation of the T_m due to prothrombin binding is half-maximal was 0.1 mM; and (d) the calcium concentration at which the half-maximal change in the molecular weight ratio occurred was 0.24 mM (compare Figures 3 and 5). Without calcium, prothrombin did not induce a change in the T_m. Since the basis of T_m measurement is very different and the location of the two probes in the membrane is different, the relative changes in the T_m monitored by either probe appear to reflect alterations in membrane structure.

Prothrombin-Induced Decreases in T_m. In the studies outlined above, the acidic phospholipid had a lower T_m than the neutral phospholipid so that phase separation caused an increase in the observed T_m. Such changes might also arise from a general "stiffening" of the membrane when protein and calcium are bound. Distinction of these two explanations is possible when one chooses the phospholipid composition such that the acidic phospholipid has a higher T_m than does the neutral phospholipid. With this system, clustering of the acidic phospholipid (which causes a shift toward the T_m of the neutral phospholipid) should result in a lower observed T_m.

Table I: Reversibility of Transition Temperature[a]

added component				
Ca^{2+} (mM)	Mn (mM)	pro-thrombin (μg/mL)	EDTA (mM)	T_m (°C)
0	0	0	0	31.50
2.0	0.2	370	0	35.00
2.0	0.2	370	6	32.25[b]
2.0	0.2	370	6	32.50[c]
2.0	0.2	370	6	31.50[d]

[a] ANS was incorporated into 20% PS and 80% DPPC vesicles at a phospholipid concentration of 0.15 mg/mL. [b] Transition temperature after mixing manganese, calcium, and prothrombin at 45 °C, cooling to 20 °C, heating back to 45 °C, and adding EDTA. [c] Manganese, calcium, and prothrombin were added to the vesicle solution at 45 °C followed by EDTA after a 30-min incubation. [d] EDTA was added to the vesicle solution at 45 °C followed by manganese, calcium, and prothrombin.

Vesicles of 30% DPPA (T_m = 58 °C at pH 8.5) and 70% DMPC (T_m = 23 °C) were prepared in Tris buffer (0.05 M, pH 8.5, 0.1 M NaCl). At this pH, phosphatidic acid is effective in prothrombin binding (Nelsestuen & Broderius, 1977). ANS was used to detect the phase transition by the methods shown above. The addition of 2 mM Ca^{2+} caused a 2 °C decrease in the observed T_m. Prothrombin [prothrombin/phospholipid = 3:1 (w/w)] plus calcium caused a 3.5 °C decrease in the observed T_m. Although the results are not as extensive as those outlined above, they are consistent with lateral phase separation and not with a general stiffening of the membrane.

Reversibility of Lateral Phase Separation. Reversibility of metal and prothrombin effects was checked by chelating the metal with EDTA. EDTA addition generally caused only about 75% reversal of the T_m change for the DPPC–PS vesicles. This degree of reversibility appeared constant for all magnitudes of change. In contrast, EDTA caused complete dissociation of the prothrombin–phospholipid complex as detected by light scattering. The results of a more detailed study on the reversal of the T_m changes are given in Table I. Addition of manganese, calcium, and prothrombin to 20% PS and 80% DPPC vesicles resulted in the elevation of the T_m from 31.5 to 35.00 °C. This solution was then warmed to 45 °C, excess EDTA was added, and a T_m of 32.25 °C was obtained. In a separate solution, manganese, calcium, and prothrombin were added to PS–DPPC vesicles at 45 °C and incubated for 15 min before the addition of excess EDTA. This also resulted in an elevated T_m (32.50 °C). Finally, excess EDTA was added to a PS–DPPC solution prior to the addition of the divalent cations and prothrombin. This yielded a T_m of 31.50 °C, unchanged from the original vesicle solution. These results indicate that the altered T_m is not due to cycling the temperature through the T_m or to nonspecific effects of the reagents or protein. Regardless of the basis of irreversibility, however, it appeared generally proportional to the extent of the T_m change. The reversible T_m changes should therefore follow the same trends documented for total changes in the T_m, and the same conclusions should hold. Changes in the T_m of DMPC–DPPA vesicles were also 75% reversible when EDTA was added.

Discussion

Previous studies on prothrombin–membrane binding have demonstrated a calcium-dependent protein transition and an additional calcium-dependent process (Nelsestuen, 1976; Nelsestuen et al., 1976). The second calcium-dependent process can be monitored by calcium-induced protein–membrane binding in the presence of manganese. The latter cation replaces calcium only in the protein transition process (Nelsestuen et al., 1976). Some evidence suggests that the second calcium-dependent process involves cation interaction with the phospholipid or a protein–phospholipid complex. The primary support for this idea is that the calcium dependence of prothrombin–membrane binding in the presence of manganese varies, depending on the phospholipid composition (Nelsestuen & Broderius, 1977).

The studies presented here utilize two very different fluorescent techniques to monitor the T_m of vesicle preparations. ANS is a charged molecule which incorporates primarily into the polar head group region of the membrane (Gulik-Krzwicki et al., 1970; Lesslauer et al., 1971; Zingsheim & Hayden, 1973). Actual changes in ANS fluorescence intensity occur during phase transition (Levine, 1972; Overath & Trauble, 1973; Trauble & Overath, 1973). DPH is a very hydrophobic probe that partitions entirely into the lipid region of the membrane. Fluorescence depolarization by this probe is very sensitive to membrane fluidity. Close correlation of the results obtained with these two probes therefore helps to validate the conclusions. Differences in the results obtained here with the two probes are not sufficient to warrant qualification of the conclusions.

This study demonstrates that calcium and, more strikingly, calcium plus prothrombin caused the observed T_m to approach that of the PC component. This results in a T_m increase in the PS–DPPC system and a T_m decrease in the DPPA–DMPC system. These changes appear to be the result of lateral phase separation or clustering of the acidic phospholipid molecules. This observation is important for understanding the structure at the prothrombin–membrane interface. For example, attempts to estimate the number of acidic phospholipids on the exterior of phospholipid vesicles per protein binding site gave a value of 9 ± 1 (Nelsestuen & Broderius, 1977). In order for simultaneous contact with several acidic phospholipids, some clustering would be necessary. The result of the present work substantiates that clustering occurs. In the phase-separated state, the T_m did not reach that of the pure neutral phospholipid (41 °C for DPPC). This suggests that phase separation is not complete. This is also consistent with the binding results; vesicles of 20% PS and 80% egg PC are able to bind 1.2 g of prothrombin per g of phospholipid (Nelsestuen & Broderius, 1977). In contrast, the vesicles of 20% PS and 80% DPPC bound only 0.6 g of prothrombin per g of phospholipid (Figures 3–5). A defined stoichiometry between clustered acidic phospholipids and protein would indicate that, in the latter system, a lower proportion of the PS molecules are capable of interacting with prothrombin. This could arise from a less severe phase separation.

The results are pertinent to defining the role of cations in prothrombin–membrane binding. The calcium-induced changes were always considerably less than those induced by calcium plus prothrombin. In the presence of manganese, structural changes induced by calcium concentrations adequate to cause prothrombin–membrane binding (e.g., 0.2 mM Ca^{2+}, Figure 3) are almost nondetectable. In total prothrombin–membrane binding, the proposed interaction of calcium with the phospholipid [K_3 of the model proposed by Nelsestuen & Lim (1977)] may consist of primarily charge neutralization to allow the approach of negatively charged protein and phospholipid.

This study also demonstrates that lateral phase separation correlates closely to the amount of protein bound to the phospholipid. This suggests that actual prothrombin–membrane interaction consists of at least two processes: protein–membrane contact and protein-induced structural reorganization in the membrane.

It has been found that prothrombin interacts with phospholipid monolayers in the absence of calcium (Lecompte et al., 1980; Lecompte & Miller, 1980). Calcium-independent binding of prothrombin to bilayer phospholipid vesicles was also observed when very acidic phospholipid compositions are used (Bull et al., 1972; Nelsestuen et al., 1978). In the present studies, neither the fluorescent probes nor the light scattering has suggested any interaction of prothrombin with the vesicle preparations used in the absence of calcium. These results are in agreement with previous findings which have utilized bilayer membranes of similar charge density and have been conducted over short time intervals (Nelsestuen & Lim, 1977).

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Interactions of Free and Immobilized Myelin Basic Protein with Anionic Detergents†

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ABSTRACT: The interaction of free and immobilized myelin basic protein (MBP) with sodium deoxycholate (DOC) and sodium dodecyl sulfate ($NaDodSO_4$) was studied under a variety of conditions. Free MBP formed insoluble complexes with both detergents. Analysis of the insoluble complexes revealed that the molar ratio of detergent/MBP in the precipitate increased in a systematic fashion with increasing detergent concentration until the complex became soluble. At pH 4.8, equilibrium dialysis studies indicated that ~15 mol of $NaDodSO_4$ could bind to the protein without precipitation occurring. Regardless of the surfactant, however, minimum protein solubility occurred when the net charge on the protein–detergent complex was between +18 and −9. Complete equilibrium binding isotherms of both detergents to the protein were obtained by using MBP immobilized on agarose. The bulk of the binding of both detergents was highly cooperative and occurred at or above the critical micelle concentration. At $I = 0.1$, saturation levels of 2.09 ± 0.15 g of $NaDodSO_4$/g of protein and 1.03 ± 0.40 g of DOC/g of protein were obtained. Below pH 7.0 the $NaDodSO_4$ binding isotherms revealed an additional cooperative transition corresponding to the binding of 15–20 mol of $NaDodSO_4$/mol of protein. Affinity chromatography studies indicated that, in the presence of $NaDodSO_4$ (but not in its absence), [^{125}I]MBP interacted with agarose-immobilized histone, lysozyme, and MBP but did not interact with ovalbumin–agarose. These data support a model in which the detergent cross-links and causes precipitation of MBP–anionic detergent complexes. Cross-linking may occur through hydrophobic interaction between detergents electrostatically bound to different MBP molecules.

The myelin basic protein (MBP)[1] is a low molecular weight, highly charged, peripheral membrane protein which constitutes ~30% of the protein of the myelin sheath in the central nervous system (Eylar et al., 1969; Eylar, 1972). In aqueous solution it exists as a highly flexible molecule with an axial ratio of approximately 10:1 (Eylar & Thompson, 1969; Epand et al., 1974). The basic protein has been reported to bind

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[1] Abbreviations used: CD, circular dichroism; cmc, critical micelle concentration; DOC, sodium deoxycholate; MBP, myelin basic protein; ORD, optical rotary dispersion; $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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