Phospholipid Lateral Organization in Synthetic Membranes As Monitored by Pyrene-Labeled Phospholipids: Effects of Temperature and Prothrombin Fragment 1 Binding†

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Received June 13, 1985

ABSTRACT: Pyrene-labeled phospholipids have been used to test for the existence of lateral domains due to temperature-induced phase separations and binding of prothrombin fragment 1 to charged lipid vesicles. When in close proximity, pyrene-containing probes can exchange excited-state energy to form excimers; the ratio of the excimer to monomer fluorescence intensity (E/M) is proportional to the local concentration of probe in the membranes, as well as to the excimer lifetime and the probe's lateral diffusion coefficient. The ability of the pyrene-labeled phospholipids to quantitatively report the coexistence of multiple environments was demonstrated in dipalmitoylphosphatidylcholine/palmitoyloleoylphosphatidylcholine multilamellar vesicle preparations of varying compositions, each of which contained coexisting fluid and gel phases. In this system, pyrene-labeled phosphatidylcholine was found to favor the fluid relative to the gel phase with a partition coefficient of 7. At 37 °C, in dioleoylphosphatidylglycerol (DOPG)/palmitoyloleoylphosphatidylcholine (POPC) large, unilamellar vesicles containing either pyrene-labeled phosphatidylglycerol (py-PG) or pyrene-labeled phosphatidylcholine (py-PC), the excimer lifetime (37 ns) and the lateral diffusion constant of the probe (5.8×10^{-8} cm^2/s) were independent of the membrane composition and the presence of fragment 1 and Ca^{2+}. Consequently, E/M was directly proportional to only the local concentration of the py-PG or py-PC probes. When saturating amounts of fragment 1 and 5 mM Ca^{2+} were added to DOPG/POPC vesicles that contained either probe, no change in E/M and hence the local probe concentration was observed. These results have been interpreted in terms of a model in which fragment 1 binds (probably via Ca^{2+} bridges) to less than 5–10 DOPG molecules, rather than inducing an extensive DOPG-rich binding domain.

The excited pyrene molecule can exist as either a monomer or an excited dimer (excimer), and the two forms of the excited molecule display different fluorescence properties, including different emission maxima. Changes in the ratio of the intensities of the excimer and monomer (E/M) have been taken as a qualitative reflection of changes in the probe's environment. Accordingly, pyrene-labeled phospholipids and fatty acids have already been used to detect temperature-induced lateral phase separations in membranes of mixed composition (Galla & Hartmann, 1980; Hresko et al., 1985) as well as changes in lipid-phase transitions associated with the interaction of membranes with Ca^{2+} (Galla & Sackmann, 1975), poly(L-lysine) (Hartmann & Galla, 1978), and certain extrinsic proteins such as the matrix protein of vesicular stomatitis virus (Weiner et al., 1983; J. R. Wierner, R. Pal, Y. Barenholz, and R. R. Wagner, unpublished results). However, since the E/M ratio is directly proportional to the probe's local concentration (Birks et al., 1963), it should be possible to interpret changes in this property of the probe quantitatively in terms of the extent of temperature-induced phase separation or of the formation of protein-induced domains in membranes.

The existence of temperature-induced lateral phase separations in mixed lipid bilayers has been established by freeze–fracture electron microscopy in several systems (Grant et al., 1974; Luna & McConnell, 1978; Lentz et al., 1980). The composition of such coexisting macroscopic domains can be documented by construction of phase diagrams (Shimshick & McConnell, 1973). In this paper, we demonstrate that the E/M ratio of pyrene-containing phospholipid probes can be used to predict correctly the presence of laterally separated phases in mixed phospholipid membranes.

Prothrombin is an extrinsic, γ-carboxyglutamic acid (Gla)[1] containing protein that, upon activation to thrombin by the prothrombinase complex, catalyzes the proteolysis of fibrinogen to fibrin in the blood coagulation cascade [for a recent review, see Zwall (1978)]. The Ca^{2+}-dependent membrane binding of prothrombin and its N-terminal activation peptide (fragment 1) requires the presence of both intact Gla residues and negatively charged phospholipids (Magnusson et al., 1974). The most widely accepted model for the mechanism of binding of prothrombin to membranes proposes that Ca^{2+} links or "bridges" the Gla residues of the fragment 1 region to the head groups of anionic phospholipids (Lim et al., 1977; Dombrose et al., 1979). Mayer & Nelsestuen (1981, 1983), after noting that prothrombin changed the phase behavior of two anionic membranes, extended the Ca^{2+}-bridge model and proposed that lateral phase separation of charged lipids occurred in conjunction with binding. We have recently shown that, although fragment 1 altered the phase behavior of phosphati-

† This work was supported in part by USPHS Grant GM 32707.

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; py-PC, 1-palmitoyl-2-(10-pyrenyldecanoyl)-3-*sn*-phosphatidylcholine; py-PG, 1-palmitoyl-2-(10-pyrenyldecanoyl)-3-*sn*-phosphatidylglycerol; REV, reverse-evaporation, large, unilamellar vesicles; LMV, large, multilamellar vesicles; EDTA, ethylenediaminetetraacetic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Gla, γ-carboxyglutamic acid; E/M, excimer to monomer fluorescence intensity ratio; HPLC, high-pressure liquid chromatography.

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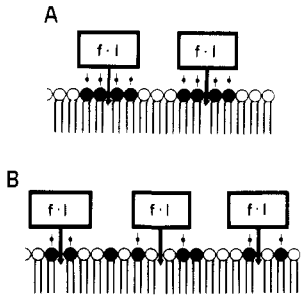

FIGURE 1: Models for interaction of prothrombin fragment 1 (f·1) with membranes containing a mixture of charged (filled head group) and neutral (open head group) phospholipids in the presence of Ca^{2+} (small closed circles with vertical bar). Both models presume some interactions involving Ca^{2+} bridging and a Ca^{2+}-independent interaction perhaps involving bilayer penetration (arrow) (Lentz et al., 1985). Model A envisions the formation of small domains (20–30 lipids) under each bound protein. Model B presumes no domain formation and binding of only a few (less than 5–10) PG molecules to each fragment 1 molecule.

dylglycerol/phosphatidylcholine (PG/PC) membranes, a complete temperature/composition phase diagram gave no indication of fragment 1 induced lateral domains containing greater than approximately 30–100 lipids (Lentz et al., 1985). We concluded that the best model for the interaction between fragment 1 and membranes includes contributions from both nonelectrostatic and Ca^{2+}-bridging mechanisms (see Figure 1) and does not involve extensive lateral reorganization of anionic phospholipids. Due to the detection limits of our thermodynamic approach, we could not distinguish between the possibilities that fragment 1 either induces the formation of small, lateral domains (roughly 30 lipids; Figure 1A) or binds tightly to too few anionic lipids to result in detectable domains (Figure 1B). In this paper, we report the use of pyrene-labeled phospholipids with either anionic or neutral head groups to probe the effect of fragment 1 binding on the lipid organization within PG/PC bilayers. We conclude from our results that fragment 1 binds to less than 5–10 molecules of DOPG (Figure 1B) rather than inducing an extensive DOPG-rich binding domain.

Materials and Methods

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC), and 1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG) were purchased from Avanti Biochemicals, Birmingham, AL. 1-Palmitoyl-2-(10-pyrenyldecanoyl)-3-*sn*-phosphatidylcholine (py-PC) and 1-palmitoyl-2-(10-pyrenyldecanoyl)-3-*sn*-phosphatidylglycerol (py-PG) were purchased from KSV Chemicals Oy, Helsinki, Finland. All lipids were judged to be greater than 98% pure on the basis of thin-layer chromatography on silica gel GHL impregnated with 0.01 M dipotassium oxalate (Analtech, Newark, DE), eluting with chloroform/methanol/water (65/25/4 v/v/v). Py-PG and py-PC exhibited extinction coefficients at 342 nm in ethanol of 51 000 and 53 000 $L \cdot mol^{-1} \cdot cm^{-1}$, respectively. Lipids were stored under an argon atmosphere in the dark in either chloroform/methanol (1/1) (POPC, DPPC, and DOPG) or toluene/ethanol (1/1) (py-PC and py-PG) at −70 °C at concentrations ranging from 3 to 30 mM.

All chemicals including ethylenediaminetetraacetic acid (EDTA) and 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) were ACS reagent grade or the best available grade; all solvents were HPLC grade. Type V poly(L-lysine) (17 000 molecular weight) was obtained from Sigma.

Protein. Bovine prothrombin fragment 1 was graciously supplied by Dr. Richard Hiskey of the UNC Chemistry Department. The protein solution was brought to 1 M in EDTA to complex free and protein-bound Ca^{2+} and then was exhaustively dialyzed against 100 mM NaCl, 10 mM TES, 25 μM EDTA, and 0.02% NaN_3, pH 7.4. All fragment 1 preparations were judged pure by polyacrylamide (11%)/sodium dodecyl sulfate (0.1%) gel electrophoresis and exhibited from 42 to 48% quenching of tryptophan fluorescence in the presence of 5 mM Ca^{2+} (Prendergast & Mann, 1977). Protein concentration was determined spectrophotometrically at 280 nm with an extinction coefficient of 1.05 $mL \cdot mg^{-1} \cdot cm^{-1}$ (Mann, 1976).

Vesicles. Phospholipid vesicles were prepared in the TES buffer (see above) and stored above their phase-transition temperatures in buffer (see above) that had been degassed with mild heating and nitrogen bubbling for at least 2 h. Large, unilamellar vesicles (REV) were prepared by reverse-phase evaporation as described by Szoka et al. (1980) and were partially fractionated by extrusions through 0.4- and 0.2-μm polycarbonate membranes. Large, multilamellar vesicles (LMV) were prepared as described by Lentz et al. (1980). During the preparation of vesicles containing py-PC or py-PG, care was taken to minimize sample exposure to light. Total phosphate concentrations were determined by using the assay of Chen et al. (1956).

Light Scattering Measurements. The binding constants for fragment 1 to DOPG/POPC vesicles at 37 °C were measured in the presence of 5 mM Ca^{2+} by the light scattering technique of Nelsestuen & Lim (1977). Prothrombin fragment 1 was preincubated with 2 mM Ca^{2+} at 37 °C for 20 min and then added sequentially with stirring to a 1-mL fluorescence cuvette containing 0.05–0.10 mM lipid and 5 mM Ca^{2+}. Scattering intensities were corrected for the scattering of unbound protein and otherwise analyzed essentially by the method of Nelsestuen & Lim (1977) by using a molecular weight of 22 000 for bovine fragment 1 (Mann, 1976). The binding constants (K_d) so obtained were used to estimate the amount of fragment 1 required to saturate the vesicles (K_d values used included 0.2 μM for >67 mol % DOPG, 0.4 μM for 60 mol % DOPG, 0.7 μM for 35 mol % DOPG, and 5 μM for 10 mol % DOPG).

Fluorescence Measurements. All fluorescence measurements were made with an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL) equipped with a modified, multitemperature cuvette holder (Barrow & Lentz, 1983). Samples and buffers were thoroughly deoxygenated and saturated with nitrogen prior to measurements, and a nitrogen atmosphere was maintained within the fluorometer sample chamber.

(*A*) *Lifetime measurements* were performed by the phase and modulation method of Birks et al. (1963), with excitation at the 313-nm mercury line of a 200-W mercury–xenon light source (Canrad-Hanovia, Newark, NJ). Emission of excimer was detected with a 3-mm high-pass KV-475 filter, and emission of monomer was detected with a combination of a 33-mm high-pass KV-389 filter and a 3-mm band-pass BG-12 filter (Schott Optical Glass, Duryea, PA). A single polarizer was set at 35° from vertical in the excitation path, and pyrene-containing samples were examined at a modulation frequency of 6 MHz at 37 °C.

(*B*) *Excimer* (*E*) *and monomer* (*M*) *fluorescence intensity measurements* were made on samples (10–12.5 μM phospholipid) containing varying amounts of pyrene-labeled lipid (normally between 0.50 and 3.25 mol %). Unless otherwise noted in the text, all measurements were made at 37 °C. The

excitation wavelength was 313 nm, and emission wavelengths were 400 (monomer) and 470 nm (excimer). Both excitation and emission slits were set at 4 nm. Measured intensities were corrected as necessary for background fluorescence of the buffer and/or unlabeled vesicles. Each measurement was repeated at least 3 times. For a given sample, the precision in the ratio of the excimer to monomer fluorescence intensities (E/M) was better than ±3%. The average standard deviation determined for several single probe concentrations in several vesicle preparations was ±8% of the mean value. We note that systematic errors could limit the accuracy of measurement beyond ±8%, but these would affect only the absolute values of estimated diffusion coefficients (see eq 2) but not our ability to detect changes in E/M reflective of domain formation.

Calculation of Diffusion Constant. E/M was related to the concentration of pyrene-labeled phospholipid in the membrane (c) by using eq 1 (Galla & Sackmann, 1974) based on the kinetic model of Birks et al. (1963). This model presumes that excimer formation is a diffusion-controlled process. Taking into account that excimer loss occurs by radiative and nonradiative decay and not by reversal of the formation process at temperatures less than approximately 60 °C (Förster, 1969), eq 1 derives from the free diffusion model. The quantities

$$E/M = [\kappa(k_f'/k_f)A]\tau k_a c \quad (1)$$

in brackets in eq 1 are constants: κ is a proportionality constant relating the measured intensity ratio to the quantum yield ratio (Birks et al., 1963); k_f'/k_f is the ratio of the probabilities of radiative decay of the excimer to the monomer and is equal to 7.14 (Sengupta et al., 1976); A is a geometric constant peculiar to a membrane, which converts the mol % concentration of probe to surface density. The quantities outside the brackets are variables in each experiment: τ is the fluorescent lifetime of the excimer; k_a is the second-order rate constant for excimer formation. The value of 0.65 was determined for κ on our spectrofluorometer by calculating the proportionality constant relating the ratio of the integrated areas of the corrected frequency spectra for monomer and excimer to the ratio of the relative intensities measured at 470 and 400 nm. The lateral diffusion constant (D_{diff}) is related to k_a as

$$D_{diff} \cong \tfrac{1}{4}k_a \quad (2)$$

assuming that the critical interaction distance for the onset of excimer formation is equal to the length of a diffusional jump in a membrane lattice and that the rate of excimer formation is totally diffusion controlled (Galla & Sackmann, 1974).

Results

Detection of Phase Transitions Using Pyrene Probes. The temperature dependencies of the E/M ratios of py-PC in fluid-phase vesicles (POPC) and in vesicles undergoing a gel-to-fluid phase transition (DPPC) are illustrated in Figure 2. For the DPPC vesicles, the midpoint in the change of E/M occurred at 39.0 °C, whereas the phase-transition temperature measured by differential scanning calorimetry is 41.3 °C (Lentz et al., 1978). The increase in E/M below the phase transition for DPPC cannot reflect more rapid lateral diffusion, since diffusion is known to be slowed in the gel phase (Wu et al., 1977). It has been suggested that the increased E/M in the gel phase reflects either local probe-rich domains or orientational effects peculiar to the highly ordered structure of the gel (Galla & Sackmann, 1974). These considerations restrict the application of eq 2 for the estimation of the probe's diffusion constant to membranes in the fluid state.

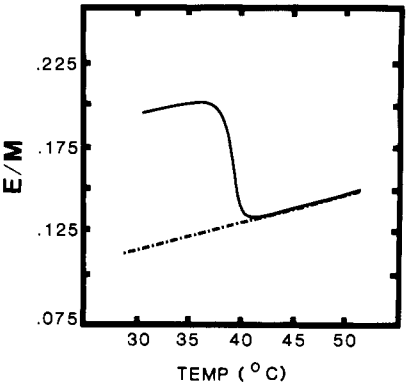

FIGURE 2: Dependence of E/M ratio on phase transition: E/M ratio of 2 mol % py-PC as a function of temperature in DPPC (solid line) and POPC (dashed line) LMV. Data were collected every 0.5 °C while cooling at a rate of 30 °C/h.

Quantitative Detection of Phase Separation. (A) Mixed Vesicle Population. For pyrenedecanoic acid, the E/M ratio observed for a vesicle in which the probe can reside in two environments obeys the empirical rule

$$(E/M)_{obsd} = x_1(E/M)_1 + x_2(E/M)_2 \quad (3)$$

where x_1 and x_2 represent the local concentrations (mole fractions) of probe in each of the two environments and where $(E/M)_1$ and $(E/M)_2$ represent the excimer to monomer fluorescence intensity ratios observed for the two different environments (Galla & Sackmann, 1975). We have tested this relationship for pyrene-labeled phospholipids by studying mixtures of gel-phase and fluid-phase vesicles, each containing py-PC. At 37 °C, vesicles containing 2 mol % py-PC in either DPPC (gel-like; see Figure 2) or POPC (fluid-like) were mixed in different ratios such that the final concentration of lipid (and hence probe) remained constant for all mixtures. Under these conditions, x_1 and x_2 of eq 3 corresponded to the mole fractions of DPPC and POPC, respectively, and $(E/M)_1$ and $(E/M)_2$ corresponded to the eximer to monomer intensity ratios for 2 mol % py-PC in DPPC and POPC, respectively. The measured E/M ratios for these different vesicle mixtures agreed with those predicted from application of eq 3 (correlation coefficient = 0.998), demonstrating the validity of eq 3 for mixtures of gel- and fluid-phase membrane environments containing known amounts of a pyrene-labeled phospholipid.

(B) Single Vesicle Population. Using a vesicle system in which temperature-induced lateral phase separation is known to occur (Davis et al., 1980), we have verified that the pyrene probe is also capable of detecting coexisting domains within a membrane (eq 3). At 37 °C, large multilamellar vesicles (LMV) composed of between 80 and 100 mol % DPPC mixed with POPC contain coexisting gel and fluid phases. The E/M ratios were obtained for vesicles composed of pure POPC, pure DPPC, 85 mol % DPPC, 90 mol % DPPC, and 95 mol % DPPC and containing varying concentrations of py-PC. These data are plotted as a function of total probe concentration in Figure 3. In the cases of vesicles containing both gel and fluid phases, E/M for a given probe concentration increased as the fraction of gel phase increased. We note that a linear relationship between E/M and probe concentration exists for gel-phase (empirical relationship), fluid-phase (predicted by eq 1), and mixed-phase membranes.

As seen in Figure 4, the E/M values for py-PC in phase-separated membranes (95, 90, and 85 mol % DPPC in DPPC/POPC LMV) closely agreed with those values predicted according to eq 3. The "observed E/M" values at 1.0, 2.0, and 3.0 mol % py-PC for each phase-separated membrane

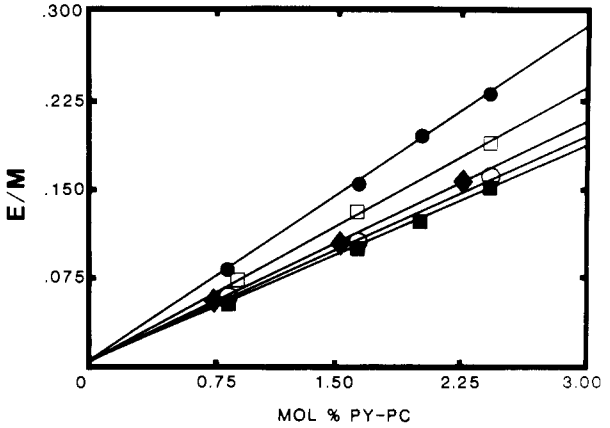

FIGURE 3: Linear dependence of E/M on pyrene probe concentration. E/M ratios at 37 °C are plotted as a function of mol % py-PC in DPPC/POPC LMV containing 100 mol % DPPC (closed circles), 95 mol % DPPC (open squares), 90 mol % DPPC (closed diamonds), 85 mol % DPPC (open circles), and 100 mol % POPC (closed squares). The least-squares line of best fit is shown for each vesicle preparation. Note that the E/M ratio for gel-phase vesicles (closed circles) is larger than that for fluid-phase vesicles (closed squares), consistent with the data in Figure 2.

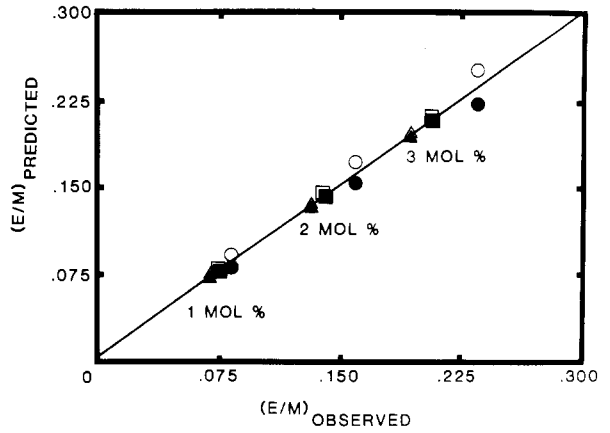

FIGURE 4: Verification of eq 3 for phases within a membrane. Observed and predicted E/M ratios are compared for py-PC in DPPC/POPC mixtures containing 95 (circles), 90 (squares), and 85 (triangles) mol % DPPC at three different probe concentrations. Predictions were made according to eq 3, assuming the gel phase was either 100 (open symbols) or 97 mol % DPPC (closed symbols) and using a fluid/gel partition coefficient of 7. The correlation coefficient is 0.99.

were taken from the appropriate line of best fit for E/M vs. probe concentration (Figure 3). "Predicted E/M" values from eq 3 were obtained with $(E/M)_1$ and $(E/M)_2$ values equal to those observed for the pure gel (DPPC) and pure fluid (POPC) phases (x_1 and x_2 of eq 3) could be obtained from the known compositions of the gel and fluid phases as determined from the published phase diagram of DPPC/POPC LMV (Davis et al., 1980) and from the partition coefficient for py-PC between fluid and gel phases. While the low-phase-transition temperature reported by py-PC for DPPC LMV (Figure 2) is consistent with preferential partitioning of the probe into the fluid phase, no quantitative estimate of the partition coefficient has been reported. For this reason, estimates for the probe partition coefficient were systematically varied, and resultant values for x_1 and x_2 were used to calculate E/M ratios. The mean square deviation between the observed and calculated E/M ratios was minimized when a partition coefficient of 7 was used, consistent with partitioning strongly in favor of the fluid phase. Our ability to predict the observed E/M ratio for phase-separated vesicles from the properties and

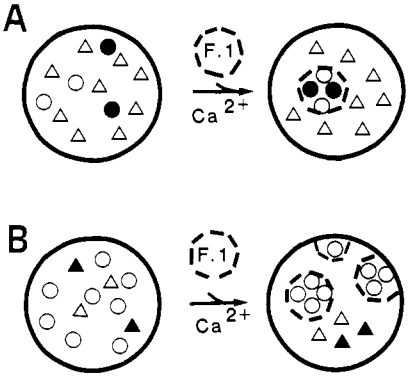

FIGURE 5: Hypothetical effect of fragment 1 (F·1) binding on lateral lipid distribution in DOPG/POPC vesicles. These illustrations assume that a lateral phase separation of only charged lipids occurs in the presence of fragment 1 and Ca^{2+} in vesicles containing DOPG (open circles) and POPC (open triangles) with either py-PG (A, closed circles) or py-PC (B, closed triangles). In both types of experiments, the final local-probe concentration is increased relative to that in the entire vesicle due to sequestering within the domain (A, py-PG) or exclusion from the domain (B, py-PC).

concentrations of the probe within the two coexisting phases, in conjunction with eq 3, confirms that pyrene-labeled phospholipids can report the presence of multiple local domain within a membrane.

Test for Fragment 1 Induced Domains. (A) Rationale. The rationale for detecting fragment 1 induced domains is that segregated pyrene-labeled phospholipids should reflect the change in local concentration by a change in the observed E/M ratio (eq 1). If fragment 1 in the presence of Ca^{2+} induces in DOPG/POPC vesicles the formation of local regions of high concentration of DOPG, experiments can be designed so as to maximize the ability to detect this sequestering of the anionic lipid. For example, in a membrane containing 10 mol % DOPG with 1 mol % py-PG, the formation of a fragment 1 induced, pure DOPG domain would increase the local concentration of py-PG 10-fold from 1 to 10 mol % (see Figure 5A). Similarly, the local concentration of py-PC (1 mol %) in a membrane containing 10 mol % POPC with 90 mol % DOPG would also increase 10-fold from 1 to 10 mol % if protein-induced, pure DOPG domains were formed (Figure 5B). In the case of py-PG, the probe would directly report the formation of the protein-induced domain; in the case of py-PC, the probe would indirectly detect the domain by virtue of its exclusion from that region. If the fragment 1 induced domains did not contain exclusively the anionic lipid species (as illustrated for simplicity in Figure 5), the current experimental approach would still reveal domain formation, with the observed E/M ratio reflecting the composition of domains (see below). For this experimental approach to be sound, the other variables of eq 1 (τ and k_a) must be independent of the presence of fragment 1 and of membrane composition.

(B) Controls. The rationale for the use of pyrene-labeled phospholipids to detect protein-induced domains presumes that the protein remains associated with the bilayer for a time much longer than the jump diffusion time for a lipid molecule moving laterally in the plane of the membrane (roughly 10^{-8} s). For prothrombin (and presumably prothrombin fragment 1 as well), the dissociation rate from an anionic membrane is roughly 3 s^{-1} (Wei et al., 1982), easily meeting this condition.

The lifetimes of both py-PC and py-PG probes were equal and independent of membrane composition (average value of 37 ± 2 ns as measured in a series of large, unilamellar vesicles composed of different mixtures of DOPG with POPC). The presence of saturating (>95% sites occupied) amounts of

fragment 1 in the presence of 5 mM Ca^{2+} also did not affect the lifetime of either py-PG in pure DOPG vesicles (37 ± 1 and 38 ± 1 ns in 99% DOPG/1% py-PG vesicles in the presence and absence of fragment 1) or py-PC in DOPG-containing vesicles (37 ± 1 and 38.5 ± 1 ns in 80% DOPG/19% DOPC/1% py-PC vesicles in the presence and absence of fragment 1).

In order to establish the influence of membrane composition on the bimolecular constant (k_a), a value for k_a was calculated according to eq 1 for vesicles composed of different DOPG/POPC molar ratios and containing varying concentrations of pyrene probe. That value was used to calculate the probe's lateral diffusion constant by eq 2. The diffusion coefficients of both py-PC and py-PG were independent of membrane composition with an average value of (5.8 ± 0.4) × 10^{-8} cm^2/s, which is within the range of values reported for diffusion of phospholipids above their phase transitions (Wu et al., 1977).

To establish whether the bimolecular rate constant for excimer formation was influenced by the presence of fragment 1, saturating amounts of the protein and 5 mM Ca^{2+} were added to pure DOPG vesicles containing py-PG. Since the binding of protein to these pure PG membranes could not be accompanied by a change in local probe concentration and since excimer lifetime was independent of the presence of fragment 1, any change in E/M would have to reflect a changed bimolecular rate constant (see eq 1) and, therefore, an altered lateral diffusion constant (see eq 2). No change in E/M was observed (0.093 and 0.097 in the presence and absence, respectively, of saturating levels of fragment 1), indicating that the diffusion of py-PG was not significantly decreased by the presence of fragment 1 and that gel-like domains did not form in response to the binding of the peptide.

Finally, light scattering binding experiments were carried out with vesicles prepared from mixtures of POPC with either 15 mol % py-PG or 15 mol % DOPG. Within experimental error, dissociation constants obtained from both systems were identical (6 ± 4 μM for py-PG and 2.4 ± 0.4 μM for DOPG), demonstrating that py-PG can substitute for unlabeled PG in terms of supporting the binding of prothrombin fragment 1.

We conclude from the control experiments that a local increase in probe concentration should be detectable by an increase in E/M since both τ and k_a are independent of the presence of fragment 1 and of membrane composition. A linear, composite plot of E/M vs. membrane pyrene phospholipid content (in mole percent) was constructed to establish the sensitivity of the E/M ratio to probe concentration (slope = 0.056 ± 0.003, intercept = 0.0016 ± 0.0002). The scatter in the data of this composite plot indicated that a change in E/M of greater than 0.03 unit (corresponding to a change in local probe concentration of 0.5 mol %) could be easily detected.

(*C*) *Test for Fragment 1 Induced Domains.* The ability of fragment 1 with Ca^{2+} to induce formation of a lateral domain was tested by comparing the E/M ratio of a vesicle alone (containing a given probe concentration) to that observed when the vesicle was saturated with protein. Complementary experiments (i.e., use of py-PG and py-PC) were used to guard against misinterpretation of results due to anomalous behavior of one of the probes.

In Figure 6, the observed E/M ratios for samples containing py-PG (frame A) and py-PC (frame B) are compared to the E/M ratio that would be expected if domains of the indicated compositions were to form in response to binding of fragment 1. The solid line of Figure 6A shows the E/M ratios predicted

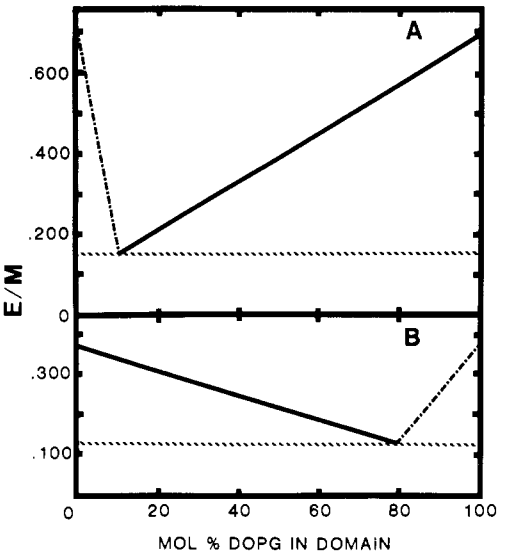

FIGURE 6: Expected E/M ratios assuming fragment 1 induced phase separation occurs to induce formation of domains of the indicated DOPG content. The horizontal hatched lines indicate the E/M ratios measured for DOPG/py-PG/POPC (7.73/2.27/90) REV (frame A) and DOPG/POPC/py-PC (80/17.85/2.15) REV (frame B) alone or in the presence of 5 mM Ca^{2+} or fragment 1 plus 5 mM Ca^{2+}. The fact that only one horizontal line is drawn per frame indicates that, well within experimental precision (±0.01), the E/M ratio did not change on addition of either Ca^{2+} alone or Ca^{2+} plus fragment 1. The predicted E/M ratios were calculated by using the established properties of these probes in membranes, under the assumptions that only the outer leaflet of the membrane would be involved in a phase separation, that all separated domains would be fluid (i.e., that E/M could increase only through local increases in probe concentration), that partitioning between domains would be random, that the vesicle preparation contained 10% contamination with bilamellar vesicles, and that the probe was either included (solid line) or excluded (dashed lines) from the domain of a given composition.

for the case in which the py-PG probe is located in fragment 1 induced domains containing varying mole percentages of DOPG (experiment diagrammed in Figure 5A). The dashed line in Figure 6A illustrates the predicted E/M ratio for the situation in which py-PG would be excluded from the fragment 1 induced domains, a situation not anticipated by the model in Figure 5A. Experiments with py-PC (Figure 6B) were designed to detect domains formed as a result of PC being excluded from PG-rich, fragment 1 induced domains (see Figure 5B). The E/M ratios predicted for one such experiment as a function of domain composition are illustrated by the dashed line in Figure 6B.

For the two experiments illustrated in Figure 6, E/M ratios measured for vesicles alone, for vesicles in the presence of 5 mM Ca^{2+}, and for vesicles in the presence of saturating amounts of fragment 1 plus Ca^{2+} were all identical and are indicated by the horizontal hatched lines intersecting the V-shaped thereotical curves at their minima. Had PG-rich domains been formed in response to fragment 1 binding, the observed E/M ratio would have increased so that the horizontal hatched lines in Figure 6 would have intersected the theoretical curves and indicated the compositions of the PG-rich and PG-poor domains, respectively. In addition to the experiments reported in Figure 6, other experiments were performed as follows: 2 mol % py-PC in vesicles containing 35 mol % DOPG; 2 mol % py-PG in vesicles containing 58 mol % DOPG; 4 mol % py-PG in vesicles containing 6 mol % DOPG. For each of these samples, the E/M ratios also were not altered by the addition of 5 mM $CaCl_2$ or saturating amounts of fragment 1 in the presence of 5 mM $CaCl_2$. Since the combination of these five complementary experiments

should have detected domains of virtually any composition, we must conclude that binding of prothrombin fragment 1 to mixed DOPG/POPC membranes does not induce lateral separation of the different phospholipid species to form domains.

Test for Poly(L-lysine)-Induced Domains. By comparison with the effects of fragment 1 on the lateral distribution of PG in membranes, we have considered another extrinsically bound polypeptide [poly(L-lysine)] that has been reported to induce lateral domain formation in phosphatidic acid containing membranes (Hartmann & Galla, 1978) and to alter the phase behavior of PG-containing membranes (Papahadjopoulos et al., 1975). In an experiment analogous to that illustrated in Figures 5B and 6B, the addition of poly(L-lysine) (equimolar concentration of protein and lipid) to DOPG/POPC (80:20) vesicles containing py-PC also failed to produce a detectable change in E/M ratio (data not shown).

Discussion

Partitioning of Pyrene-Labeled Phospholipids. Our ability to predict the E/M ratio in DPPC/POPC large, multilamellar vesicles containing coexisting phases required that the partition coefficient of the probe between gel and fluid states be known. We have previously reported the preferential partitioning of phospholipid probes with bulky groups attached to the fatty acid (diphenylhexatriene-labeled lipids) into fluid-phase domains (Parente & Lentz, 1985). In addition, our data (Figure 2) show that phase transitions monitored by pyrene-labeled lipids appear to occur at temperatures lower than those measured by differential scanning calorimetry (Lentz et al., 1978). This result is similar to that obtained with diphenylhexatriene-labeled phospholipid (Parente & Lentz, 1985) and is consistent with preferential partitioning of the pyrene phospholipid probes into the fluid phase. Our estimate of the partition coefficient ($K_{f/s} = 7$) not only agreed with these expectations but also allowed us to predict reasonably accurately the E/M ratio in membranes containing various ratios of solid-like and fluid phases (Figure 4).

We note that our predictions of E/M ratios upon binding of prothrombin fragment 1 in the presence of Ca^{2+} (Figure 6) did not take into account possible preferential partitioning of the probe between protein-associated and free lipid domains. This assumption is justified for two reasons. First, our studies of the effect of fragment 1 on pure DOPG vesicles indicated that the rate of lateral diffusion and, hence, the state of the bilayer were not affected by fragment 1 binding. Second, our use of complementary experiments involving both py-PG and py-PC (Figures 5 and 6) would have revealed any anomalies resulting from preferential partitioning.

Molecular Model for Prothrombin Fragment 1 Binding. We have shown in this work that pyrene-labeled phospholipids can be used successfully to detect lateral phase separations in membranes (Figure 4) but that such lateral domains do not form in response to the binding of prothrombin fragment 1 (Figure 6). This conclusion, based on a spectroscopic technique, extends the conclusion that we had earlier drawn from experiments using thermodynamic methods (Lentz et al., 1985) and calls into serious question the lateral phase separation model proposed by Mayer & Nelsestuen (1981, 1983) for the effect of fragment 1 binding on the local lipid organization in a mixed membrane containing both neutral and charged lipids.

(A) Limits of Resolutions. This conclusion must be considered in light of the limits of resolution inherent in our spectroscopic measurements. The pyrene probes have monomer fluorescence lifetimes of 120 ns and lateral diffusion constants (based on two different models of pyrene diffusion) of between 5.8×10^{-8} (Galla & Sackmann, 1974) and 1.3×10^{-7} cm^2/s (Galla et al., 1979).[2] Using these physical properties of the pyrene probes, we estimate that the probes can traverse between roughly 4 and 10 lattice points during their lifetime. One might expect that the lateral mobility of anionic pyrene-containing phospholipids would be slowed somewhat by their interaction with fragment 1 and would experience a jump frequency near the lower limit of this range. Assuming that a protein-induced domain were to be approximately circular, one calculates that a protein-induced, anionic lipid domain containing roughly 50 lipid molecules should cause a detectable increase in the E/M ratio. This detection limit is consistent with the observation that temperature-induced, lateral phase separations (producing domains on the order of 1000 Å in diameter) were easily detected (Figure 4). This calculation estimates the size of a domain that would appear limitless (i.e., macroscopic) to a pyrene-containing phospholipid and places an upper limit on the size of domains that would be detectable by our methods. Somewhat smaller domains should be detectable but could not be quantitatively described by eq 3.

The model that we have used to interpret observed E/M ratios in terms of phospholipid lateral diffusion coefficients assumes that excimer formation is limited only by lipid diffusion (Birks et al., 1963; Galla & Sackmann, 1974; Galla et al., 1979). This assumption has recently been challenged by Eisinger et al. (1985). However, the use of other mathematical models would not have changed our final conclusion, i.e., that no substantial domains are formed in response to fragment 1 binding. Nonetheless, different models for lateral diffusion do differ in their estimates of the local lipid lateral diffusion constant (5.8×10^{-8} and 1.3×10^{-7} cm^2/s) and, consequently, of the size of the smallest domain (50–300 lipids) for which eq 3 might be expected to be valid [i.e., the upper limit of detection (see above)]. Wu et al. (1977) have reported a lateral diffusion constant of 8×10^{-8} cm^2/s in a fluid PC membrane at 37 °C for another fluorescently labeled phospholipid. By using this value determined by fluorescence photobleaching, the upper limit for the size of a detectable domain would be close to 50 lipids.

In order to estimate the lower limit of detection, we must consider how many PG molecules would have to be gathered into a domain in order that there be a high probability of at least two probes being in the same domain. This probability will depend both on the ratio of labeled to unlabeled PG in a membrane and on the composition of the hypothetical domain. For the experiment reported in Figure 6A (2.27 mol % py-PG, 7.73 mol % DOPG), a pure PG domain of only 10 molecules should provide a high probability of excimer formation, at least immediately following excitation of one pyrene monomer. A domain composed of 50 mol % PG would have to contain 20 molecules to provide a reasonable opportunity for excimer formation. These examples illustrate that the limit of detection could be lowered by increasing the membrane content of py-PG. However, the range of usable concentrations of probe is dictated by the range of validity of eq 1. We have restricted our studies to 2–4 mol % pyrene-labeled lipid, for which eq 1 is clearly valid. Experiments performed with membranes containing 4 mol % py-PG and 6 mol % DOPG extend our lower limit of resolution down to domains containing 5–10 molecules. Attempts to extend the limits of

[2] The random walk procedure used to obtain this estimate of the diffusion coefficient has been shown to overestimate the diffusion coefficient (Eisenger et al., 1985).

resolution even lower would be of questionable worth, since eq 1 would no longer be valid, and this new lower limit would be substantially below the upper limit imposed by the lifetime and diffusion rate of the pyrene-labeled lipids.[3]

Taking into account the factors discussed above, we estimate that a fragment 1 induced PG-rich domain containing 10–50 phospholipid molecules should be detectable by the methods described here. Since the projected area of fragment 1 would cover roughly this same number of lipids in a monolayer [Dombrose et al. (1979) estimate 20–30 lipids], we conclude that fragment 1 does not induce a PG-rich domain upon binding to PG/PC membranes. Consequently, we can rule out model A in Figure 1 for the mechanism of binding.

(*B*) *Extension to Other Extrinsic Membrane Proteins.* In addition to prothrombin fragment 1 (Mayer & Nelsestuen, 1981, 1983), other charged, extrinsic membrane proteins [e.g., cytochrome *c* (Birrell & Griffith, 1976), poly(L-lysine) (Hartmann & Galla, 1978), and the matrix protein of vesicular stomatitus virus (Wiener et al., 1983, 1985)] as well as Ca^{2+} (Galla & Sackmann, 1975) have been suggested to induce the separation of anionic phospholipid domains in membranes. These suggestions have often been based on observations of shifts in phase-transition temperature in the presence of either the externally supplied protein or metal ion. As noted under Results, we have used our pyrene probe techniques to check for domain formation accompanying fragment 1, poly(L-lysine), or Ca^{2+} binding to DOPG-containing vesicles at temperatures well above their phase transition and found no evidence for PG-rich domains. Also using pyrene-containing probes, Weiner et al. (1985) report that the matrix protein induces PG-rich domains only in the region of the gel-to-fluid phase transition of DPPC/dipalmitoylphosphatidylglycerol membranes. However, as with fragment 1 (Lentz et al., 1985), these results could reflect broadening of the phase transition in response to the binding of matrix protein. Significantly, when these authors added matrix protein to fluid-phase vesicles composed of POPC/palmitoyloleoylphosphatidylglycerol, they observed no significant change in the E/M ratio of py-PC, in agreement with our results with prothrombin fragment 1 (see Figure 6). Although Weiner et al. (1985) did not measure excimer lifetime or probe diffusion coefficient values that would allow us to draw a definite conclusion, their observation of E/M ratio invariance is in agreement with our observations and suggests that the matrix protein, too, does not induce membrane domain formation. We must conclude that the general concept of lateral domain formation in response to the binding of a highly charged, extrinsic membrane protein or Ca^{2+} should be reevaluated. In particular, it is not possible to presume that lateral domains will be induced in a fluid-phase membrane on the basis of the observation of a shift in phase behavior of a mixed fluid- and solid-phase membrane.

(*C*) *Speculations on the Mechanism of Prothrombin Fragment 1 Binding to Membranes.* The results presented here as well as other recently completed studies (Lentz et al., 1985) have refined our concept of the structure and dynamics of extrinsic protein–membrane complexes in general and of prothrombin fragment 1–Ca^{2+}–PG/PC membrane complexes in particular. First, if binding of fragment 1 does induce PG-rich domains, these domains are certainly not extensive protein–lipid patches (Lentz et al., 1985). Further, the binding of fragment 1 does not appear to gather together even as many as 5–10 PG molecules, as judged by the pyrene studies reported here. A previous report has suggested that roughly 20 PG molecules are somehow associated with each bound fragment 1 molecule (Dombrose et al., 1979). This estimate was obtained from the dependence of binding capacity on membrane PG content and reflects the total membrane PG content needed to achieve binding rather than the number of PG molecules bound to or associated with each protein. This latter number, based on our results, is apparently less than 5–10 PG molecules.

If as many as 10 PG molecules were to bind to each fragment 1 molecule, the observation that fragment 1 did not alter the rate of py-PG lateral diffusion in pure DOPG bilayers would imply that PG molecules bound to fragment 1 must move off of a binding site at a rate comparable to the rate at which they move onto a binding site. This conclusion derives from the precision of our E/M measurements, which, under these conditions (i.e., 10 PGs bound per protein), would detect a 2–3-fold alteration in the diffusion rate due to protein binding relative to the rate of free lipid diffusion. Thus, PG would be expected to be bound to fragment 1 with an apparent two-dimensional binding constant (i.e., PG free $\rightleftarrows$ PG bound) of less than roughly 2 or 3. On the other hand, if only a few PG molecules were to bind to each fragment 1 molecule, these could bind with relatively high affinity (two-dimensional binding constant > 10) and move laterally in the membrane with their associated protein without this being detected by our measurements.

The picture that emerges is one in which fragment 1 binds (probably via Ca^{2+} bridges) to less than 5–10 PG molecules, each of which is bound with low to moderate affinity to fragment 1 binding sites (probably involving γ-carboxyglutamic acid residues). The free energy of fragment 1 binding is then provided by the sum of these few PG–protein binding energies along with a Ca^{2+}-independent contribution that probably involves some bilayer penetration (Lentz et al., 1985). This model is illustrated in Figure 1B.

ACKNOWLEDGMENTS

We thank Dr. Richard Hiskey for providing purified bovine fragment 1. We also thank Dr. Jan Hermans and Dr. Ken Jacobson for critically reviewing this paper.

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[3] In an experiment to extend our limit of resolution, the binding of prothrombin (plus 5 mM Ca^{2+}) was found not to alter the E/M ratio for vesicles composed solely of py-PG (15 mol %) and DOPC. The E/M ratio observed in this system (0.76) showed some negative deviation from that predicted by eq 1.

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Lipid–Protein Interactions in Cytochrome *c* Oxidase. A Comparison of Covalently Attached Phospholipid Photo-Spin-Label with Label Free To Diffuse in the Bilayer†

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Received August 2, 1985

ABSTRACT: The aim of this study was to clarify the possible origins of the motion-restricted electron spin resonance (ESR) spectral component observed in membranes. For this purpose, a phospholipid photo-spin-label was synthesized, characterized, and used to study lipid–protein interactions in beef heart cytochrome *c* oxidase. The probe was designed with a nitroaryl azide incorporated in the phospholipid head group, and a spin-label on the *sn*-2 side chain, and was radiolabeled. The resulting molecule, 1-palmitoyl-2-(14-proxyl[2-^{3}H]stearoyl)-*sn*-glycero-3-phospho-*N*-(4-azido-3-nitrophenyl)ethanolamine, was stable under subdued light and during the procedures required to reconstitute cytochrome *c* oxidase in phospholipid bilayers. Upon photolysis, the photo-spin-label reacted with the protein in high yields (50% attached). There was no detectable destruction of the spin-label. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cytochrome *c* oxidase after reaction with the photo-spin-label showed highest levels of attachment to bands I, III, and VII, with some labeling of other bands. The labeling pattern demonstrated a distribution of attachment sites, which was needed for the spin-labeling studies. ESR spectra of the attached labels at 25 °C indicated a constant fraction of motion-restricted lipid chains, independent of the lipid to protein ratio. In contrast, a spin-labeled phosphatidylcholine and the prephotolyzed photo-spin-label, both free to diffuse in the bilayer, exhibited behavior in agreement with the multiple equilibria binding model. These results, as well as data obtained with membranes frozen at −196 °C, show how several situations that lead to a motion-restricted ESR line shape can be distinguished. This study provides additional evidence that the fraction of lipids normally in contact with protein, and not aggregation artifacts, accounts for the observed motion-restricted component of ESR spectra of reconstituted cytochrome *c* oxidase in phospholipid bilayers.

Many integral membrane proteins penetrate completely through the fluid phospholipid bilayer, thus creating an interface or boundary where the proteins and the surrounding lipids interact dynamically. This interface is responsible for the high solubility of proteins in membranes and is involved in the maintenance of the permeability barrier. Lipid–protein interactions may also modulate the function of some membrane proteins, influence the organization of membrane proteins

† This work was supported by U.S. Public Health Service Research Grants GM 25698 and 34916.
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0006-2960/86/0425-0574$01.50/0