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Influence of the Peripheral Matrix Protein of Vesicular Stomatitis Virus on the Membrane Dynamics of Mixed Phospholipid Vesicles: Fluorescence Studies†

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ABSTRACT: In an effort to characterize the association of a peripheral membrane protein with mixed lipid bilayers, the basic ($pI \simeq 9.1$) matrix (M) protein of vesicular stomatitis virus was reconstituted with detergent-dialyzed vesicles and with preformed sonicated vesicles, each containing phospholipids with acidic head groups. The gel to liquid-crystalline phase transition of these reconstituted vesicles was studied by using steady-state fluorescence depolarization and differential polarized phase fluorometry of the hydrophobic membrane probes 1,6-diphenyl-1,3,5-hexatriene, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene, and *trans*-parinaric acid. Reconstitution of the M protein with detergent-dialyzed vesicles composed of 50 mol % dimyristoylphosphatidylserine (DMPS) and 50 mol % dipalmitoylphosphatidylcholine (DPPC) resulted in no significant effect on the phase transition temperature as measured by all three probes. However, the M protein appeared to increase the order of the mixed lipid bilayer gel state, as evidenced by increased fluorescence anisotropy of the three probes below the transition temperature. In addition, the phase transition of the vesicle bilayer was sharpened following reconstitution with the M protein. M protein reconstituted with preformed sonicated vesicles composed of 50 mol % DPPC and 50 mol % dipalmitoylphosphatidylglycerol (DPPG) caused a large increase in the phase transition temperature as monitored by all three probes. Depending on the probe used, the observed T_m was increased from 2 to 7 °C. The M protein dramatically increased the order of the mixed lipid gel state and sharpened the lipid phase transition. Differential polarized phase fluorometry of both vesicle systems demonstrated increased order of the gel state lipid in the presence of M protein and supported the steady-state fluorescence results. These results demonstrate that binding of the peripheral membrane protein M to lipid bilayers containing acidic phospholipids results in profound alterations in the dynamics of lipid behavior in the membrane.

Vesicular stomatitis virus (VSV)[1] is a membrane-enveloped rhabdovirus which has been extensively studied as a much simpler model system than the cellular plasma membrane from which it buds (Patzer et al., 1979). The virion contains five viral-coded proteins, three of which (N, NS, and L) are associated with the single-stranded viral RNA genome to form the enzymatically active nucleocapsid core. The remaining

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; DPPG, dipalmitoylphosphatidylglycerol; VSV, vesicular stomatitis virus; M, VSV matrix protein; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene; TPA, *trans*-parinaric acid; T_m, lipid phase transition midpoint temperature; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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two viral proteins, the glycoprotein (G) and the matrix protein (M), are intimately associated with the envelope bilayer. The G protein ($M_r \simeq 69\,000$) is an externally oriented transmembrane protein which completely spans the envelope by a sequence of 20 consecutive hydrophobic amino acids (Rose et al., 1980; Rose & Gallione, 1981). In contrast, the viral M protein ($M_r \simeq 29\,000$) is not exposed on the exterior surface of the intact virion, since it is refractory to protease digestion and ^{125}I-labeled lactoperoxidase iodination (Schloemer & Wagner, 1975; McSharry, 1977). The use of lipophilic monofunctional and bifunctional cross-linking reagents in intact virions demonstrates that the M protein is in close proximity to the inner surface of the membrane bilayer but penetrates little, if at all, into the membrane (Zakowski & Wagner, 1980). The M protein is believed to function in the maturation of the virus as a bridge between cytoplasmic viral nucleocapsids and regions of the cellular membrane into which the G protein has been inserted (Dubovi & Wagner, 1977). This process may occur through the interaction of the highly positively charged M protein (Carroll & Wagner, 1979) with phosphatidylserine polar head groups located in the inner leaflet of the plasma membrane. Analysis of the phospholipid composition of the VSV envelope revealed that PS comprises 18% of the total phospholipid and is asymmetrically distributed in the bilayer, with 85% located in the inner leaflet (Patzer et al., 1979).

It has recently been shown that purified M protein can be reconstituted with lipid vesicles, provided that the vesicle bilayer contains an acidic phospholipid component (Zakowski et al., 1981). This reconstitution occurs entirely at the surface lipid–water interface of the bilayer and thus provides a basis for the use of M protein reconstituted vesicles as a model system to study the interaction of a peripheral protein with the lipid bilayer. By use of steady-state fluorescence depolarization measurements and differential polarized phase fluorometry techniques, experiments were undertaken to study the mode of interaction of M protein with mixed lipid vesicles. Our results indicate that the binding of M protein with mixed phospholipid vesicles alters the behavior of the lipids in the bilayer.

Experimental Procedures

Materials

Lipids and Probes. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1,2-dimyristoyl-3-*sn*-phosphatidylserine (DMPS) were obtained from Avanti Biochemicals, Birmingham, AL. 1,2-Dipalmitoyl-3-*sn*-phosphatidylglycerol (DPPG) was obtained from Supelco, Inc., Belfonte, PA. [^{14}C]DPPC (100 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All lipid stocks were judged to be pure when analyzed by thin-layer chromatography in a chloroform/methanol/ammonia solvent system (65:25:4) and were used without further purification. Lipid stocks were assayed prior to each experiment for total phospholipid phosphorus content by a modification of the Bartlett method (Marinetti, 1962). 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), and *trans*-parinaric acid (TPA) were obtained from Molecular Probes, Junction City, OR, and were maintained in tetrahydrofuran at −70 °C in the dark until used.

Cells and Virus. Baby hamster kidney (BHK-21) cells were grown to 95% confluency at 37 °C in basal medium Eagle (BME) supplemented with 10% tryptose phosphate broth, 10% calf serum, and 1% each of penicillin and streptomycin. Plaque-purified VSV of the Indiana serotype was used to infect the monolayers at a multiplicity of 0.1 plaque-forming unit per cell. The viral proteins were radioactively labeled by the addition of 5 μCi/mL ^{3}H-labeled amino acid mixture (New England Nuclear, Boston, MA) to the infection medium. Following incubation for 21 h at 31 °C, bullet-shaped virions were collected from the medium and purified to homogeneity as previously described (Barenholz et al., 1976). VSV stocks were stored in phosphate-buffered saline at a concentration of 2–5 mg/mL at −80 °C.

Isolation of Viral Matrix Protein. VS viral matrix protein was isolated from purified virions exposed to 1% Triton X-100 and 0.25 M NaCl and purified by column chromatography on Whatman P11 phosphocellulose, exactly as described by Zakowski et al. (1981). Matrix protein isolated by this procedure is >98% pure, as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Lowry et al. (1951) and by the specific radioactivity of the [^{3}H]amino acid labeled M protein. The mole percentage of the M protein was calculated by assuming a molecular weight of 29 000.

Methods

Reconstitution of Viral M Protein into PS/PC Vesicles. The viral M protein was reconstituted with the phospholipids by the detergent dialysis method of Petri & Wagner (1979). To 800 nmol of phospholipid (1:1 DMPS/DPPC) in chloroform containing 0.02 μCi of [^{14}C]DPPC was added 4 mg of octyl β-D-glucoside (Calbiochem, La Jolla, CA) in chloroform. This achieves a final detergent/lipid molar ratio of 17/1. The sample was dried under a stream of nitrogen and lyophilized overnight to remove any residual solvent. The dried mixture was resuspended in 4 mL of 10 mM Tricine (pH 7.5) containing 0.65 M NaCl, purified M protein, and 10% glycerol and then dialyzed vs. 2 L of 10 mM Tricine (pH 7.5), through which N_2 had been bubbled. Control vesicle preparations received no M protein. Dialysis was continued for 48 h at 46 °C in the dark, with six changes of buffer.

Reconstitution of Matrix Protein into Preformed PG/PC Vesicles. Viral M protein was reconstituted with sonicated preformed vesicles by a modification of the method of Petri & Wagner (1980); 600 nmol each of DPPG and DPPC, containing 0.06 μCi of [^{14}C]DPPC, was dried under a stream of nitrogen and lyophilized overnight. The lipid preparation was then resuspended in 4 mL of 10 mM Tricine (pH 7.5) containing 0.65 M NaCl, bubbled with N_2, and set at 46 °C for 30 min with intermittent vortexing to ensure complete dispersion of all the lipid. Each sample was sonicated at 46 °C for 3–5 min under a nitrogen atmosphere with a Heat System W-350 sonicator. Multilamellar vesicles were removed from the lipid preparation by centrifugation at 125000*g* for 60 min at 46 °C. ^{14}C radioactivity of 50 μL of supernatant was determined by scintillation spectrometry and used to calculate the total lipid remaining as small unilamellar vesicles (SUV). M protein was then added to give the desired input ratio of M protein to phospholipid, whereas similar vesicle preparations received 10 mM Tricine (pH 7.5) containing 0.65 M NaCl and no protein. Dialysis was performed for 24 h vs. 5 L of 10 mM Tricine (pH 7.5) at 46 °C with two changes of buffer.

Sonicated vesicle preparations were subjected to quasi-elastic light scattering by using a laser scattering spectrometer, Model HN 5-90, and a computing autocorrelator, Model 6864 (NiComp Instruments), to determine vesicle diameter (Wong & Thompson, 1982). Small sonicated vesicles composed of 1-palmitoyl-2-oleoylphosphatidylcholine and prepared as described above were used as control preparations, their average

diameters being 240 Å. The spectrometer laser wavelength used for measurement was 632.8 nm.

Density Gradient Centrifugation. Vesicle suspensions prepared as described above and used for the analysis of M binding to the membrane were concentrated vs. Aquacide-1-A (Calbiochem, La Jolla, CA), mixed with an equal volume of 65% sucrose in 0.15 M NaCl and 10 mM Tris (pH 7.5), and overlaid with a continuous gradient of 0–30% sucrose in 0.15 M NaCl and 10 mM Tris (pH 7.5). The gradients were centrifuged at 200000*g* for 16 h at 46 °C and then fractionated into 0.4-mL aliquots. The relative amounts of M protein and phospholipid contained in each fraction were determined by scintillation spectrometry. Final (output) ratios of M protein to phospholipid were calculated by using the protein and phospholipid specific radioactivities. Densities of gradient aliquots were determined by refractometry.

Fluorescence Studies. (A) Steady-State Fluorescence Depolarization. Steady-state fluorescence depolarization experiments were performed exactly as described by Barenholz et al. (1976) on a modified Perkin-Elmer MPF3 spectrofluorometer with polarizers in the excitation and emission beams. Cuvette temperature was monitored continuously by means of a Yellow Springs Instrument thermistor probe connected to a digital ohmmeter. When fluorescence depolarization was measured as a function of temperature, the sample was heated to 60 °C and then cooled at a rate of 25 °C/h.

Three different fluorescent probes were used: 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylammonio)-phenyl]-6-diphenyl-1,3,5-hexatriene (TMA-DPH), and *trans*-parinaric acid (TPA). All of the probes used were dissolved in tetrahydrofuran and added to the vesicle suspensions at a ratio of one probe molecule per 200 phospholipid molecules. To allow complete incorporation of the probe, the preparations were incubated for 1 h under nitrogen at 40 °C prior to the experiments. Light scattering of the vesicle preparations in the absence of probe was never more than 3–5% of the fluorescence intensity perpendicular to the plane of the excitation beam.

Fluorescence measurements of DPH and TMA-DPH were performed by excitation at 360 nm; fluorescence was detected by monitoring emission at 430 nm by using the MPF3 390-nm cutoff filter to reduce scattered light. The fluorescence of vesicle preparations containing TPA was measured in a similar fashion, except that TPA was excited at 320 nm, and the emission monitored at 420 nm, by using the MPF3 410 nm cutoff filter to reduce scattered light.

(B) Emission Lifetime. Emission lifetime was measured directly in an updated version of the cross-correlation phase and modulation fluorometer from SLM Instruments, Urbana, IL. For DPH and TMA-DPH the excitation wavelength (360 nm) was selected from a 450-W xenon arc lamp by a monochromator (0.5-mm band path) and modulated at 30 MHz by a Sears–Debye ultrasonic light modulator. Lifetime was measured both by phase shift and by the demodulation of the fluorescence of the probe-loaded vesicle suspension relative to a scattering suspension of DPPC fused-unilamelar vesicles of approximately equal emission intensity (Spencer & Weber, 1969). Measurements were made continuously until stable values were obtained.

(C) Differential Phase Fluorescence Measurement. The theory and experimental techniques of differential phase fluorometry have been described elsewhere (Lakowicz & Prendergast, 1978; Lakowicz et al., 1979). The differential lifetime of vertically and horizontally polarized components of the modulated fluorescence was measured on the same instrument as used for emission lifetime measurement. The nonzero limiting anisotropy (r_∞) at time long compared to the fluorescence lifetime was calculated by the combined use of lifetime, differential lifetime, and steady-state anisotropy measurements as described elsewhere (Lakowicz et al., 1979; Lakowicz, 1981).

Results

Reconstitution of M Protein with Mixed Phospholipid Vesicles. In order to characterize the association of the matrix (M) protein of VSV with mixed phospholipid bilayers, the protein was reconstituted with lipid vesicles by two different methods: (1) with detergent-dialyzed mixed phospholipid vesicles containing 50 mol % dimyristoylphosphatidylserine as the acidic phospholipid component and (2) with preformed sonicated mixed phospholipid vesicles containing 50 mol % dipalmitoylphosphatidylglycerol as the acidic phospholipid component. Since attempts to prepare PG-containing vesicles by detergent dialysis were unsuccessful, the PG/PC vesicles used in this study were prepared by sonication.

(A) Reconstitution of M Protein with DMPS/DPPC Vesicles. The M protein of VSV was reconstituted with mixed lipid vesicles composed of DPPC and DMPS by the octyl glucoside detergent dialysis method (Petri & Wagner, 1979). Preparations of reconstituted vesicles formed by dialysis were made 32% with respect to sucrose and floated in a 0–30% sucrose density gradient which contained 0.15 M NaCl, as described under Experimental Procedures.

Figure 1 illustrates the flotation profiles obtained from vesicle preparations composed of 50 mol % DMPS/50 mol % DPPC alone (Figure 1A) or reconstituted with the M protein at a protein/phospholipid molar ratio of 0.5 mol % (Figure 1B). Vesicles reconstituted with the M protein displayed an increase in density of $\sim$0.02 g/cm^3. Vesicles composed solely of DPPC did not bind the M protein and therefore exhibited no change in density when dialyzed in the presence of the M protein (data not shown). Density gradient centrifugation of M protein alone in the absence of lipid resulted in pelleting of the protein in the centrifuge tube (data not shown).

Reconstitution experiments performed with DMPS/DPPC (1:1) detergent-dialyzed vesicles containing varying amounts of the M protein indicated that the capacity of the vesicles to bind M protein reached a saturation level at a predialysis molar ratio of protein to phospholipid of $\sim$1.3 mol %. Vesicles reconstituted in the presence of $>$1.3 mol % M demonstrated no further protein binding and subsequent density increase and resulted in increased pelleting of any unbound protein in the centrifuge tube (data not shown). Negative-stain electron microscopy of sample vesicle preparations containing no M protein showed a distribution of unilamellar vesicles of heterogeneous size with a mean diameter of $\sim$900 Å and a size range of 700–1100 Å (data not shown). The addition of the M protein did not significantly change the mean vesicle diameter.

(B) Reconstitution of M Protein with Preformed DPPG/DPPC Vesicles. Preformed sonicated vesicles composed of 50 mol % DPPG/50 mol % DPPC alone or reconstituted with the M protein were made 32% with respect to sucrose and floated in density gradients similar to those performed for the DMPS-containing vesicles formed by detergent dialysis. Figure 1 shows the flotation profiles obtained with sonicated DPPG/DPPC vesicles alone (Figure 1C) or reconstituted with 0.5 mol % M protein (Figure 1D). The binding of the M protein to vesicles increased the vesicle density $\sim$0.03 g/cm^3. Typically, 80–90% of the M protein remained bound to the

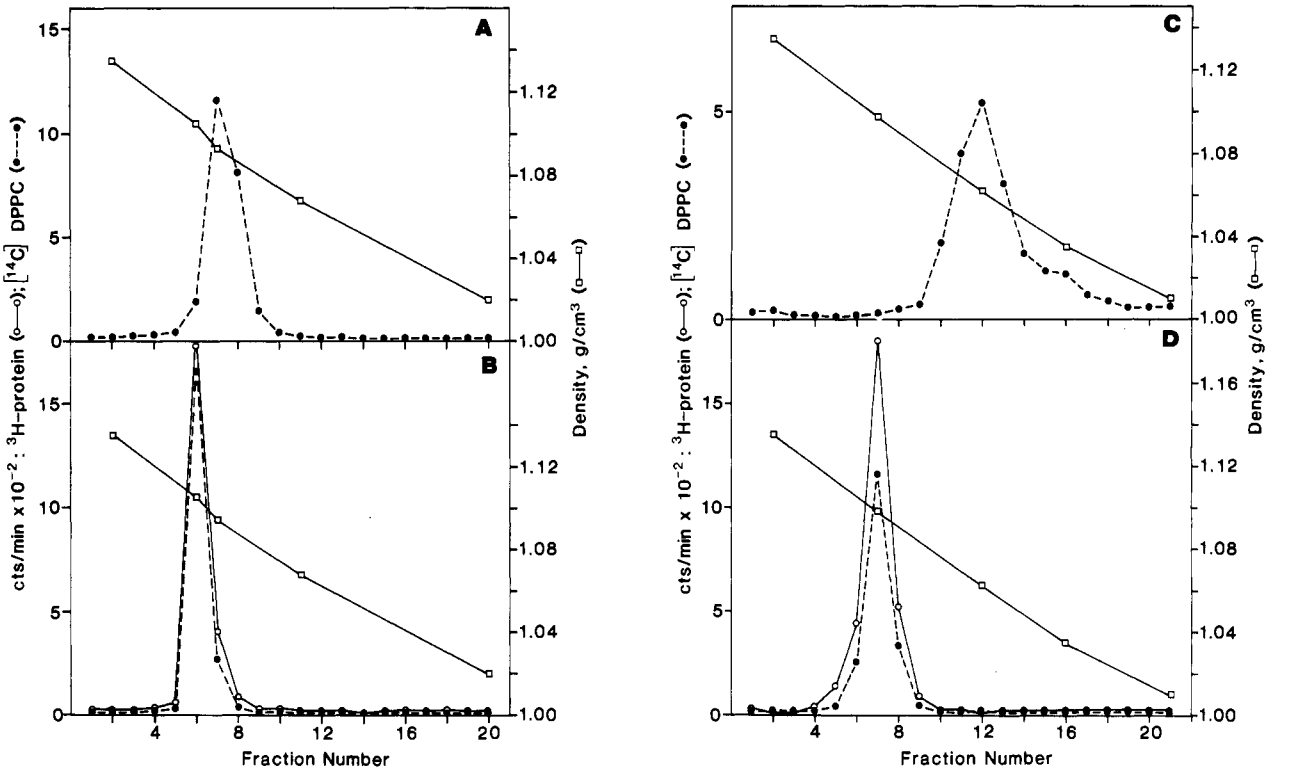

FIGURE 1: Comparative buoyant density of detergent-dialyzed DMPS/DPPC (1:1) vesicles or sonicated DPPG/DPPC (1:1) vesicles reconstituted in the absence or presence of M protein of VSV. (A) DMPS/DPPC vesicles alone; (B) DMPS/DPPC vesicles containing 0.5 mol % M protein; (C) DPPG/DPPC vesicles alone; (D) DPPG/DPPC vesicles containing 0.5 mol % M protein. All vesicle preparations contained [^{14}C]DPPC in trace quantity to label the lipid, and the M protein was metabolically labeled with ^{3}H-labeled amino acids. As described under Experimental Procedures, vesicles were prepared either by detergent dialysis in the presence or absence of M protein or by sonication followed by dialysis in the presence or absence of M protein. Buoyant density was determined by flotation upward through a 0–30% sucrose gradient subjected to centrifugation at 200000*g* at 46 °C for 16 h. Gradients were fractionated and 0.4-mL aliquots counted for ^{3}H and ^{14}C. (○) ^{3}H-Labeled protein; (●) [^{14}C]DPPC; (□) density (g/cm^{3}).

vesicles following centrifugation in the gradients, which contained 0.15 M NaCl. As with the larger DMPS-containing vesicles, the capacity to bind the M protein reached the saturation level at an input protein/phospholipid molar ratio of ~1.3 mol %.

Quasi-elastic light scattering (QELS) performed on sonicated vesicle preparations gave values for the average vesicle diameter of ~408 Å. Reconstitution of the vesicles with the M protein increased the average vesicle diameter to ~438 Å.

Figure 2 illustrates a comparison of the binding efficiency (Figure 2A) and subsequent change in density (Figure 2B) of the PS/PC and PG/PC vesicles reconstituted with varying amounts of the M protein. The results in Figure 2A demonstrate that the capacity of the PG-containing vesicles to bind M protein is clearly superior to that of the PS-containing vesicles given equivalent input (predialysis) mole percent M protein. In addition, binding of the equivalent mole percent M protein results in a larger increase in vesicle density in the PG system (Figure 2B).

Fluorescence Studies. The probes used in this study were 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), and *trans*-parinaric acid (TPA). DPH partitions equally well in gel or liquid-crystalline state phospholipids and is believed to occupy and report the hydrophobic core region of the membrane (Shinitzky & Barenholz, 1978). In contrast to DPH, its synthetic derivative TMA-DPH contains a quaternary cationic group; this hydrophilic nonfluorescent moiety anchors the probe at the lipid–water interface. The fluorescent hydrophobic portion of the TMA-DPH molecule resides in the upper fatty acyl chain region of the bilayer (Prendergast et al., 1981) and thus reports on a region different from, and more defined than, that of the parent DPH molecule. *trans*-Parinaric acid is a naturally occurring fluorescent polyene fatty acid which preferentially partitions into gel state phospholipids and shows an increased fluorescent intensity in such regions (Sklar et al., 1979).

(*A*) *Steady-State Fluorescence Depolarization.* We have used the steady-state fluorescence depolarization of three fluorescent membrane probes to monitor protein-induced perturbations in mixed lipid vesicle bilayers reconstituted with the M protein. Preliminary experiments indicated that interaction of the M protein with each of the probes used was negligible and did not contribute to the fluorescence anisotropy observed in the vesicle systems (data not shown).

(*1*) *Detergent-Dialyzed DMPS/DPPC Vesicles.* Figure 3 illustrates the fluorescence anisotropy of DPH, TMA-DPH, and TPA (parts A, B, and C of Figure 3, respectively) in DMPS/DPPC (1:1) detergent-dialyzed vesicles which have been reconstituted in the presence or absence of 0.5 mol % M protein. All three probes reported increased fluorescence anisotropy below the lipid phase transition, as well as an increase in the transition line slope, in vesicles reconstituted with the M protein. The lipid phase transition midpoint temperature (T_m) detected by any of the probes was not significantly altered in the presence of the protein. In contrast to DPH and TPA, TMA-DPH reported decreased anisotropy in the lipid liquid-crystalline state in vesicle bilayers containing the protein.

(*2*) *Preformed Sonicated DPPG/DPPC Vesicles.* Preformed sonicated vesicles composed of DPPG/DPPC (1:1) prepared by the method of Barenholz et al. (1977) are homogeneously sized unilamellar vesicles. It was of interest to compare the effect of M protein binding to the vesicle bilayer upon the fluorescence depolarization of the three membrane

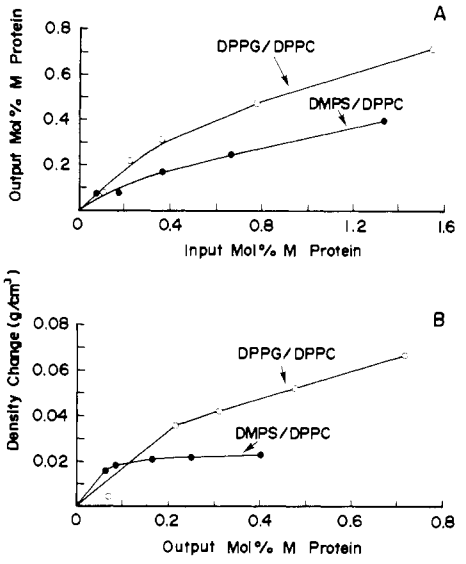

FIGURE 2: (A) Fractional association of M protein (Output) with mixed phospholipid vesicles (Input) and (B) buoyant density changes in vesicles that associate with increasing amounts of M protein (Output). As described under Experimental Procedures, increasing amounts of M protein were mixed with constant amounts of DMPS/DPPC (800 nmol of total phospholipid; 1:1 molar ratio) in the presence of octyl glucoside and dialyzed to form detergent-dialyzed vesicles, or with constant amounts of sonicated preformed DPPG/DPPC vesicles (600 nmol of total phospholipid; 1:1 molar ratio). Buoyant density was determined by flotation in a 0–30% sucrose gradient subjected to centrifugation at 200000*g* for 16 h. Mole percent M protein was determined by specific radioactivity of M protein labeled with ^{3}H-labeled amino acids, assuming a molecular weight of 29 000 for M protein. Vesicles were labeled with [^{14}C]DPPC.

probes, which presumably report on different regions of the bilayer.

The fluorescence depolarization of DPH, TMA-DPH, and TPA in sonicated vesicles composed of DPPG and DPPC (1:1) with or without 0.5 mol % M protein is illustrated in Figure 4 (parts A, B, and C, respectively). Binding of the M protein to the mixed lipid bilayers resulted in a dramatic increase in fluorescence anisotropy for all three probes below the T_m, and sharpened the transition, in a manner similar to that observed for the detergent-dialyzed vesicle preparations. However, each probe reported a large increase in the T_m in bilayers reconstituted with the M protein. DPH and TMA-DPH reported an increase in T_m of ~7 and ~5 °C, respectively, while TPA detected an increase of ~3 °C. None of the probes reported a marked change in anisotropy in the bilayer liquid-crystalline state in the presence of the protein.

Figure 5 shows the steady-state fluorescence depolarization of DPH (Figure 5A), and TMA-DPH (Figure 5B), in sonicated DPPG/DPPC (1:1) vesicles reconstituted with the M protein in varying M protein/phospholipid ratios. In the absence of the M protein, the vesicle phospholipids underwent a phase transition which centered at 41.7 °C. Vesicles reconstituted with 0.125 mol % M protein exhibited a transition temperature of 45.7 °C. With each subsequent increase in mole percent M protein, the slope of the transition was substantially larger, and the anisotropy in the gel state was markedly higher than in vesicles lacking the M protein. Vesicles reconstituted with the M protein at the saturation level of M protein binding (~1.3 mol %) showed a phase transition which centered at 46.9 °C, with an increase in anisotropy that was similar to the curves obtained at M protein/phospholipid molar ratios of 0.25 or 0.5 mol % (data not shown).

It has been reported that the bivalent cation Ca^{2+} increases the phase transition temperature of lipid dispersions composed

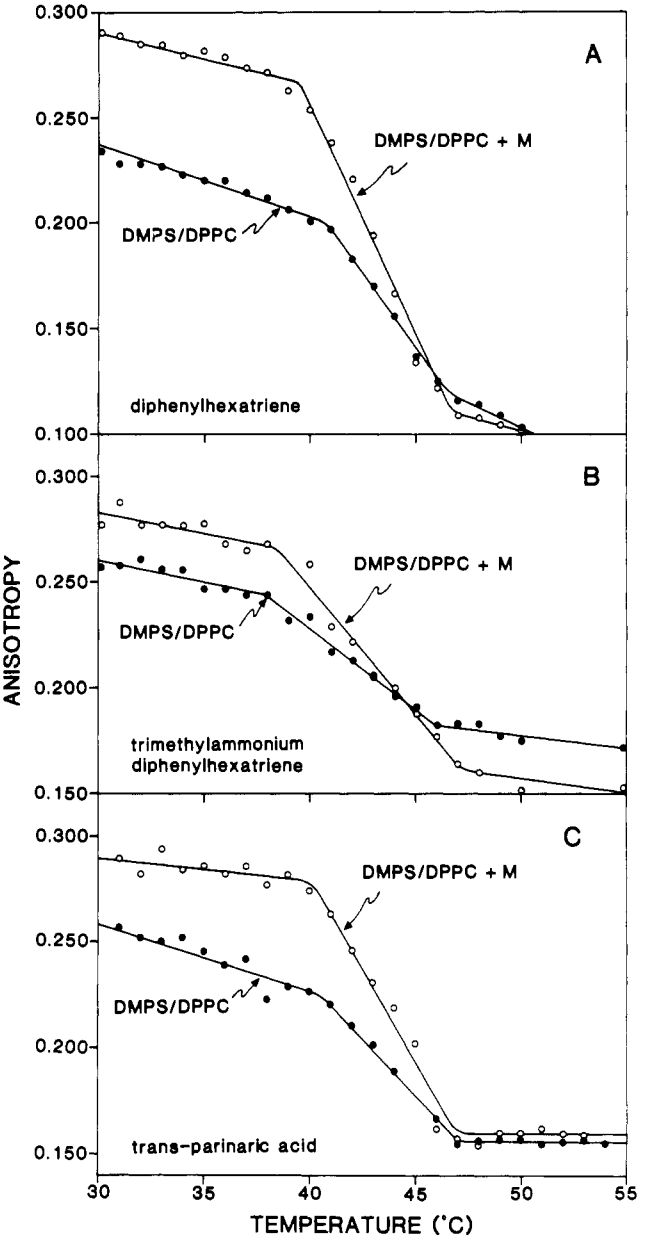

FIGURE 3: Fluorescence anisotropy as a function of temperature of (A) 1,6-diphenyl-1,3,5-hexatriene, (B) 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene, and (C) *trans*-parinaric acid in DMPS/DPPC (1:1) vesicles reconstituted by octyl glucoside detergent dialysis in the presence or absence of VSV M protein. The detergent-dialyzed vesicles contained either no M protein (●) or 0.5 mol % M protein (○).

of didodecanoyl-PG and didodecanoyl-PC (1:1) at constant pH (Verkleij et al., 1974). It was of interest to compare the effect of reconstitution of the M protein with sonicated DPPG/DPPC (1:1) vesicles with the effect induced in identical DPPG/DPPC vesicles by the presence of Ca^{2+}. We found that sonicated vesicles in the absence of Ca^{2+} exhibited a phase transition reported by DPH which centered at 40.7 °C; addition of 8.0 mM (final concentration) $CaCl_2$ increased the transition midpoint to 42.0 °C with a minimal increase in anisotropy compared to the control (data not shown). The presence of 78.0 mM $CaCl_2$ in the vesicle suspension dramatically increased the T_m to 49.5 °C (data not shown). The transition line slope was significantly increased, although the anisotropy was only minimally increased in the gel state compared to control vesicle preparations. The effect of varying the Ca^{2+} concentration upon the phase transition temperature

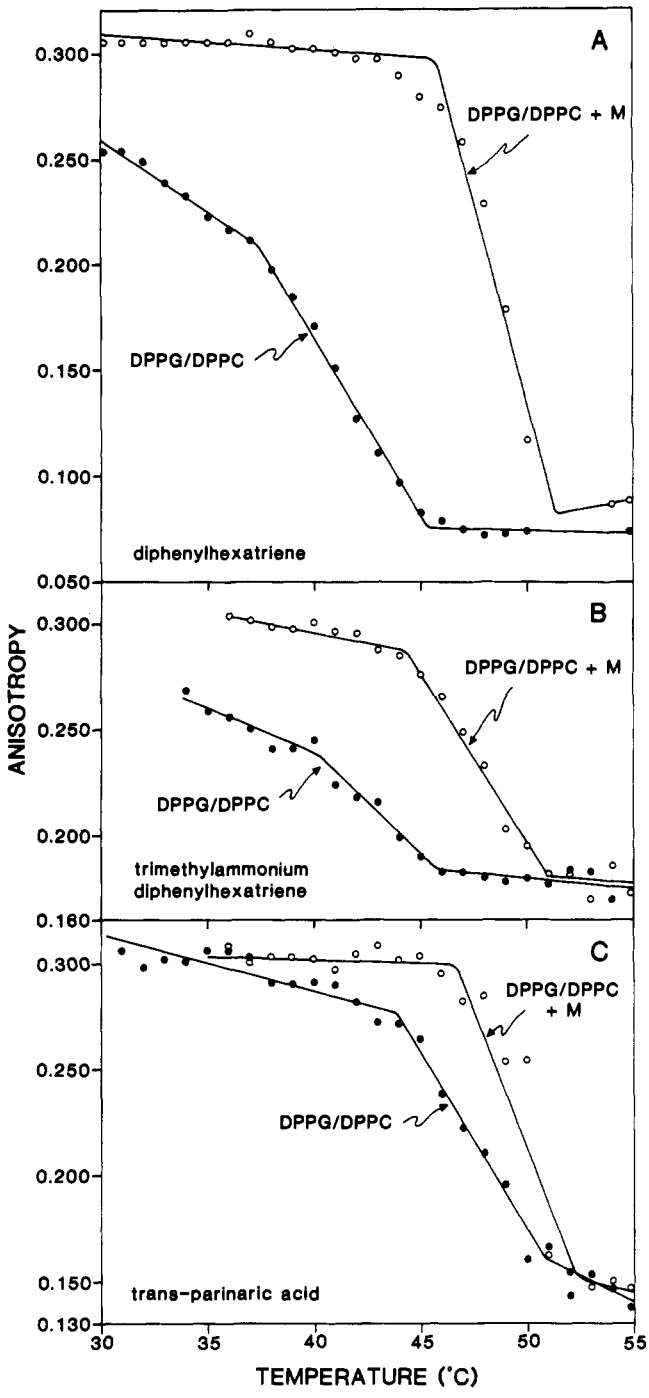

FIGURE 4: Fluorescence anisotropy as a function of temperature (A) 1,6-diphenyl-1,3,5-hexatriene, (B) 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene, and (C) *trans*-parinaric acid in sonicated DPPG/DPPC (1:1) vesicles reconstituted with or without VSV M protein. The preformed sonicated vesicles contained either no M protein (●) or 0.5 mol % M protein (○).

was linear until apparent saturation occurred at ~100 mM Ca^{2+} (data not shown).

(*B*) *Differential Polarized Phase Fluorometry.* Using differential phase fluorometric techniques, we have determined the limiting anisotropy of DPH and TMA-DPH incorporated into the phospholipid bilayer in the presence of the M protein of VSV. Both DPH and TMA-DPH were used in this study for such measurements; the differential polarized phase behavior of these two probes is well characterized (Lakowicz et al., 1979; Prendergast et al., 1981).

(*1*) *Detergent-Dialyzed DMPS/DPPC Vesicles.* The fluorescence lifetime of DPH in PS/PC vesicles with or

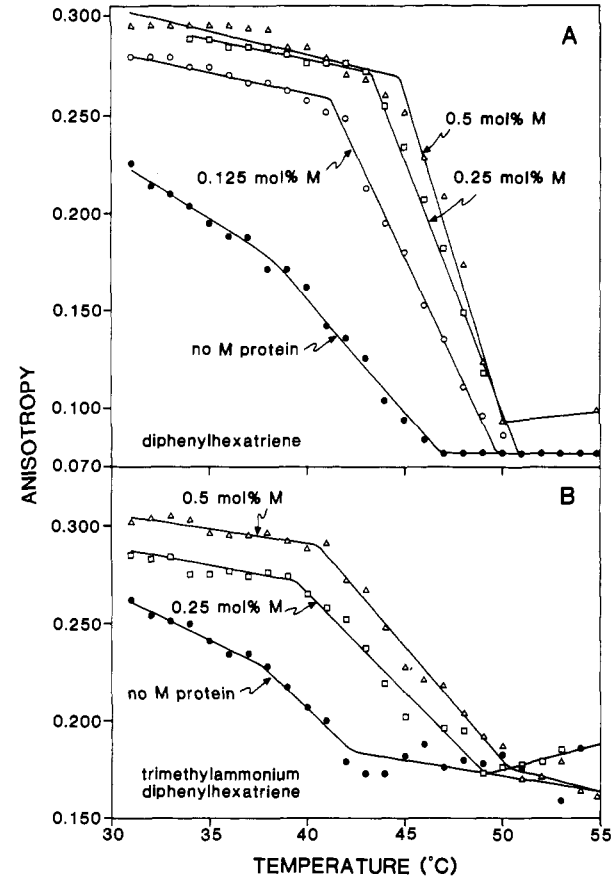

FIGURE 5: Fluorescence anisotropy as a function of temperature of (A) 1,6-diphenyl-1,3,5-hexatriene and (B) 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene in sonicated DPPG/DPPC (1:1) vesicles reconstituted with varying concentrations of VSV M protein. The vesicles were reconstituted with no M protein (●), 0.125 mol % M protein (○), 0.25 mol % M protein (□), or 0.5 mol % M protein (△).

without M protein is presented in Table I. The lifetime of DPH was influenced significantly by temperature in both types of vesicles, increasing from nearly 7 to ~11 ns as the temperature was lowered from 50 to 31 °C. The presence of the M protein in the vesicles further increased the fluorescence lifetime of DPH to only a small extent.

Table I also shows the observed differential tangents (tan Δ) and theoretically calculated differential tangents (tan Δ_{max}) of DPH as a function of temperature for PS/PC (1:1) vesicles in the absence or presence of M protein. A large tangent defect was observed in both vesicles, suggesting that the depolarizing rotations of DPH are highly hindered. The presence of M protein in PS/PC vesicles decreased the tan Δ values both in the gel state and in the liquid-crystalline state. The limiting anisotropy (r_∞) values calculated from lifetime, steady-state anisotropy, and tan Δ are shown in Figure 6A. The r_∞ values in vesicles were lower in the liquid-crystalline state, suggesting that the probe was less restricted in its motion in that state. However, in the gel state, the r_∞ values showed a steady increase, suggesting that the rotational motion of the probe was hindered. The presence of M protein in the vesicles appeared to increase the r_∞ value in the gel state but not significantly in the liquid-crystalline state, suggesting that the order of the bilayer increased in the presence of the protein mainly in the gel state.

(*2*) *Preformed Sonicated Vesicles DPPG/DPPC (1:1)* were reconstituted with the M protein at a protein/lipid molar ratio of 0.5 mol %, as described under Experimental Procedures. The fluorescence lifetime of DPH and TMA-DPH in the

Table I: Lifetime [τ (ns)], Differential Tangent (tan Δ), and Theoretically Calculated Differential Tangent (calcd tan Δ_{max}) of DPH and TMA-DPH in Lipid Vesicles Reconstituted with or without the M Protein of VSV[a]

	DPH in Detergent-Dialyzed Vesicles					
	DMPS/DPPC			DMPS/DPPC/M		
temp (°C)	τ (ns)	tan Δ	calcd tan Δ_{max}	τ (ns)	tan Δ	calcd tan Δ_{max}
50.0	7.65	0.120	0.279	7.80	0.111	0.282
45.0	8.10	0.128	0.288	8.20	0.115	0.290
40.0	9.10	0.100	0.315	9.75	0.080	0.318
35.0	10.30	0.045	0.327	10.75	0.032	0.334
30.0	10.75	0.031	0.334	11.10	0.022	0.339
	DPH in Sonicated Vesicles					
	DPPC/DPPG			DPPC/DPPG/M		
temp (°C)	τ (ns)	tan Δ	calcd tan Δ_{max}	τ (ns)	tan Δ	calcd tan Δ_{max}
52.5	6.63	0.10	0.255	7.40	0.090	0.274
50.0	7.09	0.10	0.266	8.02	0.090	0.287
48.0	7.36	0.10	0.273	8.80	0.060	0.302
45.3	7.92	0.10	0.285	9.87	0.012	0.321
35.5	8.17	0.07	0.290	10.28	0.010	0.326
32.5	8.24	0.06	0.291	10.20	0.010	0.327
28.8	9.60	0.051	0.316	11.43	0.009	0.344
25.4	9.73	0.035	0.318	11.54	0.008	0.345
	TMA-DPH in Sonicated Vesicles					
	DPPC/DPPG			DPPC/DPPG/M		
temp (°C)	τ (ns)	tan Δ	calcd tan Δ_{max}	τ (ns)	tan Δ	calcd tan Δ_{max}
55.0	3.18	0.050	0.145	3.44	0.044	0.155
50.0	3.50	0.053	0.158	4.14	0.045	0.181
47.4	3.49	0.060	0.157	6.05	0.003	0.240
43.0	3.83	0.062	0.170	7.70	0.003	0.270
34.9	4.57	0.030	0.196	7.78	0.003	0.282
30.7	4.84	0.027	0.205	7.67	0.002	0.279
25.6	4.86	0.026	0.205	7.38	0.002	0.273

[a] The tan Δ_{max} was calculated as described elsewhere (Lakowicz et al., 1979).

PG/PC vesicles with or without the M protein are shown in Table I. In general, the lifetime of TMA-DPH was shorter than that of DPH. The lifetime of both DPH and TMA-DPH was affected by temperature to a significant extent. The presence of M protein increased the lifetime of the probe in the vesicles to a great extent; the effect was most pronounced with TMA-DPH as the lifetime increased from 3.44 ns at 55 °C to 7.7 ns at 43 °C. The observed increase in lifetime of both probes in the presence of M protein may suggest that the protein increases the order of the lipids in the bilayer. Increased order of the lipid matrix may exclude water from the bilayer and reduce fluorophore nonradiative phenomena. This would result in increased probe fluorescence lifetime.

Table I also shows the differential tangents (tan Δ) of DPH and TMA-DPH in DPPG/DPPC (1:1) vesicles with or without M protein. As before, a large tangent defect was observed in both vesicles. The presence of M protein in these vesicles had a marked effect on the tan Δ. For both probes, the value of tan Δ showed a sharp drop around 46 °C in the presence of M protein, whereas a slower decline was observed for DPPG/DPPC vesicles alone beginning at 41 °C. Further, in the gel state, the tan Δ values in DPPG/DPPC vesicles associated with M protein were significantly lower than those in the absence of M protein.

The temperature profiles of r_∞ in these vesicles are shown in parts B and C of Figure 6 for DPH and TMA-DPH, respectively. It is apparent from the curves that the rotation of both probes is hindered significantly in the gel state as compared to the liquid-crystalline state. Furthermore, TMA-DPH appeared to reside in a rather restricted area as compared to the DPH molecule. The presence of M protein in DPPG/DPPC vesicles sharpened the transition of the lipids and had a large affect on the r_∞ of the bilayer. The limiting anisotropy for both probes was slightly higher in the presence of the protein in the liquid-crystalline state. However, in the gel state, the protein appeared to increase the limiting anisotropy markedly by increasing the order of the lipid bilayer. The transition temperature of the lipids in the vesicles was also shown to increase by nearly 5–7 °C in the presence of the M protein.

All these studies clearly demonstrate that the matrix protein of VSV has a large effect on the order of the lipid bilayer. The major effect was observed in mixed vesicles composed of DPPG/DPPC. The hindrance to the motion of both DPH and

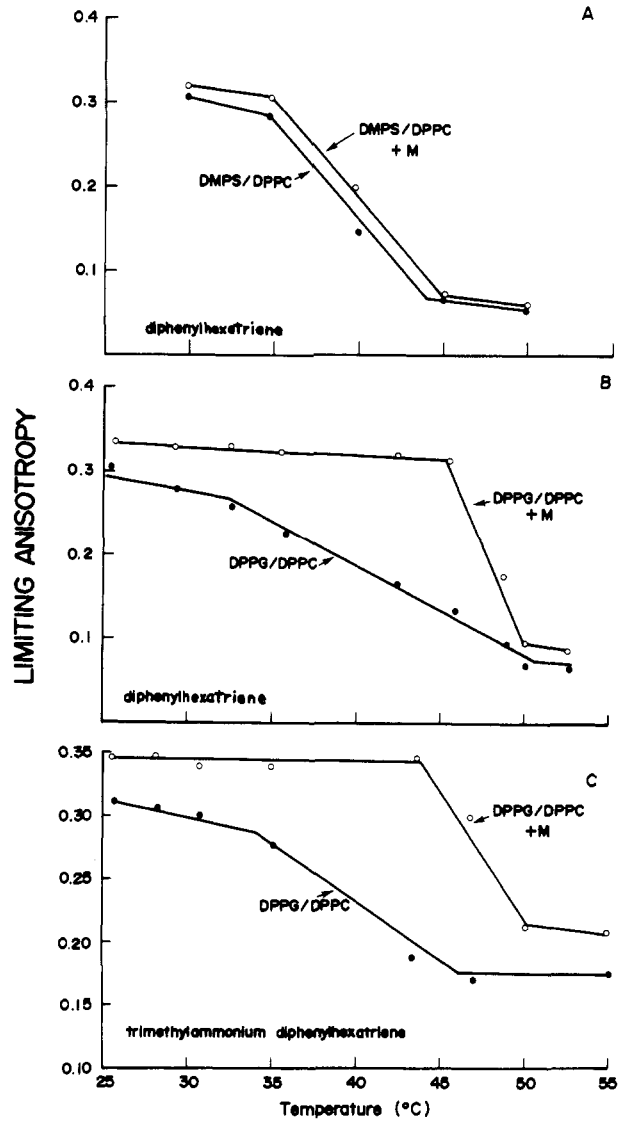

FIGURE 6: Limiting anisotropy of (A) DPH in DMPS/DPPC detergent-dialyzed vesicles containing either no M protein or 0.5 mol % M protein. (B) DPH in DPPG/DPPC sonicated vesicles containing either no M or 0.5 mol % M protein. (C) TMA-DPH in DPPG/DPPC sonicated vesicles containing either no M or 0.5 mol % M protein. Limiting anisotropy at different temperatures was calculated from differential tangent, lifetime, and steady-state anisotropy as outlined under Experimental Procedures and described elsewhere (Lakowicz et al., 1979).

TMA-DPH increased markedly mainly in the gel state which was a result of increased order in the bilayer in the presence of the protein.

Discussion

The matrix (M) protein of VSV is a peripheral membrane protein which is believed to line the interior leaflet of the virion envelope (Dubovi & Wagner, 1977; Zakowski & Wagner, 1980). In order to characterize the effect of a model peripheral membrane protein on the dynamic properties of mixed lipid bilayers, the M protein was reconstituted with detergent-dialyzed vesicles composed of DMPS and DPPC and with sonicated preformed vesicles composed of DPPG and DPPC.

The fluorescence studies presented here demonstrated that reconstitution of mixed lipid vesicles with the M protein sharpened the lipid phase transition and increased the order of the bilayer lipids in the gel state. All three fluorescent probes used in this study reported increased steady-state anisotropy in the gel state for DMPS-containing vesicles reconstituted with the M protein. Differential polarized phase fluorometry measurements indicated that this change is due to an increase in the steric hindrance of probe motion (compare Figure 3A and Figure 6A as well as tan Δ data in Table I). The degree of lipid ordering was most dramatic for the two probes which report on the hydrophobic fatty acid chain region of the bilayer core, TPA and DPH. In contrast, the increase in anisotropy reported by TMA-DPH was less than that for the other two probes, a finding which reflects the relative rigidity of the upper acyl chain and polar head group region on which this probe reports (Prendergast et al., 1981). Furthermore, all three probes reported negligible change in the T_m of DMPS/DPPC vesicle bilayers reconstituted by detergent dialysis with the M protein. All three probes indicated that the phase transition is sharpened in DMPS/DPPC bilayers reconstituted with the M protein.

Association of the M protein with sonicated preformed vesicles containing DPPG and DPPC resulted in more pronounced changes in the dynamic behavior of the lipid bilayer than was observed for the DMPS-containing vesicles. Reconstitution of DPPG-containing mixed lipid vesicles with 0.5 mol % M protein resulted in a large increase in the transition temperature. This was most dramatically illustrated in the $\sim$7.0 °C increase in T_m reported by DPH. The observed increase in T_m was shown to be directly proportional to the protein/phospholipid molar ratio in the reconstituted vesicles. All three probes reported a marked sharpening of the lipid phase transition for the bilayers reconstituted with M protein, as well as increased anisotropy in the gel state.

The increase in steady-state anisotropy, as well as the increase in limiting anisotropy observed in both vesicle systems reconstituted with the M protein, is evidence that this peripherally bound membrane protein ordered the phospholipid fatty acyl chains in the gel state. In addition, association of the M protein with the bilayer appeared to increase the cooperativity of the lipid phase transition, as evidenced by an increase in the transition line slope. The most dramatic alteration of bilayer lipid behavior, however, was the large increase in the T_m in sonicated DPPG/DPPC vesicles reconstituted with M protein. Previous studies have demonstrated that small unilamellar vesicles with diameters in the size range of 200–700 Å exhibit a transition temperature which is a function of their diameter; the size dependence of the T_m is most pronounced for vesicles with a diameter of $\sim$400 Å and is nearly insignificant for vesicles >700 Å in diameter (Lichtenberg et al., 1981; Figure 6). In the results presented here, the large increase in T_m in the DPPG-containing vesicles, as compared with the less dramatic alteration of T_m in the DMPS-containing vesicles (diameter $\sim$900 Å), is not a result of size dependence for the following reasons: (a) Reconstitution of the sonicated DPPG-containing vesicles with 0.5 mol % M protein results in an increase in vesicle diameter from $\sim$410 to $\sim$440 Å, as detected by laser light scattering. This increase in size would be expected to cause an experimentally observed increase in T_m of only $\sim$0.4 °C (Lichtenberg et al., 1981; Figure 6). (b) Fused unilamellar vesicles (diameter $\sim$1100 Å) containing 50 mol % each of DPPG and DPPC displayed an increase in T_m of $\sim$3–5 °C when reconstituted with 0.5 mol % M protein (data not shown). We postulate that the increase in T_m observed is most likely due to the formation of domains in the bilayer which are in direct contact with the M protein. The strict requirement of acidic phospholipids for M binding to bilayers suggests that these domains could be enriched in PS or PG, at the expense of the neutral PC lipid component. Increased order of the phospholipids in these domains would result in an increase in T_m and transition cooperativity, both of which are observed in this study.

The amino acid sequence of the M protein has been predicted from a cDNA clone derived from the mRNA for M protein (Rose & Gallione, 1981). In support of previous studies, which showed by isoelectric focusing that the M protein has a p*I* of $\sim$9.1 (Carroll & Wagner, 1979), the predicted sequence reveals the presence of 21 lysines, 10 arginines, and 8 histidines in a total of 229 amino acids. The amino-terminal segment of the M protein is highly basic, containing 8 lysines within the first 19 residues (Rose & Gallione, 1981). The initial interaction of the M protein with bilayers containing an acidic phospholipid is electrostatic in nature and appears to require the free ϵ-amino groups of the lysine residues of the protein (Zakowski et al., 1981). It can be postulated, therefore, that the M protein, and specifically the amino-terminal domain, initially associates with the acidic polar head group of phosphatidylglycerol or phosphatidylserine in a bilayer in a manner similar to that of polylysine. However, unlike polylysine, the association of M protein with mixed lipid bilayers appears to involve a secondary nonelectrostatic interaction (Zakowski & Wagner, 1980). Although the protein does not contain any lengthy sequences of consecutive hydrophobic amino acids (Rose & Gallione, 1981), binding of the protein to the bilayer may induce a change in the tertiary conformation of the polypeptide chain, which could result in the formation of membrane-associated hydrophobic domains.

Lateral phase separation induced by Ca^{2+} in bilayers composed of phosphatidylserine and phosphatidylcholine has been reported (Jacobsen & Papahadjopoulos, 1975; Hoekstra, 1982). In addition, the association of polylysine (Galla & Sackmann, 1975), cytochrome *c* (Birrell & Griffith, 1976), or human myelin basic protein (Boggs et al., 1977) with mixed phospholipid bilayers has been observed to cause lateral phase separation of the lipid components. The interaction of myelin basic protein with the bilayer appears to be very similar to that of M protein and relies strongly on an initial electrostatic attraction with an acidic phospholipid component, followed by a secondary interaction of undefined nature (Papahadjopoulos et al., 1975). The results presented here show that binding of the M protein to the acidic phospholipid component of a DPPG/DPPC mixed lipid bilayer induced an increase in the observed T_m in a manner similar to that of the bivalent cation Ca^{2+} or of polylysine. An increase in the observed T_m, in conjunction with an increase in lipid order in the gel state, is insufficient in itself to conclude that a protein-induced phase separation has occurred. We postulate, however, that the M

protein neutralizes the charge repulsion of acidic phospholipid head groups and allows tighter packing of the lipids in the bilayer. This can be visualized by assuming that the basic residues of the protein electrostatically cross-link the charged phospholipid head groups of the bilayer and thereby form domains which are enriched in the acidic phospholipid component. These domains may be composed of pure acidic phospholipids or mosaics of mixed lipid that are enriched in the acidic species. Our current investigations are directed to examining the effects of membrane binding of M protein on the lateral redistribution of phospholipids in the bilayer plane.

Acknowledgments

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Registry No. DPPC, 2644-64-6; DMPS, 64023-32-1; DPPG, 4537-77-3; Ca, 7440-70-2.

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