platelets may be required by, and is consistent with, the relatively high lipid metabolism in this cell [for a review on lipid metabolism in platelets, see Holub (1984)].

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Protein Redistribution in Model Membranes: Clearing of M13 Coat Protein from Calcium-Induced Gel-Phase Regions in Phosphatidylserine/Phosphatidylcholine Multilamellar Vesicles†

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ABSTRACT: A model system of M13 bacteriophage coat protein reconstituted into multilamellar vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) is used to examine protein redistribution in membranes in the presence of Ca^{2+}. The reconstitution procedure is analyzed by using protease digestion and gel permeation chromatography of radioactively labeled coat protein and is found to incorporate coat protein into lipid vesicles predominantly in the in vivo orientation and without aggregation. Quenching of protein tryptophanyl fluorescence by spin-labeled PC is used to determine the local lipid environment of the coat protein in binary lipid mixtures. The distribution of coat protein between fluid liquid-crystal (LC) and Ca^{2+}-induced gel (G) phases in PS/PC multilayers, expressed as a concentration ratio $R_{LC/G}$, is found to be 25 ± 5 in favor of the fluid phase, indicating significant clearing of membrane protein from Ca^{2+}-induced gel-phase regions.

Protein-free membrane contact regions in fusing biological membranes have been observed by electron microscopy by use of fixed and stained thin sections and cryoprotected freeze–fracture (Lawson et al., 1977; Orci et al., 1977; Peixoto de Menezes & Pinto da Silva, 1978; Chi et al., 1979; Kalderon & Gilula, 1979). This finding of extensive, protein-free membrane areas is controversial, being disputed by some workers using rapidly frozen membrane samples without cryoprotectant (Chandler & Heuser, 1979, 1980; Ornberg & Reese, 1981) but verified by others (Morris et al., 1983). Freeze–fracture studies of reconstituted model membranes containing Ca^{2+}-ATPase from sarcoplasmic reticulum (Kleeman & McConnell, 1976) or glycophorin (Grant & McConnell, 1974) showed particle-free regions thought to correspond to patches of thermotropic gel-phase lipid. Portis et al. (1979) have suggested that a rigid complex of Ca^{2+} with acidic phospholipids could form a membrane region that excludes proteins. Numerous studies have confirmed that Ca^{2+} can induce phase separation in mixed lipid systems containing negatively charged phospholipids [see Düzgünes (1985) for a recent review]. A question remains as to the behavior of

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the membrane proteins when gel and fluid lipid phases coexist.

In a previous report from this laboratory, a simple system consisting of the membrane-bound polypeptide gramicidin A′ in 1,2-diacyl-*sn*-glycero-3-phosphatidic acid/1,2-diacyl-*sn*-glycero-3-phosphocholine (PA/PC)[1] vesicles ± Cd^{2+} was studied in order to investigate cation-induced redistribution of membrane components. The model used to analyze the experiments is that Cd^{2+} binding results in coexisting gel and fluid lamellar phases, with the polypeptide distributed in a characteristic way between the phases. EPR spectroscopy and fluorescence quenching measurements were used to establish the identity, composition, and relative amounts of the phases. Fluorescence quenching of the tryptophanyl fluorescence of the polypeptide established a 3-fold difference in polypeptide concentration favoring the fluid phase (Feigenson, 1983). This way of studying cation-induced redistribution of membrane components was then extended to a system of more biological interest, PS/PC multilamellar vesicles ± Ca^{2+}, as a model for the bilayer contact region of fusing biological membranes. Our findings indicate that Ca^{2+}-induced lipid phase separation into a $Ca(PS)_2$ gel phase and a fluid liquid-crystal PS/PC phase results in a pronounced partitioning of several small membrane-bound fluorophores out of the gel phase and into the fluid phase. Only a fluorescent-labeled PS showed significant concentration in both phases (Florine & Feigenson, 1987).

In this paper, we report results of experiments with PS/PC multilamellar vesicles ± Ca^{2+} which contain the membrane-bound coat protein from M13 bacteriophage. M13 coat protein is a 50 amino acid residue polypeptide, containing a single (fluorescent) tryptophanyl residue in the hydrophobic core (Asbeck et al., 1969; Nakashima & Konigsberg, 1974). It is easily obtained from the phage (Makino et al., 1975; Wickner, 1975) and, using a reconstitution method developed in this laboratory (Bayer & Feigenson, 1985), can be readily incorporated into preformed phospholipid vesicles with a high efficiency, without aggregation, and with most molecules spanning the membrane in their native orientation and conformation. We describe a modification of this reconstitution procedure for obtaining multilamellar vesicles without apparent alteration of the physical state of the protein in the bilayers. Using the quenching of protein tryptophanyl fluorescence by spin-labeled phospholipids (London & Feigenson, 1981a,b), we determine the partition behavior of M13 coat protein between coexisting $Ca(PS)_2$ and fluid lipid phases in PS/PC multilayers in the presence of Ca^{2+}.

Experimental Procedures

Materials. Dioleoylphosphatidylserine (DOPS), bovine brain PS (BBPS), and egg PC were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Spin-labeled PC was synthesized with a doxyl free radical on the eighth carbon of the 2-position fatty acyl group by condensation of (7,6)-palmitic acid with egg lyso-PC as described previously (London & Feigenson, 1981a). All lipids were judged to be >98% pure by thin-layer chromatography of 50 μg on Adsorbosil Plus P plates (Applied Science, State College, PA) using chloroform/methanol/water (65:25:4 v/v) and chloroform/methanol/concentrated ammonium hydroxide (66:30:6 v/v). M13 bacteriophage strain mp2 and *Escherichia coli* strain 71-18 were gifts of Dr. Ray Wu of Cornell University. L-[4-^{3}H(N)]Proline, L-[4,5-^{3}H(N)]lysine, L-[^{35}S]methionine, and L-[*methyl*-^{14}C]methionine were obtained from New England Nuclear (Boston, MA). Protease type XI (proteinase K) and Hepes buffer were from Sigma Chemical Co. (St. Louis, MO). Cholic acid from Sigma was decolorized with activated charcoal and recrystallized from 70% ethanol. Water was purified by using a Milli-Q system (Millipore Corp., Bedford, MA). Formic acid (88%) was from Mallinckrodt (Paris, KY), and absolute ethanol was from U.S. Industrial Chemicals Co. (Tuscola, IL). Other chemicals were reagent grade.

Sample Preparation. Phage growth and protein purification were performed as described by Bayer and Feigenson (1985). Both unlabeled and radioactively labeled phage were grown on *E. coli* 71-18 in M9 medium + 10 mM $MgCl_2$ and purified, after centrifugation in order to pellet cells, by precipitation from 5% (w/v) poly(ethylene glycol) + 0.5 M NaCl, followed by CsCl density gradient centrifugation. Cholate-solubilized coat protein was prepared by incubation of buffer solutions of phage with added cholate and chloroform at 37 °C for 1 h, with occasional vortex mixing, followed by chromatography on Sephacryl S-200 equilibrated with cholate-containing buffer to separate solubilized coat protein from DNA. Coat protein containing fractions with an absorbance ratio A_{280}/A_{260} greater than 1.5 were combined, concentrated to 2.5 mg/mL by ultrafiltration, and then stored at 4 °C for up to 1 week.

Coat protein was incorporated into preformed phospholipid vesicles by the freeze–thaw–cholate reconstitution technique (Bayer & Feigenson, 1985). Phospholipid in chloroform was placed in a 13 × 100 mm Pyrex tube and then dried to a thin film under a stream of argon gas followed by 3–4 h of vacuum pumping in the dark. Buffer A (10 mM Hepes, 100 mM KCl, and 0.1 mM EDTA, pH 7.0) was added to give a lipid concentration of 27 mM, and the sample was sonicated to near clarity under argon in a bath sonicator (Laboratory Supplies Co., Inc.). Coat protein in cholate buffer (buffer A + 12 mM cholate) was added such that the protein:lipid molar ratio was 1:100, and the solution was then vortexed. Additional buffer A was added to give a total lipid concentration of 6.75 mM, and the solution was then frozen in liquid N_2, thawed at room temperature, and sonicated for 30 s. Sample volumes were 0.6 mL for fluorescence experiments using unlabeled coat protein and 1 mL for chromatography experiments using labeled coat protein. Cholate was removed by dialysis for 48 h of 5–10 samples against 1–2 L of buffer A at 4 °C in the dark, with 4 changes of buffer. For fluorescence experiments using Ca^{2+}, an additional 24 h of dialysis with two changes of buffer A′ (10 mM Hepes/100 mM KCl, pH 7.0) was used to remove EDTA. Lipid blanks for fluorescence measurements were prepared identically (except that buffer A was substituted for coat protein in cholate buffer in the initial vesicle preparation) and contributed typically 5% of the sample fluorescence signal.

Multilamellar vesicles were prepared from the mostly unilamellar sonicated vesicles by placing the dialyzed samples in 10 × 75 mm borosilicate culture tubes, lyophilizing, and rehydrating in buffer A′ to 6.75 mM lipid. For fluorescence experiments using DOPS/(7,6)PC ± Ca^{2+}, multilamellar dispersions were divided and diluted either with buffer A′ alone or else with buffer A′ + $CaCl_2$, in 10 × 75 mm tubes, to a final concentration of 1 mM lipid ± 20 mM Ca^{2+}. Typical

[1] Abbreviations: PA, 1,2-diacyl-*sn*-glycero-3-phosphatidic acid; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; BBPS, 1,2-diacyl-*sn*-glycero-3-phosphoserine derived from bovine brain; egg PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine derived from hen egg yolks; (7,6)-palmitic acid, 2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy; (7,6)PC, 1-acyl-2-[(7,6)-palmitoyl]-*sn*-glycero-3-phosphocholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid disodium salt; EPR, electron paramagnetic resonance; $R_{LC/G}$, ratio of fluorophore concentration in the fluid liquid-crystal phase to that in the gel phase.

sample volume was 600 μL. The tubes were sealed under argon, placed in a KCl/ice bath (1:4 w/w) at −10 °C (above the highest lipid gel- to fluid-phase transition temperature) for 2 min, and then frozen by nucleating with a pipe cleaner dipped in liquid N_2, touching the outside of the tube just above the meniscus. After an additional 2 min in the KCl/ice bath, samples were thawed in a water bath. A total of 15 freeze–thaw cycles were performed to equilibrate the Ca^{2+} (Feigenson, 1986) and to form a fine dispersion that did not settle during the fluorescence measurements.

Protease Digestions and Gel Chromatography. Following dialysis, 1-mL samples of sonicated vesicles containing radioactively labeled M13 coat protein were incubated with proteinase K (0.5 mg/mL) overnight at 37 °C and then evaporated to dryness in a Savant Speedvac vacuum centrifuge. Digests were then taken up in 210 μL of formic acid and incubated at 37 °C to dissolve PS, after which 490 μL of ethanol was added and the sample loaded on a Fractogel TSK HW40(s) column and eluted with 88% formic acid/ethanol (30:70 v/v) as described by Bayer and Feigenson (1985). Collected fractions were assayed for radioactivity.

Fluorescence Spectroscopy. Fluorescence measurements were performed with a home-built spectrofluorometer (previously described; Caffrey & Feigenson, 1981) utilizing conventional 90° optics and equipped with double monochromators in both excitation and emission optics. Excitation and emission wavelengths were 287 and 340 nm, respectively. Nominal bandwidths were 2 nm for excitation and 16 nm for emission. Fluorescence measurements were made by diluting 1 mM lipid samples into acrylic cuvettes of 1 cm^2 cross section containing the same buffer (buffer A′ ± 20 mM Ca^{2+}) to a final volume of 2 mL and lipid concentration of 100 μM. There was no contribution to the fluorescence signal from light scattering at this lipid concentration. The use of acrylic cuvettes for fluorescence measurements, as well as polypropylene pipet tips (Eppendorf) for sample transfers, was necessitated by the fact that samples, particularly lipid blanks, containing high mole fractions of PS in the presence of excess Ca^{2+} stick to glass (Florine & Feigenson, 1987). Lipid concentration in the fluorescence cuvette was determined by phosphate analysis of aliquots removed from the cuvettes immediately after fluorescence measurements, according to a modification of the methods of Bartlett (1959) and Chen et al. (1956) as described by Kingsley and Feigenson (1979).

Results

Orientation and Aggregation State of Reconstituted M13 Coat Protein. The configuration of coat protein incorporated into sonicated PS vesicles was determined by use of digestion of doubly labeled coat protein with proteinase K followed by gel permeation chromatography on Fractogel TSK in formic acid/ethanol. Figure 1A shows the amino acid sequence of the protein as determined by Asbeck et al. (1969) and Nakashima and Konigsberg (1974). The hydrophobic region which lies within the lipid bilayer and is inaccessible to protease includes Trp-26 through Lys-40 (Chamberlain et al., 1978). Coat protein was labeled at the N-terminus with [^{3}H]proline and at the hydrophobic core with [^{35}S]- or [^{14}C]methionine. In order to quantitate digestion at the C-terminus, coat protein was also prepared labeled with [^{3}H]lysine and [^{14}C]methionine. The elution profiles of doubly labeled coat protein in PS vesicles following protease digestion are shown in Figure 1B–D. On the basis of molecular weight as well as charge effects (Bayer & Feigenson, 1985), aggregated coat protein elutes at the void volume centered at fraction 14, while intact nonaggregated coat protein (in the dimeric form), as well as coat protein with only the C-terminus removed, elutes primarily at fraction 17. The major hydrophobic core peptide, representing digestion at both ends, and also coat protein with only the N-terminus removed elute mainly at fraction 20.

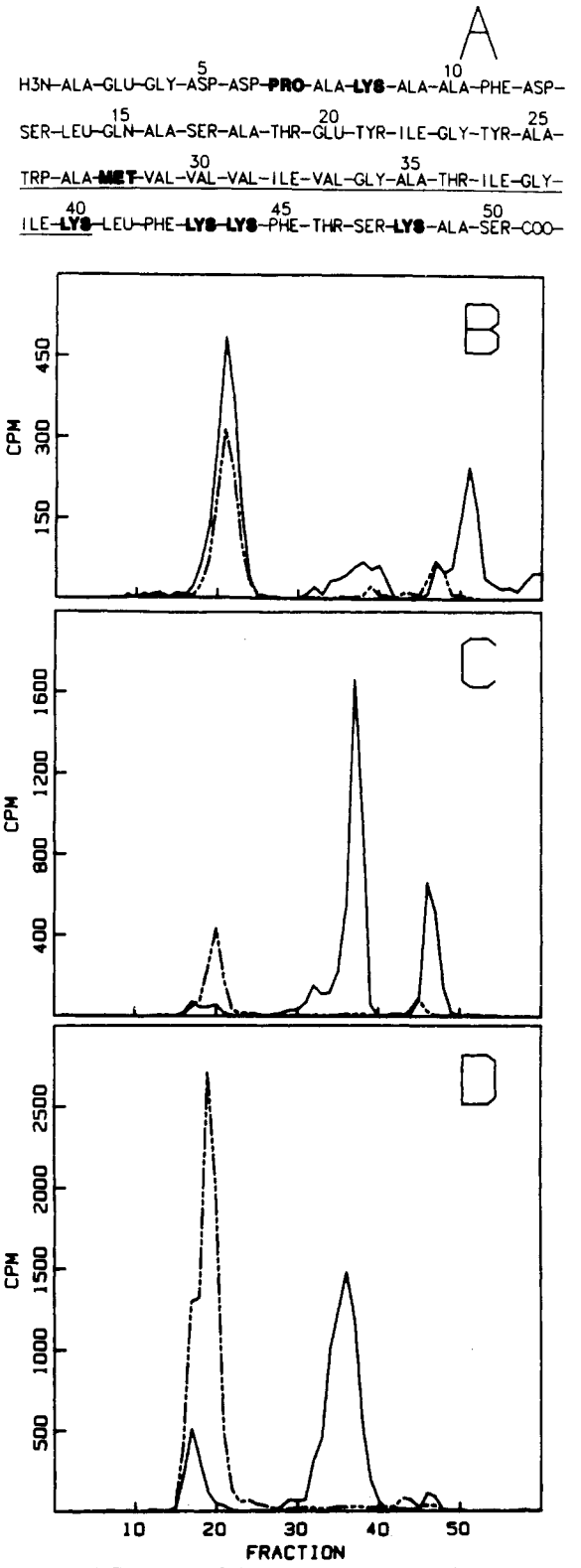

FIGURE 1: (A) Sequence of the M13 coat protein with the lipid-associated hydrophobic core underlined and labeled amino acids used in phage growth shown in boldface type. (B–D) Proteinase K digests of doubly labeled coat protein reconstituted into sonicated vesicles: [^{3}H]Lys,[^{14}C]Met-labeled coat protein in BBPS (B); [^{3}H]-Pro,[^{35}S]Met-labeled coat protein in BBPS (C); [^{3}H]Pro,[^{14}C]-Met-labeled coat protein in DOPS (D). Solid lines represent [^{3}H]lysine or [^{3}H]proline, and broken lines represent [^{35}S]- or [^{14}C]methionine.

Panels B and C of Figure 1 are digests of, respectively, [^{3}H]Lys,[^{14}C]Met-labeled and [^{3}H]Pro,[^{35}S]Met-labeled coat protein incorporated into sonicated BBPS vesicles. In both digests, a negligible amount of material (<1%) elutes at the void volume, indicating little or no coat protein aggregation. The amount of intact (inaccessible) coat protein plus that digested at the C-terminus, contained in fractions 17–18, is in both digests less than 5%. From the ^{3}H/^{14}C ratio of fraction 18 (Figure 1B), no lysine has been removed; hence, virtually no coat protein was oriented with the C-terminus outward. The peak centered at fractions 20–21 has 97% of the proline removed (judging from the ^{3}H/^{35}S ratio) and 34% of the lysine removed. Because coat protein digested only at the N-terminus should have 20% of the lysine removed whereas that digested at both termini should have 80% of the lysine removed, this peak consists mostly of coat protein which spanned the membrane with the N-terminus outward, as in vivo, with a smaller amount incorporated in a U-shape configuration (Wickner, 1976) on the outside of the vesicle. Figure 1D shows a digest of [^{3}H]Pro,[^{14}C]Met-labeled coat protein incorporated into sonicated DOPS vesicles. The amount of proline released (fractions 28–48) is 85%, compared to 95% for the BBPS sample (Figure 1C). The amount of inaccessible coat protein in a digest of 50% BBPS/50% egg PC vesicles was 25% (data not shown), consistent with the results of Bayer and Feigenson (1985) for egg PC and egg PC/egg PA vesicles in which the presence of charged lipid increased the amount of accessible coat protein.

In order to determine the effects of lyophilization and repeated freeze–thaw cycles on the aggregation state of reconstituted coat protein, column runs were performed on multilamellar vesicles not subjected to protease, with and without 15 freeze–thaw cycles. For the protein:lipid molar ratios examined, 1:200 and 1:100, intact (undigested) coat protein in all cases eluted as a single peak centered at fraction 17, with no significant amount of aggregated material eluting at the void volume (data not shown).

Fluorescence Quenching of M13 Coat Protein in Multilamellar Vesicles Containing (7,6)PC. The local lipid environment of a membrane-bound fluorophore can be determined from the contact quenching of fluorescence by nitroxide spin-labeled phospholipids. A fluorescence quenching curve is generated by measuring fluorescence as the mole fraction of spin-labeled lipid in a binary lipid mixture is varied from 0 to 1. In a one-phase fluid system in which there is no exchange of neighboring phospholipids during the fluorescence lifetime of the fluorophore and in addition there is no selective binding of the fluorophore to one of the component lipids, the quenching curve reflects the random distribution of spin-labeled and unlabeled phospholipids around the fluorophore, with the curve described by

$$(F - F_{min})/(F_0 - F_{min}) = (1 - [(7,6)PC])^n \quad (1)$$

where F is the observed fluorescence, F_0 is the fluorescence in the absence of (7,6)PC, F_{min} is the residual fluorescence in pure (7,6)PC, and n is the number of lipids close enough to the fluorophore to result in quenching, i.e., the number of lattice sites adjacent to the fluorophore (London & Feigenson, 1981a).

Figure 2 shows fluorescence quenching data for M13 coat protein in fluid-phase multilamellar vesicles composed of egg PC/(7,6)PC or DOPS/(7,6)PC. The similarity of the two data sets indicates that the coat protein does not show strong binding selectivity between PC and PS, the difference in binding affinities being about 5% in favor of PC. [See London and Feigenson (1981b) for a discussion of binding affinity determination from fluorescence quenching curves.] For both binary lipid systems, the fractional coat protein fluorescence in pure (7,6)PC is $F_{min}/F_0 = 0.01$. Thus, eq 1 becomes

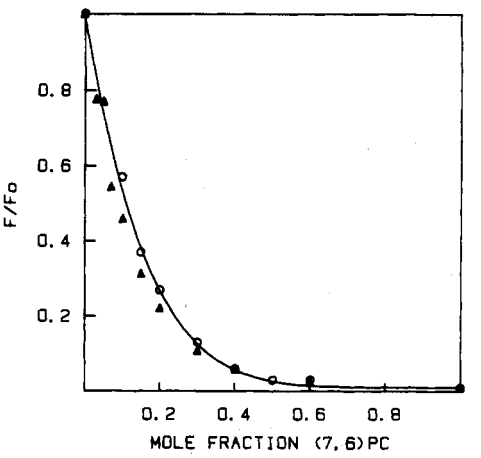

FIGURE 2: Fluorescence quenching of M13 coat protein in egg PC/(7,6)PC (○) or DOPS/(7,6)PC (▲) multilamellar vesicles. The abscissa is the mole fraction of (7,6)PC in the vesicles, and the ordinate is the ratio of fluorescence in the presence (F) and absence (F_0) of (7,6)PC. Symbol height represents the range of triplicate F measurements. The solid line is a theoretical curve calculated according to $F/F_0 = 0.99(1 - [(7,6)PC])^n + 0.01$ for $n = 6$ (see text). Protein:lipid molar ratio is 1:100.

$$F/F_0 = 0.99(1 - [(7,6)PC])^n + 0.01 \quad (2)$$

The data for coat protein in egg PC/(7,6)PC are well fit by $n = 6$ (solid line in Figure 2). For the DOPS/(7,6)PC system, the data fall slightly below the $n = 6$ curve due to the slightly greater binding affinity of coat protein for PC (the quenching lipid) over PS.

Redistribution of M13 Coat Protein in PS/PC Vesicles in the Presence of Ca^{2+}. In PS/PC multilamellar vesicles, Ca^{2+} induces lipid phase separation into a $Ca(PS)_2$ gel phase which excludes PC and a PC-rich fluid liquid-crystal phase. The boundaries of the phase coexistence region for DOPS/(7,6)PC vesicles in excess Ca^{2+} at 23 °C, determined by EPR spectroscopy and X-ray diffraction, are $[(7,6)PC]_G = 0.00$ and $[(7,6)PC]_{LC} = 0.55$–0.60 (Florine & Feigenson, 1987). For M13 coat protein in DOPS/(7,6)PC vesicles, Ca^{2+}-induced lipid phase separation yields a quenching curve that reflects, in the two phase region, the distribution of the protein between the two coexisting phases. The lipid composition of each phase is found to determine the quantum yield of tryptophanyl fluorescence for the protein in that phase. The fluorescence of coat protein in egg PC/(7,6)PC is unaffected by the presence of Ca^{2+}, indicating no direct interaction of the coat protein with Ca^{2+}, nor is it affected by the 15-cycle freeze–thaw process itself. Fluorescence quenching data for coat protein in DOPS/(7,6)PC ± excess Ca^{2+} are shown in Figure 3. In the presence of excess Ca^{2+}, a significant drop in tryptophanyl fluorescence is observed at mole fractions of (7,6)PC from 0 to 0.4, which includes most of the region of coexistence of two lipid phases. Because this change in fluorescence can be described as partitioning of coat protein out of the rigid $Ca(PS)_2$ phase and into the (7,6)PC-rich fluid lipid phase (where tryptophanyl fluorescence is highly quenched), the ratio of the fluorophore concentration in the fluid liquid-crystal phase to that in the gel phase, $R_{LC/G}$, can be found from the fluorescence quenching data according to

$$F = F_{LC} + \frac{[G]}{R_{LC/G}(1 + [G]) + [G]}(F_G - F_{LC}) \quad (3)$$

where F is the measured or calculated fluorescence intensity,

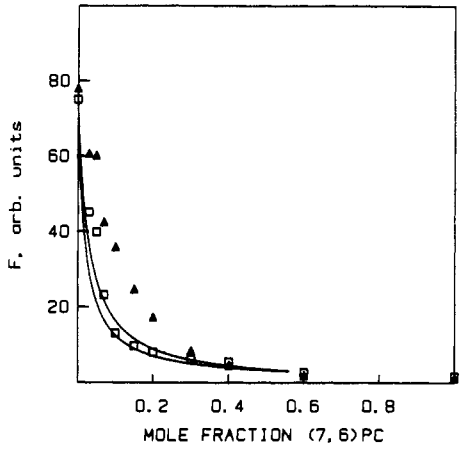

FIGURE 3: Fluorescence quenching of M13 coat protein in 100 μM DOPS/(7,6)PC multilamellar vesicles with (□) or without (▲) 20 mM Ca^{2+}. Symbol height represents the range of triplicate F measurements. Solid lines are theoretical curves calculated by use of eq 3 (text) for values of the concentration ratio of coat protein in a liquid-crystal phase of (7,6)PC mole fraction 0.56 to that in a rigid phase of (7,6)PC mole fraction 0.00, $R_{LC/G}$, of 20 and 30. Protein:lipid molar ratio is 1:100.

F_G or F_{LC} is the fluorescence in a membrane of the composition at the gel or liquid-crystal phase boundary, and

$$[G] = \frac{[(7,6)PC]_{LC} - [(7,6)PC]}{[(7,6)PC]_{LC} - [(7,6)PC]_G}$$

(London & Feigenson, 1981b). $R_{LC/G}$, the only unknown, is determined by fitting the experimental data to theoretical curves of F vs. [(7,6)PC] in the two-phase region calculated from eq 3 by using $[(7,6)PC]_{LC} = 0.56$ and $[(7,6)PC]_G = 0.00$. As shown in Figure 3, the fluorescence quenching data for M13 coat protein in DOPS/(7,6)PC in excess Ca^{2+} are best fit with $R_{LC/G} = 25 \pm 5$, indicating strong partitioning into the liquid-crystal phase.

Discussion

In examining the effects of Ca^{2+}-induced gel-phase formation on membrane-bound proteins, we use as a model system the coat protein of M13 bacteriophage incorporated into PS/PC multilamellar phospholipid vesicles. We use a reconstitution procedure that inserts the protein into the vesicles in its native configuration and without protein aggregation. As Figure 1 illustrates, the method of adding cholate-solubilized coat protein to preformed vesicles followed by freezing in liquid N_2, thawing, and dialysis to remove cholate, efficiently incorporates coat protein with most molecules spanning the membrane in their native orientation (N-terminus outward) and without aggregation. The elution positions of the digestion fragments indicate that the protein is probably dimeric in the vesicles (Bayer & Feigenson, 1985). The digest results for BBPS and DOPS are in good agreement with those obtained by Bayer and Feigenson (1985) for egg PC and egg PC/egg PA vesicles, suggesting that the details of coat protein incorporation are independent of these phospholipid variations. Chamberlain et al. (1978) have reported the lack of effect of negatively charged phospholipids on a variety of coat protein reconstitutions. Lyophilization of vesicles to obtain multilayers, followed by repeated freezing at −10 °C and thawing, appears to have no effect on the aggregation state of the coat protein in PS or PS/PC vesicles.

The fluorescence quenching experiments reveal the local lipid environment of the coat protein in the vesicles. The data shown in Figure 2 for coat protein in fluid-phase egg PC/(7,6)PC or DOPS/(7,6)PC multilamellar vesicles indicate that M13 coat protein binds egg PC and DOPS with about equal affinity. According to the theoretical curve shown in Figure 2, coat protein is surrounded by a random distribution of unlabeled and labeled phospholipids, with about six lipid molecules adjacent to each protein tryptophanyl residue. Wolber and Hudson (1982), using fluorescence polarization anisotropy decay of parinaric acid in M13 coat protein/dimyristoyl-PC vesicles, report six to seven lipids perturbed per monolayer per coat protein which spans the membrane.

In DOPS/(7,6)PC multilamellar vesicles, Ca^{2+} induces lipid phase separation, with coat protein partitioning strongly out of the $Ca(PS)_2$ gel phase and into the PS/PC fluid liquid-crystal phase. From the data shown in Figure 3, and using phase boundaries determined from EPR spectroscopy and X-ray diffraction, we calculate a ratio of coat protein concentration between the two phases of $R_{LC/G} = 25 \pm 5$. This result is similar to our findings for several small organic fluorophores, which, except for labeled PS, partition out of Ca^{2+}-induced gel-phase regions in PS/PC multilayers.

These observations of protein clearing from Ca^{2+}-induced gel-phase regions in a simple model system might be of biological relevance. However, studies of real fusing biological membranes would be required in order to determine whether such gel-phase formation and protein clearing are actually involved in membrane fusion.

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Electrostatic Coupling of Spectrin Dimers to Phosphatidylserine Containing Lipid Lamellae†

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ABSTRACT: We studied the interaction of spectrin dimers from human erythrocytes with (bilayer and monolayer) model membranes of mixtures of dimyristoylphosphatidylethanolamine, dimyristoylphosphatidylcholine, and dimyristoylphosphatidylserine (DMPS) by (1) densitometric evaluation of phase transitions and phase boundaries, (2) film balance experiments, and (3) microfluorescence. We demonstrate that spectrin readily adsorbs to mixed bilayers and monolayers even in the presence of small DMPS concentrations (30 mol %) whereas no appreciable interaction with lamellae containing zwitterionic lipids alone is observed. The selectivity of the DMPS/spectrin interaction is established by quantitative evaluation of the shifts of the phase boundaries (liquidus and solidus line) caused by the lipid/protein interaction as a function of the composition of the binary lipid mixtures. Quantitative information about the free energy of the lipid/protein interaction is obtained by computer simulation of the phase diagram of the lipid mixture in the absence or in the presence of a very small molar fraction of the protein and comparison of calculated and measured shifts. A binding energy of about 10^{-17} J per spectrin molecule is found. The present perturbation method can be generalized to study selective lipid/protein interaction mechanisms in ternary or higher component mixtures. The present results provide evidence that in addition to the binding to band III, spectrin may also couple directly to the lipid moiety of the inner monolayer of erythrocytes. The spectrin/phosphatidylserine interaction energy is, however, not large enough to account solely for the asymmetric distribution of this lipid in erythrocytes. Since spectrin is flexible, it is expected to be highly folded and can thus act as an entropy spring, the stiffness of which may be controlled by the lipid/protein interaction.

The deformability (Sackmann et al., 1986; Stokke et al., 1986a,b), the microscopic structural and dynamic properties of the erythrocyte membrane (Elgsaeter et al., 1976), and also the cell shape (Branton et al., 1981; Lieber et al., 1984) are determined to a large extent by the coupling of the spectrin/actin network to the lipid/protein bilayer.

It is generally assumed now that the filamentous spectrin dimers are interconnected by self-association and by actin oligomers and that the network formed in this way is primarily coupled to the lipid/protein bilayer by binding of part of the spectrin dimers to the membrane proteins such as to band III via ankyrin and to glycophorin C via band 4.1 (Haest, 1982; Branton et al., 1981). The coupling of the network to the bilayer is, however, rather weak as follows from the findings that the membrane bending elastic modulus is higher by at most about a factor of 5 than the value measured for fluid lipid bilayers (Fricke et al., 1986) and that the bilayer lipid molecules and proteins not coupled to the cytoskeleton exhibit fast lateral diffusion (Schindler et al., 1980; Kapitza & Sackmann, 1980).

The first evidence for an additional mode of coupling, namely, a direct binding of the spectrin filaments into the lipid bilayer moiety by electrostatic binding to the charged phosphatidylserine, was provided by Mombers et al. (1977, 1980).

In the present work, the binding of spectrin to bilayers and monolayers composed of mixtures of (synthetic) zwitterionic lipids (phosphatidylcholine and phosphatidylethanolamine) and phosphatidylserine is studied by densitometry, film balance experiments, and microscopic techniques. Evidence is provided that spectrin interacts selectively with the charged lipid by Coulomb forces whereas evidence for a hydrophobic interaction as postulated by Mombers et al. (1980) could not be found.

A further purpose of the present work is to show that quantitative information about the selectivity of the lipid/protein interaction as well as about protein binding energies is obtained by analyzing the shifts of the phase boundaries of

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