Thrall, C. L., & Spelsberg, T. C. (1980) *Biochemistry 19*, 4130–4138.
Thrall, C. L., Webster, R. A., & Spelsberg, T. C. (1978) *Cell Nucl. 6*, 461–529.
Webster, R. A., & Spelsberg, T. C. (1979) *J. Steroid Biochem. 10*, 343–351.
Webster, R. A., Pikler, G. M., & Spelsberg, T. C. (1976) *Biochem. J. 156*, 409–418.
Woodcock, C. L. F. (1977) *Science (Washington, D.C) 195*, 1350–1352.
Zama, M., Olins, D. E., Wilkinson-Singley, E., & Olins, A. L. (1978) *Biochem. Biophys. Res. Commun. 85*, 1446–1452.

Calcium-Dependent and Calcium-Independent Interactions of Prothrombin Fragment 1 with Phosphatidylglycerol/Phosphatidylcholine Unilamellar Vesicles†

Barry R. Lentz,* Dennis R. Alford, Marcie E. Jones, and Frederick A. Dombrose‡
Department of Biochemistry, The University of North Carolina, Chapel Hill, North Carolina 27514
Received March 25, 1985

ABSTRACT: We have measured the phase behavior of mixed dipentadecanoylphosphatidylglycerol ($DC_{15}PG$)/dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles (SUV) in the presence of saturating (>98% occupancy of binding sites) concentrations of bovine prothrombin fragment 1 and 5 mM Ca^{2+}. Binding of fragment 1 in the presence of Ca^{2+} was verified by an increase in 90° light scattering. Only in the cases of $DC_{15}PG$/DMPC SUV below their phase transition and of pure DMPC SUV were such light scattering measurements not reversible upon addition of ethylenediaminetetraacetic acid to complex Ca^{2+}. Phase-behavior changes of $DC_{15}PG$/DMPC SUV as monitored by diphenylhexatriene fluorescence anisotropy occurred in concert with the binding of fragment 1. The major effects of peptide binding on SUV phase behavior were to raise the phase-transition temperature by 2–15 °C, depending on vesicle composition, and, in general, to make the phase diagram for these small vesicles closely resemble that of large vesicles. No evidence was obtained for the existence of lateral membrane domains with distinct compositions induced by the binding of prothrombin fragment 1 plus Ca^{2+}. Surprisingly, fragment 1 without Ca^{2+} also altered the phase behavior of $DC_{15}PG$/DMPC SUV. Most striking was the effect of fragment 1 (with or without Ca^{2+}) on DMPC SUV phase behavior. Freeze–fracture electron microscopy demonstrated that pure DMPC vesicles were induced to fuse in the presence of fragment 1, while vesicles containing $DC_{15}PG$ remained intact. The rate of DMPC SUV fusion (followed by 90° light scattering) increased with increasing fragment 1 concentration but was not saturable up to 40 μM fragment 1, suggesting a weak, nonspecific interaction between fragment 1 and the neutral phospholipid vesicle. We conclude that Ca^{2+}-dependent binding of prothrombin fragment 1 to $DC_{15}PG$/DMPC vesicles does not induce the formation of thermodynamically stable $DC_{15}PG$-rich domains but does involve Ca^{2+}-independent interactions in addition to the commonly accepted electrostatic one inherent in the Ca^{2+}-bridging model.

The activation of prothrombin to thrombin is a membrane-mediated process requiring the tight association of both the substrate (prothrombin) and enzyme (factor Xa) with a negatively charged membrane surface (Suttie & Jackson, 1977; Zwaal, 1978). While platelets probably provide the requisite membrane surface in vivo, synthetic phospholipid vesicles provide a more convenient model system for examining this association in vitro.

Ca^{2+} is required for tight binding of either prothrombin or its N-terminal proteolytic fragment (fragment 1) to a charged membrane surface (Jackson et al., 1975; Nelsestuen, 1976). The dissociation constants for binding of either prothrombin or prothrombin fragment 1 to PG-containing membranes have been reported to be identical (Dombrose et al., 1979). The ion presumably acts to "bridge" γ-carboxyglutamic acid residues of prothrombin fragment 1 to negatively charged lipids within a membrane (Nelsestuen, 1978; Dombrose et al., 1979). This view has spawned a detailed molecular picture in which prothrombin binding is seen to induce formation of membrane domains that are rich in charged lipids (Dombrose et al., 1979; Nelsestuen, 1978; Barton & Findley, 1976). While this view is currently the most popular, others feel that nonelectrostatic interactions may be involved (Madar et al., 1982). Figure 1 summarizes the several possible models for the interaction of prothrombin fragment 1 with mixed-phospholipid membranes that will be considered in this paper. These models all recognize the probable contribution of Ca^{2+} bridging but differ as to the size of possible membrane domains and as to the occurrence of nonelectrostatic interactions.

In this paper, we begin to distinguish between these possible models of prothrombin binding by determining the effect of prothrombin fragment 1 with and without Ca^{2+} on the phase behavior of small unilamellar vesicles (SUV)[1] composed of mixtures of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$) and dimyristoylphosphatidylcholine (DMPC). The results

†Supported by grants from the U.S. Public Health Service (HL22771 and GM 32707). While conducting this research, B.R.L. was the recipient of an Established Investigator Award from the American Heart Association with funds contributed in part by the North Carolina Heart Association.
‡Present address: Director, Coagulation and Hematology R&D, American Dade, Miami, FL 33152.

[1] Abbreviations: DMPC, 1,2-dimyristoylphosphatidylcholine; $DC_{15}PG$, 1,2-dipentadecanoylphosphatidylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; LUV, large unilamellar vesicles; LMV, large multilamellar vesicles; SUV, small unilamellar vesicles.

0006-2960/85/0424-6997$01.50/0

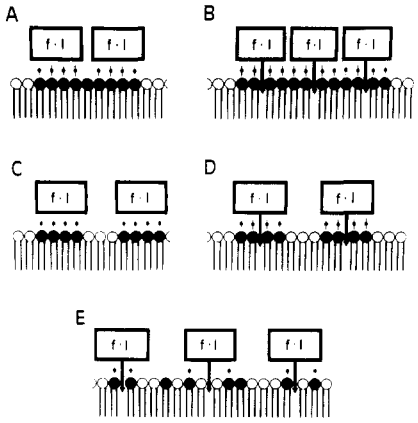

FIGURE 1: Possible models for the interaction of prothrombin fragment 1 (f·1) with membranes containing a mixture of charged (filled head group) and neutral (open head group) phospholipids, in the presence of Ca^{2+} (small closed circles with a vertical bar). All models presume some electrostatic interactions involving Ca^{2+} bridging, but certain models include a nonelectrostatic interaction perhaps involving bilayer penetration (illustrated by the arrows in models B, D, and E). Models A and B presume extensive charged-lipid-rich domains (containing roughly 30–100 or more phospholipid molecules) as well as protein patching. Models C and D presume less extensive lipid domains (involving less than roughly 30 lipid molecules) and no protein patching. Model E envisions no domain formation or lipid patching.

have been used to construct temperature–composition phase diagrams. The rationale of this approach has been that a peptide-induced special lipid domain should be evident as a compositionally unique phase (defined by the shape of the phase diagram) not present in the phase diagram previously reported for the vesicles alone (Lentz et al., 1982). Our results demonstrate that extensive fragment 1 induced domains (Figure 1, models A and B) do not occur. In addition, our results shows that fragment 1 can interact with membranes containing either charged or neutral lipids even in the absence of Ca^{2+}, suggesting that model C is incomplete and that a nonelectrostatic mode of interaction exists (e.g., Figure 1, model B, D, or E). Therefore, either model D or E contains what we believe to be the essential features of the interaction between prothrombin fragment 1 and negatively charged membranes.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC) and the sodium salt of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$) were purchased from Avanti Biochemical (Birmingham, AL). Associated divalent metal ions were removed from $DC_{15}PG$ as described previously (Lentz et al., 1982a), followed by recrystallization from acetone (Lentz et al., 1976). Residual levels of metal ions [detected by atomic absorption (Lentz et al., 1982a)] were <0.5 mol/mol of phospholipid. Phospholipids were >98% pure as judged by thin-layer chromatography (Lentz et al., 1976). [^{14}C]DMPC was synthesized (Patel et al., 1979) from [1-^{14}C]myristic acid (RPI Corp., Elk Grove, IL; lot 1280) and added to stock solutions of DMPC in $CHCl_3$. Vesicular DMPC content was then established by scintillation counting. Total phosphate content was determined by the assay of Chen et al. (1956). Water was distilled from basic potassium permanganate and then redistilled from glass, bubbled with argon, and stored under argon at 4 °C. High-purity 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes (Junction City, OR). All other chemicals were reagent-grade or better.

Prothrombin Fragment 1. Prothrombin was isolated by barium citrate precipitation from bovine plasma followed by QAE-Sephadex (Pharmacia) chromatography, as described by Dombrose et al. (1979). Prothrombin was cleaved by thrombin (Owen et al., 1974), and the resulting prothrombin fragment 1 was isolated by ion-exchange chromatography on QAE-Sephadex A-50 in 0.35 M NaCl, 0.05 M Tris, pH 7.5, and 0.02% NaN_3, followed by gel filtration on Sephadex G-100 in the same buffer (Dombrose et al., 1979). Na_2EDTA (5 mM) was added to selected preparations at this point to assure low levels of free Ca^{2+}. The Tris buffer (with or without added EDTA) was replaced with the buffer used for fluorescence studies by gel filtration over a Bio-Gel P-4 (Bio-Rad) column. Tris buffer could not be used for fluorescence scans both because of the large temperature dependence of its pK_a and because of background fluorescence associated with commercial preparations. All protein preparations exhibited quenching of tryptophan fluorescence by 42–50% in the presence of 5 mM Ca^{2+} (Prendergrast & Mann, 1977; Nelsestuen et al., 1981). Purity of fragment 1 preparations was established by high-pressure liquid chromatography on a TSK 125 column (Bio-Rad, Richmond, CA) and by electrophoresis in the presence of sodium dodecyl sulfate on polyacrylamide gels (11%) followed by staining with Kodavue (Eastman Kodak, Rochester, NY). Finally, protein solutions were sterile filtered (Millipore, 0.45–1 μm) prior to storage at 4 °C under argon.

Phospholipid Vesicles. Vesicles were prepared from quantitative mixtures of stock solutions of DMPC and $DC_{15}PG$ in $CHCl_3/CH_3OH$. For small unilamellar vesicles (SUV), the lipid mixture was dried in a thin film on the wall of a 10-mL ampoule, dispersed in warm (40–45 °C) buffer (0.1 M NaCl, 10 mM TES, 0.02% NaN_2, 0.1 mM Na_2EDTA), and sonified in a Heat Systems W350 Sonicator equipped with a Heat Systems Cup Horn (Heat Systems-Ultrasonics, Inc., Plainview, NY) as described (Lentz et al., 1982b). Vesicles were maintained above their phase transition at all times and were fractionated by ultracentrifugation (Lentz et al., 1980a) within 2–14 h before use. Large, unilamellar vesicles (LUV) were prepared by the reverse-phase evaporation procedure described by Szoka et al. (1980) or by slow dilution and dialysis of an octylglucoside-solubilized lipid sample as described by Parente & Lentz (1984).

Prothrombin Fragment 1/Vesicle Mixtures. Prothrombin fragment 1 (2.35 mg) was incubated at 48 °C for 10 min in 0.75 mL of TES buffer containing either 5 mM $CaCl_2$ or 0.1 mM Na_2EDTA and then added to phospholipid vesicles (2.75 mL) containing DPH. Preincubation was necessary to allow the fragment 1 time to assume its ion-dependent conformation (Marsh et al., 1979). The final solution (3.5 mL) contained 36 μM fragment 1, 0.1 mM phospholipid, 0.1 M NaCl, 10 mM TES, pH 7.5, 0.02% NaN_2, and either 5 mM Ca^{2+} or 0.1 mM Na_2EDTA. This mixture was allowed to equilibrate at 48 °C for roughly another 10–20 min before beginning a fluorescence experiment (see below). Results were reversible (see Results), independent of whether samples were incubated for a total of 10 or 30 min at 48 °C. The incubations at high temperatures (48 °C) required for these experiments should not have resulted in fragment 1 denaturation, as prothrombin fragment 1 is reported to denature at 58.5 and 64.5 °C in the absence and presence of Ca^{2+}, respectively (Ploplis et al., 1981). In addition, Pletcher et al. (1981) have shown that storage of prothrombin fragment 1 even at 80 °C (above the denaturation temperature) for 10–20 min did not prevent its recovery of native Ca^{2+} binding response and spectral properties upon being cooled to a lower temperature.

Fragment 1 Binding. Light scattering measurements (Nelsestuen & Lim, 1977) were performed at a 90° angle on an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL) to obtain a measure of the binding of prothrombin fragment 1 to $DC_{15}PG/DMPC$ vesicles. Experiments were carried out with both emission and excitation monochromators at 313 nm and with emission and excitation slits set at 1 and 4 nm, respectively. Prior to light scattering experiments, buffers (see above) were filtered through 0.22-μm GS-type filters (Millipore Corp., Bedford, MA). Prothrombin fragment 1 (1.0 mg/mL) was preincubated with 2–5 mM Ca^{2+} at the temperature of the experiment and then added sequentially and with stirring to a 1-mL fluorescence cuvette containing 0.05–0.1 mM SUV and 5 mM Ca^{2+}. Light scattering intensity measurements were analyzed on a Southwestern Technical Products 6809 microcomputer interfaced with the SLM spectrofluorometer. Scattering intensities were corrected for the scattering of unbound protein and otherwise analyzed essentially by the method of Nelsestuen & Lim (1977). Following completion of a binding isotherm, Na_2EDTA was added to a final concentration of 43 mM to chelate Ca^{2+} and determine the reversibility of the measurement. Irreversible increases in scattering intensity were interpreted in terms of fusion of SUV to yield larger vesicles.

Fluorescence Measurements. DPH was introduced into vesicle suspensions at 48 °C by injecting, with vigorous vortexing, a small volume (0.2–0.4 μL) of 2 mM DPH dissolved in tetrahydrofuran. The final dye:lipid ratio for most preparations was 1:300. Measurements of DPH fluorescence anisotropy were made in cooling and then heating scans at rates of ±30 °C/h with an SLM 4800 spectrofluorometer. Details of the measurements as well as of calculation of the "microviscosity" and "microviscosity activation energy" parameters are given in the literature (Lentz et al., 1978, 1980b). As previously noted (Lentz et al., 1980b), these quantities have both practical and historical advantages for the detection of lipid-phase transitions but do not necessarily reflect a simple, macroscopic viscosity. The validity of these techniques as procedures for detecting phospholipid phase transitions has been established for a variety of lipid mixtures by comparison with calorimetric data (Lentz et al., 1976, 1978, 1980b).

Calorimetry. Differential scanning calorimetric measurements were performed on a specially constructed (by Roger Hart, currently of Hart Scientific, Orem, UT), high-sensitivity, heat flow calorimeter designed for biological samples. This instrument is based on the design of Suurkuusk et al. (1976) but with improvements of increased sensitivity and the ability to cool as well as to heat. Specificiations of this calorimeter as well as a discussion of the treatment of data have been given previously (Lentz et al., 1982a).

Freeze–Fracture Electron Microscopy. Samples were incubated between thin copper sheets for 4–6 min at the indicated temperatures before rapid freezing by the jet-freezing procedure described previously (Lentz et al., 1980b; Parente & Lentz, 1984). Jet freezing has been shown (Lentz et al., 1980b) to minimize artifacts in fracture face morphology due to slow freezing rates. Frozen samples were fractured on a Balzers BA360M freeze–etching device at −110 °C and 10^{-6} Torr and shadowed with platinum (~10-Å deposit) before viewing on a JEM 100 CX electron microscope operating at 100 kV.

RESULTS

Binding Studies. Figure 2 presents relative light scattering data demonstrating the binding of bovine prothrombin fragment 1 to $DC_{15}PG/DMPC$ (67/33) SUV. These experiments were carried out at 44 °C, a temperature above the phase transition of these vesicles (see below). At this temperature, addition of Na_2EDTA (to chelate Ca^{2+}) completely returned the light scattering intensity to the level expected for unassociated vesicles plus peptide (Table I). Light scattering intensity is extremely sensitive to small amounts of large vesicle contamination in an SUV sample (Barrow & Lentz, 1980). Therefore, the return to original light scattering intensity upon the chelation of Ca^{2+} demonstrates that large vesicles were not formed as a result of binding of fragment 1 to $DC_{15}PG/DMPC$ SUV above the phase transition in the presence of Ca^{2+}.

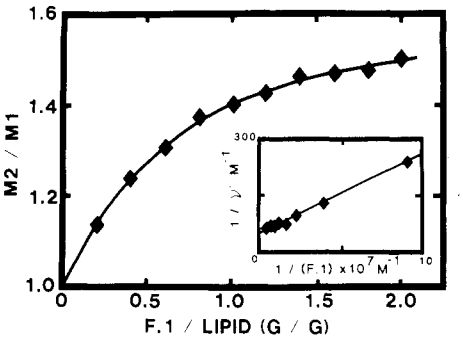

FIGURE 2: Light scattering at 90° from a solution containing prothrombin fragment 1, $DC_{15}PG/DMPC$ (67/33) SUV (0.1 mM), and 5 mM Ca^{2+}. The ratio of the masses of protein/vesicle complexes to vesicles alone (M_2/M_1) is plotted as a function of the weight ratio of protein to lipid in the solution. Data were collected at 44 °C. The inset shows the data transformed and plotted in a Hildebrand format, from which intrinsic dissociation constants and binding stoichiometries were obtained.

Table I: Binding of Fragment 1 to $DC_{15}PG/DMPC$ (67/33) Small Vesicles

temp (°C)	K_d (μM)[a]	stoichiometry (lipids/protein)[a]	% contamination by LUV[b]
44	0.37–0.46	70–74	<2
37	0.23–0.39	31–35	16
23	0.26–0.32	618–656	25
37	0.21[c]	18[c]	

[a] Range of values obtained for Hildebrand and Scatchard analyses of experimental data. [b] Mole percent contamination by phospholipid presumed to be present in the form of LUV is given by $[(I_{total} - I_{fl} - I_{SUV})/SI_{LUV}] \times 100$, where I_{total} = total scattering intensity from sample containing 0.1 mM $DC_{15}PG/DMPC$ SUV, 5 mM Ca^{2+}, a saturating concentration of fragment 1, and 43 mM Na_2EDTA added to reverse binding, I_{fl} = scattering intensity from a sample containing only the appropriate concentration of fragment 1, I_{SUV} = scattering intensity from a sample containing 0.1 mM $DC_{15}PG/DMPC$ SUV, and SI_{LUV} = specific scattering intensity of LUV obtained by incremential addition of LUV to a suspension of SUV. [c] Binding was monitored in terms of the change in membrane phase behavior as reported by DPH fluorescence anisotropy (r). Dissociation constant (K_d) and stoichiometry (N/n) were obtained from $1/(f[PL]) = (1/K_d)[[F1]/(b[PL])] - (n/N)(1/K_d)$, where $b = 1 - f$ = fraction of binding sites occupied = $(r_b - r)/(r_b - r_f)$, r_b = anisotropy in the presence of saturating fragment 1, r_f = anisotropy in the absence of fragment 1, [PL] = total phospholipid concentration, [F1] = total fragment 1 concentration.

Binding experiments were also performed at temperatures within (37 °C) and below (23 °C) the phospholipid phase transition of the vesicles (see phase behavior results reported below). These studies are summarized in Table I. Unfortunately, the interpretation of light scattering data below the phase transition was obscured by irreversible changes in light scattering (see Table I), presumably due to vesicle fusion. Such irreversible increases in light scattering intensity typically produce anomalously large stoichiometries (as evidenced in Table I) but reasonably appropriate K_d values, as judged from

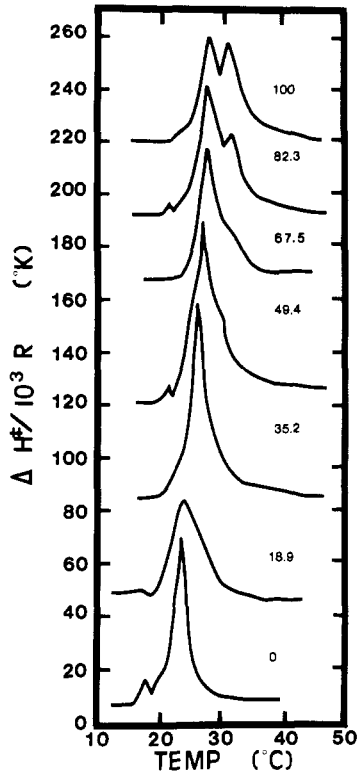

FIGURE 3: Temperature dependence of the DPH-derived microviscosity activation energy ($\Delta H^{\ddagger}$) in small, unilamellar vesicles composed of mixtures of $DC_{15}PG$ (mole percentage indicated) and DMPC (total lipid concentration 0.1 mM) in the presence of 36 μM prothrombin fragment 1. Data for cooling scans are shown; heating scans were essentially identical except for pure $DC_{15}PG$, as noted in text. The 0% data are plotted according to the units of the ordinate while successive curves are displaced upward by arbitrary amounts.

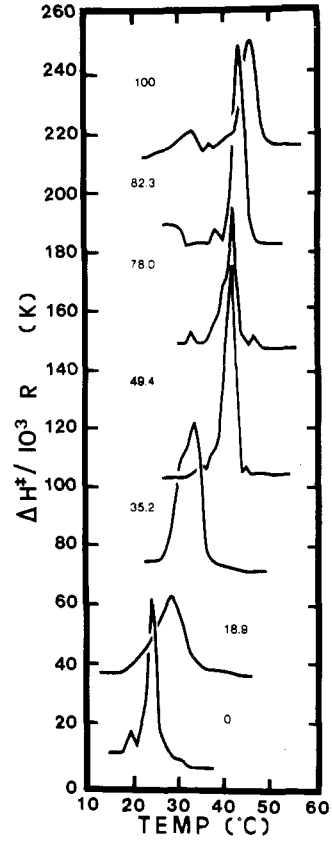

FIGURE 4: Temperature dependence of the DPH-derived microviscosity activation energy ($\Delta H^{\ddagger}$) in small, unilamellar vesicles composed of mixtures of $DC_{15}PG$ (mole percentage indicated) and DMPC (total lipid concentration 0.1 mM) in the presence of 36 μM prothrombin fragment 1 and 5 mM Ca^{2+}. Data for cooling scans are shown; heating scans were essentially identical. Data are located relative to the ordinate as in Figure 3.

independent experiments with different vesicle preparations. Within the level of uncertainty associated with the irreversible changes in vesicle light scattering, these results demonstrate that binding occurred with roughly the same dissociation constant below the phase transition as above.

Fluorescence Studies. The phase behavior of mixed $DC_{15}PG$/DMPC SUV in the presence of prothrombin fragment 1 (with and without Ca^{2+}) was determined by monitoring changes in the fluorescence polarization of DPH to detect lipid phase transitions. Representative plots of the DPH-derived microviscosity activation energy (Lentz et al., 1978) vs. temperature are given in Figure 3 for samples containing fragment 1 without Ca^{2+}. Only cooling scans are shown, since heating and cooling experiments produced essentially the same temperature profiles except for pure $DC_{15}PG$. In addition, except for DMPC, results were independent of whether heating or cooling scans were performed first, as long as vesicles were preincubated for 5–10 min with fragment 1 plus Ca^{2+} at 45–48 °C before cooling to start a heating scan. This was necessary because $DC_{15}PG$/DMPC SUV, by themselves, are unstable below their phase transition temperature (Lentz et al., 1982b). The heating scan of pure $DC_{15}PG$ showed only a single peak at 32 °C, corresponding to the high-temperature peak of the cooling scan. These phase transitions were all considerably different from those observed for $DC_{15}PG$/DMPC SUV in the absence of fragment 1 (Lentz et al., 1982a), with the major difference being a substantially more narrow transition due mainly to an onset temperature 6–8 °C higher in the presence of fragment 1. The upper limit of the phase transition was barely affected by the presence of fragment 1. The most notable and surprising effect of fragment 1 was on the phase behavior of pure DMPC vesicles, which showed a much sharper phase transition in the presence of fragment 1 (Figure 3).

Incubation of fragment 1 with 5 mM Ca^{2+} before combination with the $DC_{15}PG$/DMPC SUV in the presence of 5 mM Ca^{2+} led to an appreciable increase in the lamellar phase transition temperature for the phosphatidylglycerol-rich samples, as evidenced by the data in Figure 4. For a sample containing 67 mol % $DC_{15}PG$ and maintained at 37 °C, the corresponding increase in the DPH fluorescence anisotropy was titratable and saturable and exactly paralleled the change in light scattering (see Figure 2) induced by fragment 1 binding in the presence of Ca^{2+} (data not shown). As in Figure 3, only cooling scans are shown in Figure 4, since subsequent heating scans were essentially unchanged. The reversible phase behavior observed for phosphatdiylglycerol-rich SUV in the presence of both fragment 1 (Figure 3) and fragment 1 plus Ca^{2+} (Figure 4) contrasts markedly with the irreversible behavior of these vesicles observed in the absence of peptide (Lentz et al., 1982a). Another notable feature of the data in Figure 3 is the appearance of a low-temperature transition in $DC_{15}PG$-rich samples, reminiscent of the pretransition commonly observed in large multilamellar (Surkuusk et al., 1976) and large unilamellar (Parente & Lentz, 1984) vesicles but not in small unilamellar vesicles (Suurkuusk et al., 1976).

To determine to what extent high vesicle curvature accounts for the effects of fragment 1 on SUV phase behavior, we also measured the phase behavior of vesicles with low curvature (67 mol % $DC_{15}PG$/DMPC LUV; data not shown). Fragment 1 alone had no effect on the main phase transition of LUV.

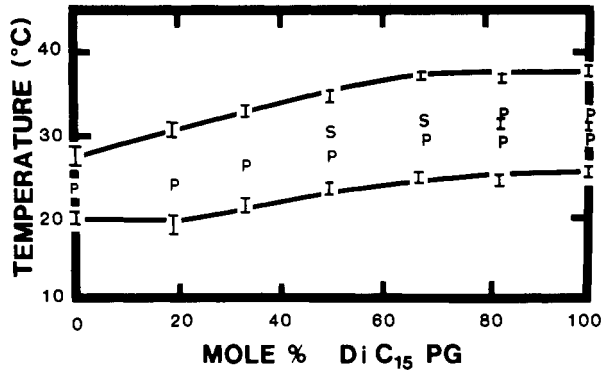

FIGURE 5: Temperature–composition diagram for $DC_{15}PG$/DMPC small unilamellar vesicles in the presence of 36 μM prothrombin fragment 1. Temperature extremes taken from the data in Figure 3 are indicated by vertical bars, peak temperatures are indicated by P, and the edge of peak shoulders is indicated by S. In both cases, composite results from cooling and subsequent heating scans are presented, with the length of the bar indicating the range of uncertainty between heating and cooling results.

Fragment 1 plus Ca^{2+} shifted the transition to higher temperature by roughly 11 °C but did not appreciably alter transition width. In both cases, LUV phase behavior was quite similar to that of SUV exposed in similar fashion to fragment 1 or fragment 1 plus Ca^{2+}. Thus, fragment 1 interacted with $DC_{15}PG$/DMPC SUV so as to relieve the packing constraints caused by high bilayer curvature, whereas fragment 1 plus Ca^{2+} interacted with the SUV both to relieve the effect of curvature and to alter the phospholipid packing (and, hence, phase transition temperature) of the bilayer.

As a control, $DC_{15}PG$/DMPC SUV phase behavior was also examined in the presence of Ca^{2+} alone. The results for vesicles containing $DC_{15}PG$ (data not shown) revealed broad changes in DPH fluorescence anisotropy that were not reproducible between cooling and subsequent heating scans. These profiles were entirely different in shape from those in Figure 4, demonstrating that Ca^{2+} alone cannot be responsible for the changes in phase behavior portrayed by the data of Figure 4. Ca^{2+} had no effect on pure DMPC phase behavior.

Finally, we tested whether observed shifts in phase behavior indeed reflected binding of fragment 1, as opposed to the influence of impurities in our fragment 1 preparation. To do this, we monitored, in terms of DPH fluorescence anisotropy, the shift in SUV (67 mol % $DC_{15}PG$) phase behavior at constant temperature (37 °C) upon sequential addition of fragment 1. The resultant increase in DPH anisotropy was analyzed to obtain apparent binding parameters, as recorded in Table I (last row and legend). The resultant dissociation constant agreed well with that obtained by out light scattering analysis (Table I) and reasonably well with that reported for similar membranes in the literature ($K_d \simeq 0.55$ μM; Dombrose et al., 1979), demonstrating that the observed shift in phase behavior indeed reflects fragment 1 binding.

The effects of prothrombin fragment 1 and fragment 1 plus Ca^{2+} on the phase behavior of $DC_{15}PG$/DMPC SUV are summarized in temperature–composition diagrams shown in Figures 5 and 6. These diagrams were obtained by plotting the peak temperatures and the upper and lower limiting temperatures of the transition (from data in Figures 3 and 4) vs. the composition of the vesicles in which the transitions were observed. The insert to Figure 6 gives an interpretation of our data in terms of a phase diagram drawn to be consistent with our data and with the Gibbs phase rule.

Calorimetric Experiments. In order to confirm the surprising and dramatic effect of prothrombin fragment 1 plus

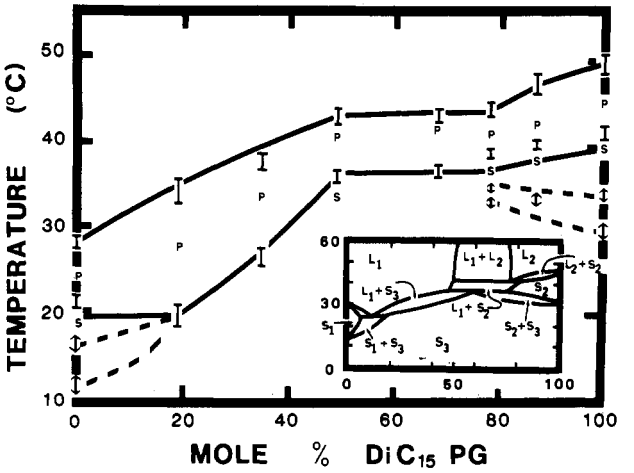

FIGURE 6: Temperature–composition diagram for $DC_{15}PG$/DMPC small unilamellar vesicles in the presence of 36 μM prothrombin fragment 1 plus 5 mM Ca^{2+}. Symbols are as in Figure 5, with the addition of vertical bars with arrows (and dashed lines) to indicate gel-phase transitions. The inset shows an interpretation of the data in terms of a hypothetical phase diagram for this phospholipid mixture in the presence of fragment 1 plus Ca^{2+}. In the inset, S indicates a solid-like phase and L indicates a liquid-like phase. This phase diagram differs from one proposed previously for $DC_{15}PG$/DMPC large multilamellar vesicles (Lentz et al., 1982a) only in the current proposal of immiscible gel phases and in the somewhat higher phase transition temperatures in the presence of fragment 1 plus Ca^{2+}.

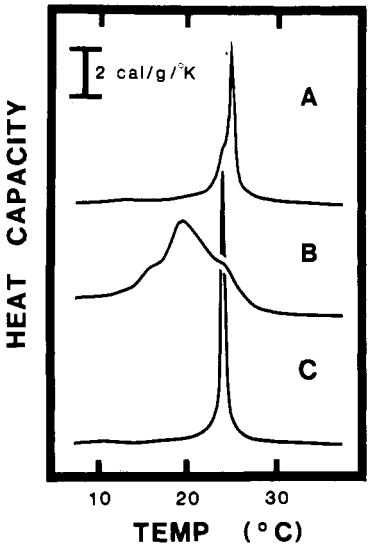

FIGURE 7: Temperature dependence of the specific heat capacity of DMPC vesicle samples. Trace A was obtained by heating a mixture of DMPC SUV (1.7 mM), prothrombin fragment 1 (0.38 mM), and Ca^{2+} (5 mM) at a rate of 15 °C in our high-sensitivity differential scanning calorimeter. The previous cooling scan (−16.8 °C/h) showed a single peak at 26 °C. The reason for the low-temperature shoulder observed on heating is not known, although it was reproducible in subsequent heating scans. Trace B was recorded during a cooling experiment performed on DMPC SUV (6.2 mM) at a rate of −16.8 °C/h. Subsequent heating scans showed a dramatic increase in the minor 24 °C shoulder due to fusion of SUV below their phase transition (Suurkuusk et al., 1976). Trace C (heating scan) shows the specific heat capacity of DMPC large unilamellar vesicles prepared by octyl glucoside dialysis (Parente & Lentz, 1984) so as to demonstrate the similarity to the behavior of DMPC SUV in the presence of fragment 1 plus Ca^{2+} (trace A). The cooling scan was essentially identical. Sample volumes were in all cases 1.5 mL. Curves A and C are plotted according to the scale marker shown in the figure, while curve B is plotted according to a 5-fold expanded scale.

Ca^{2+} on pure DMPC SUV, differential scanning calorimetric measurements were performed on DMPC SUV in the presence and absence of fragment 1 plus Ca^{2+}. The results are presented in Figure 7 along with results obtained, for comparison,

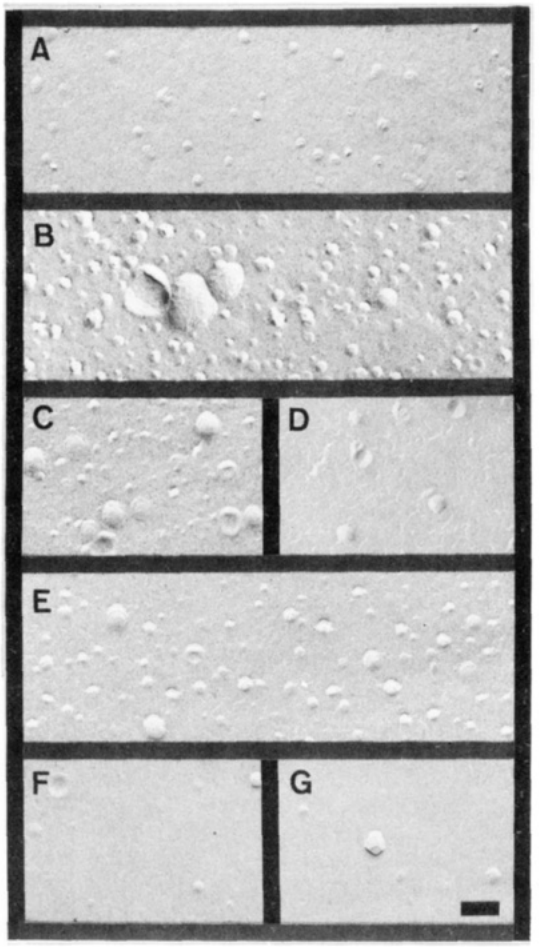

FIGURE 8: Electron micrographs of freeze–fracture replicas prepared from SUV vesicles quenched from 55 °C. Magnification 37500×. Samples were all 10 mM in phospholipid and, where indicated, 0.1 mM in fragment 1 and 5 mM in Ca^{2+}. (A) DMPC SUV; (B) DMPC SUV plus fragment 1; (C) DMPC SUV plus fragment 1 plus Ca^{2+}; (D) 50 mol % $DC_{15}PG$/DMPC SUV plus fragment 1; (E) 50 mol % $DC_{15}PG$/DMPS SUV; (F and G) 50 mol % $DC_{15}PG$/DMPC SUV plus fragment 1 plus Ca^{2+}. Fractured samples were platinum shadowed at an angle of 45°. Micrographs are shown with shadowing from below.

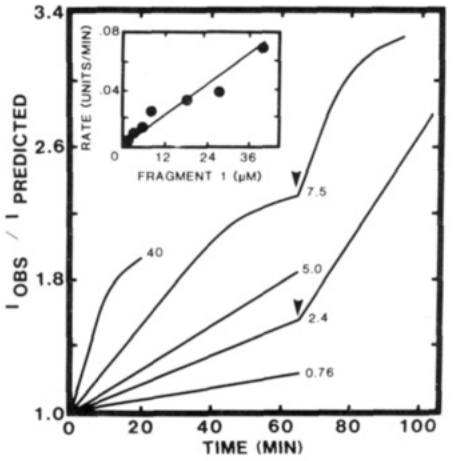

FIGURE 9: Time course of increase in 90° light scattering from DMPC SUV preparation following addition of prothrombin fragment 1 (plus Ca^{2+} at 5 mM) to the concentrations indicated in the figure. The quantity plotted on the ordinate is the observed intensity of scattered light divided by the expected intensity in the absence of any interaction between fragment 1 and the vesicles. The inset shows a plot of the initial rate of increase in scattering intensity (arbitrary units) vs. fragment 1 concentration. At the points indicated by arrows, Na_2-EDTA was added to a final concentration of 83 mM.

with DMPC LUV. These data corroborate the fluorescence results shown in Figure 4 in demonstrating the substantial influence of fragment 1 plus Ca^{2+} on the phase behavior of pure DMPC SUV. Indeed, comparison of traces A and C of Figure 7 reveals that DMPC SUV in the presence of fragment 1 plus Ca^{2+} behave much more like LUV than SUV.

Freeze–Fracture Electron Microscopy. The dramatic change in phase behavior occasioned by the presence of the protein could be due to vesicle fusion or aggregation induced by fragment 1 or fragment 1 plus Ca^{2+}. To test for this possibility, SUV samples were incubated as described for fluorescence experiments with fragment 1 or fragment 1 plus Ca^{2+}, jet frozen from 55 °C (incubation time 4–6 min), and fractured. Electron micrographs of platinum-shadowed replicas of fractured samples are shown in Figure 8. Frames A–C of this figure reveal clearly that fragment 1 (with or without Ca^{2+}) caused DMPC SUV to fuse to LUV. By contrast, frames D–G demonstrate that the peptide caused no appreciable aggregation or fusion of vesicles containing an equimolar mixture of DMPC and $DC_{15}PG$. This result is consistent with the report of Dombrose et al. (1979) that the size of dioleoylphosphatidylglycerol/dioleoylphosphatidylcholine SUV was not altered as a consequence of fragment 1 binding in the presence of Ca^{2+}. Frames F and G of Figure 8 contrast the appearance of concave and convex fracture faces, respectively, of 50 mol % $DC_{15}PG$/DMPC SUV in the presence of fragment 1 and Ca^{2+}. As seen by comparing frames F and G with D or E, convex fracture faces displayed a "ridged" morphology only in the presence of fragment 1 plus Ca^{2+}. We observed the same ridged, convex fracture–face morphology in the case of 67 mol % $DC_{15}PG$/DMPC LUV, for which the ridges were even more pronounced (data not shown). Ridged fracture–face morphology was not observed for pure DMPC vesicles in the presence of fragment 1 plus Ca^{2+}.

To test for fusion products below the phase transition, 67 mol % $DC_{15}PG$/DMPC SUV were incubated and fractured at 22.5 °C (data not shown). In agreement with previous results (Lentz et al., 1982a), these were almost universally converted to LUV (600–900-Å diameter) under these conditions. However, in the presence of fragment 1 or fragment 1 plus Ca^{2+}, the majority of vesicles (95%) remained as small vesicles (200–300-Å diameter). The remaining vesicles (5%) had diameters of 800–1200 Å.

Light Scattering To Detect Fragment 1 Induced Fusion of DMPC SUV. Since 90° light scattering was used successfully to detect irreversible changes in $DC_{15}PG$/DMPC SUV preparations upon binding of fragment 1 below the phospholipid phase transition (Table I), the technique was applied to define the circumstances that led to fragment 1 induced DMPC vesicle fusion above the phase transition temperature. Substantial changes in 90° light scattering intensity occurred over a period of 20–60 min when fragment 1 plus Ca^{2+} was added to 0.1 mM DMPC SUV in the presence of 5 mM Ca^{2+} (Figure 9). Similar results were obtained with three separate preparations of fragment 1, one prepared by us and two by Dr. Richard Hiskey's laboratory (Chemistry Department, UNC). However, additional and more rapid increases in light scattering intensity were recorded after adding Na_2EDTA (final concentration 83 mM) to the same samples (see Figure 9). These observations qualitatively agree with the freeze–fracture electron micrographs shown in Figure 8, where fragment 1 in the presence of 0.1 mM Na_2EDTA produced more extensive fusion (Figure 8B) in a 20–30-min incubation period than did fragment 1 plus 5 mM Ca^{2+} (Figure 8C). In similar light scattering experiments, Na_2EDTA alone produced

no change in scattering intensity of DMPC SUV after 3 h.

The initial rate of DMPC SUV fusion (detected by light scattering) increased as a function of fragment 1 concentration (Figure 9, insert). The effect of fragment 1 was not saturable up to 40 μM fragment 1, suggesting that a low-affinity, nonspecific interaction of fragment 1 with DMPC SUV accounts for the observed fusion.

Discussion

Our analysis of the interactions of prothrombin fragment 1 with anionic phospholipid membranes has taken advantage of the phase behavior of these membranes as a tool for detecting and characterizing the interactions. Our studies have addressed two major issues: first, whether and to what extent lateral phospholipid domains form as a consequence of fragment 1 binding and, second, whether the interaction has a Ca^{2+}-independent or nonelectrostatic component. As will be discussed below, our results argue against the occurrence of extensive lateral domains in response to fragment 1 binding but strongly support the existence of a heretofore undemonstrated nonelectrostatic component to the interaction.

Lack of Lateral Domains. A principal focus of this work has been to test the hypothesis that the Ca^{2+}-mediated binding of either prothrombin or prothrombin fragment 1 to charged phospholipid vesicles induces the formation of lateral domains of specific composition that are best suited to protein binding (Barton & Findlay, 1976; Nelsestuen, 1978; Dombrose et al., 1979). This hypothesis has its origin in the "Ca^{2+}-bridge" model for the interaction of prothrombin with a negatively charged bilayer (Nelsestuen, 1978; Dombrose et al., 1979). Our approach has been to test whether thermodynamically defined, compositionally distinct phases can be shown to form in response to Ca^{2+}-mediated binding of fragment 1 to $DC_{15}PG$/DMPC SUV (e.g., see Figure 1A). If such phases were to form, a temperature–composition phase diagram for the $DC_{15}PG$/DMPC vesicles in the presence of Ca^{2+} and prothrombin fragment 1 should show one of several possible shapes that define a special compositional domain. The most obvious possibilities are (1) a simple maximum or minimum in the phase diagram for situations in which fragment 1 binding does not produce coexisting solid or liquid-phase solutions or (2) a eutectic point for a situation in which fragment 1 binding induces two partially miscible solid phases—i.e., with and without fragment 1 bound. For both possibilities, one would expect to find the phase diagram in the presence of fragment 1 plus Ca^{2+} substantially different from the phase diagram characterizing the phase behavior of pure $DC_{15}PG$/DMPC SUV.

The phase diagram (Figure 6) obtained in the presence of prothrombin fragment 1 and Ca^{2+} was different in several ways from that previously reported (Lentz et al., 1982a) for small vesicles alone. While these differences have important implications in terms of understanding the nature of fragment 1/membrane interactions (see below), none of these differences clearly point to the occurrence of specific compositional phases induced by fragment 1 plus Ca^{2+}. Membrane phase behavior should reveal structurally altered bilayer regions containing as few as 30–100 phospholipid molecules (Lentz et al., 1985). On this basis, we may conclude that the binding of fragment 1 in the presence of Ca^{2+} does not cause the formation of extensive membrane regions (larger than about 30–100 lipid molecules) having a unique structure and composition specifically suited to prothrombin binding. This effectively eliminates models A and B of Figure 1 from further consideration. This does not imply that fragment 1 cannot gather negatively charged lipids into local, microscopic domains under individual protein molecules (e.g., models C or D of Figure 1). These microdomains might be too small (containing less than roughly 30 lipid molecules and having a cross-section of roughly 40–50 Å) or too transient to be detected as separate phases, even by DPH, which is capable of reporting local membrane structure (Lentz et al., 1978). The question of whether fragment 1 induces lateral inhomogeneities in the bilayer on this scale remains uncertain until it is addressed with other techniques.

We note that others have used shifts in lipid-phase behavior to conclude that prothrombin (Mayer & Nelsestuen, 1981), protrhombin fragment 1, and blood coagulation factors X and V (Mayer & Nelsestuen, 1983) induce laterally separated phases upon binding to phosphatidylserine/phosphatidylcholine vesicles. As for our results, those of Mayer and Nelsestuen are thermodynamic in nature and, therefore, bear only on the formation of extensive membrane regions. Rather than constructing a complete phase diagram, however, those authors reported the phase behavior of vesicles of mainly a single composition (at most, two compositions). It is clear from our complete phase diagrams (Figures 5 and 6) that a shift in phase behavior may occur without there being evidence for lateral phase separation.

For the analysis outlined above to be valid, sufficient fragment 1 must be present in a vesicle suspension to saturate surface binding sites. Under these conditions, at every composition, all potentially affected lipid will be associated with bound prothrombin fragment 1. In this way, complications of interpretation associated with variations in the degree of saturation with changes in membrane composition are avoided. To assure that saturating conditions were used, a ratio of fragment 1 concentration to lipid concentrations was chosen on the basis of published binding constants to dioleoylphospholidylglycerol/dioleoylphosphatidylcholine membranes (Dombrose et al., 1979)), so that charged membranes of all compositions studied were between 95 and 99% saturated with bound fragment 1. Using light scattering techniques, we have shown (Table I) that binding to our $DC_{15}PG$/DMPC vesicles is nearly 1 order of magnitude tighter than reported by Dombrose et al. for the dioleoyl phospholipids. Thus, sufficient fragment 1 was used in all experiments that better than 98% of potential binding sites on vesicle surfaces should be occupied. In addition, our results have shown that the dissociation constant for binding of fragment 1 to $DC_{15}PG$/DMPC vesicles was essentially the same whether the binding occurred at a temperature above, within, or below the phospholipid phase transition (Table I). As such, the shapes of $DC_{15}PG$/DMPC vesicle phase transitions reflect the interaction of fragment 1 plus Ca^{2+} with the membrane rather than the dissociation or association of the peptide as a result of the membrane passing through its phase transition.

As an aside, we note that there have been several reports of extrinsic membrane proteins inducing lateral phospholipid domains upon binding to mixed charged/neutral bilayers [e.g., Birrell & Griffith (1976), Hartmann & Galla (1978), and Wiener et al. (1983)]. Our results demonstrate that a simple shift in phase transition temperature [e.g., Wiener et al. (1983) and Mayer & Nelsestuen (1981, 1983)] cannot be taken as evidence for such domain formation and that the general concept of lateral domain formation in response to extrinsic membrane proteins should be more carefully examined.

Nonelectrostatic Interactions. Fragment 1 (with or without Ca^{2+}) interacts with and mofifies the phase behavior of $DC_{15}PG$/DMPC SUV in several ways, which can be summarized as follows. First, in the presence or absence of Ca^{2+},

fragment 1 increased the cooperativity of the order/disorder phase transition experienced by $DC_{15}PG$/DMPC SUV, so that they underwent a transition comparable to that observed for $DC_{15}PG$/DMPC LUV. This suggests that fragment 1 interacts with SUV in such a way as to relieve the constraints of high membrane curvature. Second, in the presence of Ca^{2+}, fragment 1 shifted the main phase transition of both $DC_{15}PG$/DMPC SUV and LUV to higher temperature. This means that fragment 1 (plus Ca^{+}) enhances the lateral packing of phospholipids on the bilayer. The phase transition temperature of uncharged DMPC vesicles remained unchanged relative to its value with fragment 1 alone. Third, fragment 1 (in the presence of Ca^{2+}) prevents fusion of $DC_{15}PG$/DMPC SUV when cooled below their phase transition. This explains the low contamination of SUV by LUV detected by freeze–fracture electron microscopy below the phase transition (5%; see Results). However, note that SUV first cooled below their phase transition and then titrated with fragment 1 plus Ca^{2+} became substantially contaminated by LUV (25%; see Table I), due to the documented (Lentz et al., 1982b) instability of PG-rich SUV below their phase transition. Finally, in the presence or absence of Ca^{2+}, but more rapidly in the absence of Ca^{2+}, fragment 1 caused DMPC (but not $DC_{15}PG$/DMPC) SUV to fuse to form LUV. Altogether, these observations imply a set of interactions of prothrombin fragment 1 with negatively charged membranes that are much more complex than predicted by the commonly accepted Ca^{2+}-bridging model.

It is widely accepted that both Ca^{2+} and negatively charged phospholipids are necessary for the binding of prothrombin fragment 1 with a membrane (Jackson et al., 1975; Zwaal, 1978; Nelsestuen, 1978). For this reason, it has generally been assumed that the interaction between fragment 1 and a membrane involved a Ca^{2+}-bridging mechanism (Resnick & Nelsestuen, 1980; Dombrose et al., 1979). Two features of our results, however, argue that Ca^{2+} bridging is not the sole mechanism of interaction of fragment 1 with membranes. First, fragment 1 alone (in the presence of 0.1 mM Na_2EDTA) substantially increased the cooperativity of the $DC_{15}PG$/DMPC SUV phase transition [compare Figure 5 with Figure 6 of Lentz et al. (1982a)], implying that there is an aspect to the interaction of fragment 1 with a charged membrane that is independent of Ca^{2+} and, therefore, cannot involve Ca^{2+} bridging. Because this interaction so completely alleviated the effects of membrane curvature in SUV, the mechanism of interction may well involve some penetration by fragment 1 of at least the upper portions of the bilayer (see schematic models B, D, and E of Figure 1). Presumably, these interactions are also responsible for the shoulder and dual peaks apparent in the DPH microviscosity activation energy plots (Figure 3) for vesicles of high $DC_{15}PG$ content. These dual peaks may reflect different interactions of fragment 1 with the inner and outer leaflets of small unilamellar vesicles or simply partial saturation of the potential interaction sites.

Second, and equally intriguing, is the observation that fragment 1, in the presence or absence of Ca^{2+}, caused uncharged DMPC SUV to fuse into large unilamellar vesicles (Figure 7 and Figure 8). Fusion occurred more rapidly in the absence of Ca^{2+} than in its presence (Figure 8), which may reflect the self-association of bovine prothrombin fragment 1 in the presence of Ca^{2+} (Prendergast & Mann, 1979), making it less available for interaction with the membrane. In any case, the fragment 1 must have interacted with the DMPC SUV in order to have induced their fusion, demonstrating that even charged phospholipids are not necessary for certain aspects of the prothrombin fragment 1 membrane interaction. It is worth noting that the enhancement of transition cooperativity and the induction of DMPC SUV fusion were caused by fragment 1 in both its Ca^{2+}-bound and unbound conformational states (Marsh et al., 1979). It has been suggested previously that fragment 1 might interact with a lipid bilayer through a hydrophobic portion of the peptide exposed only in its Ca^{2+}-bound conformation (Madar et al., 1982). This suggestion appears inconsistent with our observations.

In summary, our results argue strongly for a nonelectrostatic, Ca^{2+}-independent contribution to the mechanism of interaction of fragment 1 with a charged membrane (i.e., models B, D, and E of Figure 1). This does not deny the existence of an electrostatic contribution (i.e., Ca^{2+} bridging). In addition, our results also argue against the formation of expensive lipid domains in response to binding of fragment 1 in the presence of Ca^{2+} (i.e., models A and B of Figure 1). Therefore, we conclude that models D and E of Figure 1 best summarize our current understanding of the mechanism of interaction of prothrombin (or prothrombin fragment 1) with a negatively charged phospholipid membrane.

ACKNOWLEDGMENTS

We are grateful to Dr. Dan Powers for reading and criticizing the manuscript and to Dr. Richard Hiskey for a gift of fragment 1 used in the later stages of this work. We especially thank Dr. Matt Höechli for help with the electron microscopy and Brett Ayers for help with some experiments.

Registry No. DMPC, 13699-48-4; $DC_{15}PG$, 98361-88-7; Ca, 7440-70-2; prothrombin fragment 1, 72270-84-9.

REFERENCES

Barrow, D. A., & Lentz, B. R. (1980) *Biochim. Biophys. Acta 597*, 92–99.

Barton, P. G., & Findlay, E. J. (1976) in *The Significance of Platelet Function Tests in the Evaluation of Hemostatic and Thrombotic Tendencies* (Day, H. J., Holmsen, H., & Zucker, M. B., Eds.) pp 461–470, DHEW Publication NIH 78-1087, DHEW, Washington, DC.

Birrell, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925–2929.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Dombrose, F. A., Gitel, S. N., Zawalich, K., & Jackson, C. M. (1979) *J. Biol. Chem. 254*, 5027–5040.

Hartmann, W., & Galla, H.-J. (1978) *Biochim. Biophys. Acta 509*, 474–490.

Jackson, C. M., Esmon, C. T., Gitel, S. N., Owen, W. G., & Henriksen, R. A. (1975) in *Prothrombin and Related Coagulation Factors* (Hemker, H. C., & Veltkamp, I. J., Eds.) pp 59–88, Lieden University Press, Leiden, The Netherlands.

Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4521–4537.

Lentz, B. R., Friere, E., & Biltonen, R. L. (1978) *Biochemistry 17*, 4475–4480.

Lentz, B. R., Alford, D. R., & Dombrose, F. A. (1980a) *Biochemistry 19*, 2555–2559.

Lentz, B. R., Barrow, D. A., & Hoechli, M. (1980b) *Biochemistry 19*, 1943–1954.

Lentz, B. R., Alford, D. R., Hoechli, M., & Dombrose, F. A. (1982a) *Biochemistry 21*, 4212–4219.

Lentz, B. R., Madden, S., & Alford, D. R. (1982b) *Biochemistry 21*, 6799–6807.

Lentz, B. R., Clubb, K. W., Alford, D. R., Höchli, M., & Meissner, G. (1985) *Biochemistry 24*, 433–442.

Madar, D. A., Sarasua, M. M., Marsh, H. C., Pedersen, L. G., Gottschalk, K. E., Hiskey, R. G., & Koehler, K. A. (1982) *J. Biol. Chem. 257*, 1836–1844.
Marsh, H. E., Scott, M. E., Hiskey, R. G., & Koehler, K. A. (1979) *Biochem. J. 183*, 513–517.
Mayer, L. D., & Nelsestuen, G. L. (1981) *Biochemistry 20*, 2456–2463.
Mayer, L. D., & Nelsestuen, G. L. (1983) *Biochim. Biophys. Acta. 734*, 48–53.
Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648–5656.
Nelsestuen, G. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2621–2625.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.
Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H., & Bloomfield, V. A. (1981) *Biochemistry 20*, 351–358.
Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594–605.
Parente, R., & Lentz, B. R. (1984) *Biochemistry 23*, 2353–2362.
Patel, K. M., Morrisett, J. D., & Sparrow, J. T. (1979) *J. Lipid Res. 20*, 674–677.
Pletcher, C. H., Bouhoutsos-Brown, E. F., Bryant, R. G., & Nelsestuen, G. L. (1981) *Biochemistry 20*, 6149–6155.
Plopis, V. A., Strickland, D. K., & Castellino, F. J. (1981) *Biochemistry 20*, 15–21.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.
Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028–3033.
Suttie, J. P., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1–70.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393–1401.
Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., & Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta 610*, 559–571.
Wiener, J. R., Wagner, R. R., & Freire, E. (1983) *Biochemistry 22*, 6117–6123.
Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta 515*, 163–205.

Human α-Thrombin Binding to Nonpolymerized Fibrin–Sepharose: Evidence for an Anionic Binding Region†

Lawrence J. Berliner* and Yoshiaki Sugawara‡
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
John W. Fenton II
The Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201
Received April 26, 1985

ABSTRACT: In order to investigate ligand binding sites in α-thrombin that interact with nonpolymerized fibrin, fibrinogen was conjugated (with CNBr) to Sepharose 4B and converted to the nonpolymerized fibrin resin with α-thrombin. Human α-thrombin was bound to the resin at 22 °C and eluted with a linear NaCl gradient [50–300 mM in 50 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.6] with midpeak elution occurring at an ionic strength that corresponds to 170 ± 5 mM NaCl. Among various ligands examined, ATP and its analogues caused α-thrombin to elute with 125 mM or less salt. Apparent dissociation constants were estimated by the dependence of elution volume on ligand concentration. The most potent ligands for desorption from the column were anionic (e.g., adenine nucleotides), which also inhibit thrombin esterolytic/amidolytic and clotting activity [Conery, B. G., & Berliner, L. J. (1983) *Biochemistry 22*, 369–375]. The desorption series was at 10 mM concentrations: ATP = ADP > pyrophosphate > citrate > oxalate > PO_4^{3-}. Contrastingly, serotonin and related apolar compounds did not cause dissociation of α-thrombin from the fibrin resin, even though several of these substances inhibit fibrinogen clotting and esterolytic/amidolytic activities of the enzyme. These data imply that independent sites for apolar and anionic binding in α-thrombin are required for converting fibrinogen into clottable fibrin and that α-thrombin–fibrin binding involves an anionic site.

Thrombin has central bioregulatory functions in thrombosis and hemostasis. Upon generation during vascular injury, procoagulant α-thrombin interacts with platelets, endothelial cells, fibrinogen, and other components of the coagulation system (factors V, VIII, and XIII, protein C, etc.) to cause the formation of a hemostatic plug (Seegers, 1977; Fenton, 1981).

The conversion of fibrinogen by α-thrombin into clottable fibrin monomers occurs first at the Arg-16–Gly-17 bond in the Aα chains and subsequently at the Arg-14–Gly-15 bond in the Bβ chains with the release of fibrinopeptides A and B, respectively (Blombäck et al., 1978). Whereas the fibrinopeptides are small and formed as the second products of enzymic catalysis, the fibrin monomer is large and constitutes the first product of peptide bond cleavage. The monomer subsequently polymerizes to form fibrin strands, where polymerization is generally the rate-limiting process of clotting

†Supported in part by USPHS Grants HL 24549 (L.J.B.) and HL 13160 (J.W.F.) from the National Heart, Lung and Blood Institute. A preliminary report of this work was presented at the Xth International Society of Thrombosis and Hemostasis Congress, San Diego, CA, July 1985.
‡On leave from the Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu, Japan.

0006-2960/85/0424-7005$01.50/0