ability and presumably contained no functional band 3 molecules. Hence

$$P(0,\mu) = 0.80 \pm 0.04$$

$$\frac{\mu^0}{0!}e^{-\mu} = 0.80 \pm 0.04$$

$$\mu = 0.22 \pm 0.05$$

After vesicle treatment, only 54 ± 4% of the cells remained impermeable to anions, corresponding to an average of 0.62 ± 0.07 functional band 3 molecule per cell. Hence, vesicle treatment produced approximately a 3-fold increase in the mean number of band 3 molecules per cell.

The validity of this model can be tested by using the Poisson distributions corresponding to $\mu = 0.22$ and 0.62 (Figure 8) to reconstruct influx curves analogous to those in Figure 6. Assuming that influx is first order in $[Cl^-]$

$$\% \text{ equilibrium}(t) = 100[1 - \sum_{x=0}^{\sim 10^6} P(x,\mu)e^{-k(x)t}] \quad \text{(A2)}$$

where $k(x)$ is the apparent rate constant for a fraction of cells containing x band 3 molecules per cell. For small x, $k(x)$ should be a linear function of x (eq A3):

$$k(x) = kx = x/\tau \quad \text{(A3)}$$

Hence, for eosin MA inhibited cells

$$\% \text{ equilibrium}(t) \cong 100[1 - 0.800 - 0.178e^{-t/\tau} - 0.020e^{-2t/\tau} - 0.001e^{-3t/\tau} - 0.0001e^{-4t/\tau}] \quad \text{(A4)}$$

Similarly, for cells treated with band 3–vesicle complexes

$$\% \text{ equilibrium}(t) \cong 100[1 - 0.540 - 0.330e^{-t/\tau} - 0.103e^{-2t/\tau} - 0.021e^{-3t/\tau} - 0.003e^{-4t/\tau}] \quad \text{(A5)}$$

These curves, plotted in Figure 9, agree well with the experimental data from Figure 6. It is noteworthy that although these curves explicitly contain four exponential components with different characteristic times, they are indistinguishable from single-component exponentials for the time scales plotted (inset, Figure 9). The ratio of the apparent rate constants before and after vesicle treatment, taken from Figure 9, is 1.20 ± 0.30 (where the standard error is derived from the uncertanties in μ), in agreement with the experimental value of 1.01.

Thermotropic Behavior of Mixed Phosphatidylcholine–Phosphatidylglycerol Vesicles Reconstituted with the Matrix Protein of Vesicular Stomatitis Virus†

Jon R. Wiener, Robert R. Wagner,* and Ernesto Freire

ABSTRACT: The peripheral matrix (M) protein of vesicular stomatitis virus reconstituted with fused unilamellar vesicles containing equimolar amounts of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol has been studied by high-sensitivity differential scanning calorimetry and steady-state fluorescence spectroscopy. The association of the basic (p$I \simeq 9.1$) matrix protein with the mixed neutral/acidic phospholipid bilayer induced a dramatic upward shift in the phospholipid phase transition temperature even at M protein/phospholipid molar ratios as low as 1/12000. Despite the large effect of the matrix protein on the phase transition temperature and the shape of the heat capacity function, the enthalpy change associated with the phospholipid gel to liquid-crystalline transition remained constant even at saturating protein concentrations. Steady-state fluorescence depolarization measurements indicated that association of the M protein with the phospholipid bilayer increased the apparent order of the bilayer both below and above the phospholipid phase transition temperature; this effect may be responsible for the observed changes in thermotropic behavior. At high protein concentrations, the matrix protein induced lipid phase separation, probably due to its tight association with the acidic phospholipid component of the membrane.

Vesicular stomatitis virus (VSV)[1] is a negative-stranded RNA virus which is composed of a transcriptase-containing nucleoapsid core surrounded by a lipoprotein envelope. The virion obtains the lipid component of the envelope as it buds from the host cell plasma membrane during viral maturation (Wagner, 1975). Two viral-coded proteins are intimately associated with the envelope: (i) a transmembrane glycoprotein (G) ($M_r \sim 69000$), which comprises the spikes that protrude from the external surface of the membrane (Schloemer & Wagner, 1975), and (ii) a nonglycosylated peripheral matrix protein (M) ($M_r \sim 29000$), which is quite basic (p$I \sim 9.1$; Carroll & Wagner, 1979) and appears to line the internal surface of the viral membrane (Zakowski & Wagner, 1980). Both viral membrane proteins have been purified and reconstituted with phospholipid vesicles of defined composition (Petri & Wagner, 1979; Zakowski et al., 1981).

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[1] Abbreviations: VSV, vesicular stomatitis virus; M, VSV matrix protein; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; SUV, small unilamellar vesicles; FUV, fused unilamellar vesicles; MLV, multilamellar vesicle(s); T_m, lipid phase transition temperature; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; p*I*, isoelectric pH; DPH, 1,6-diphenyl-1,3,5-hexatriene; SDS, sodium dodecyl sulfate; ESR, electron spin resonance.

0006-2960/83/0422-6117$01.50/0

The interaction of the G protein with vesicles composed of dipalmitoylphosphatidylcholine (DPPC) has been studied by using steady-state fluorescence depolarization (Petri et al., 1981), differential scanning calorimetry (Petri et al., 1980), and, more recently, differential polarized phase fluorometry (Pal et al., 1983). The results of these studies indicated that insertion of the G protein in the bilayer lowered the lipid gel to liquid-crystalline phase transition temperature (T_m), broadened the phase transition, and significantly lowered the enthalpy change associated with the phospholipid transition. In contrast to the integral G protein, the peripheral M protein requires the presence of acidic phospholipid as a prerequisite for reconstitution with the bilayer (Zakowski et al., 1981). Presumably, the association of the M protein with phospholipid bilayers is initiated by an electrostatic attraction between the numerous lysine residues of the M protein (Rose & Gallione, 1981) and the acidic lipid components of the bilayer. However, secondary nonelectrostatic forces also appear to be involved since, once bound, the protein cannot be removed by the presence of high salt concentration (Zakowski et al., 1981).

The capacity to reconstitute phospholipid bilayers of defined composition with purified M protein of VSV provides a model system to study the interaction of a peripheral membrane protein with a mixed-lipid bilayer. We have previously demonstrated that reconstitution of the M protein with DPPG/DPPC (1:1) mixed phospholipid sonicated unilamellar vesicles (SUV) profoundly altered the lipid gel to liquid-crystalline phase transition, when analyzed by steady-state fluorescence depolarization and differential polarized phase fluorometry (Wiener et al., 1983). In this paper, we present the results of a high-sensitivity calorimetric characterization of the perturbing effects of the M protein on the thermotropic behavior and organization of mixed dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol fused unilamellar vesicles (FUV).

Experimental Procedures

Materials

Cells and Virus. Plaque-purified VS virus of the Indiana serotype was grown on baby hamster kidney (BHK-21) cells at 31 °C in the presence of a 5 μCi/mL ^{3}H-labeled amino acid mixture (New England Nuclear, Boston, MA). Bullet-shaped virions released from infected cells were purified to homogeneity by using differential, rate zonal, and equilibrium centrifugation in sucrose and potassium tartrate gradients as previously described (Barenholz et al., 1976). Viral stocks prepared in this manner were stored at −80 °C in phosphate-buffered saline (pH 7.4) at a concentration of 2–5 mg/mL.

Lipids. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-3-*sn*-phosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Each lipid stock yielded a single spot by thin-layer chromatography in a chloroform/methanol–ammonia (65:25:4 v/v/v) solvent system. Stock lipid preparations were assayed prior to each experiment for total phospholipid phosphorus content by a modification of the Bartlett procedure (Marinetti, 1962).

Isolation of VSV M Protein. The ^{3}H-amino acid labeled matrix protein of VSV was purified by the method of Zakwoski et al. (1981). Briefly, purified virus was solubilized in 10 mM Tricine (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl, and 1.2 mM dithiothreitol at room temperature. Following centrifugation to pellet the nucleocapsid material, the supernatant containing the viral G and M proteins and viral phospholipids was chromatographed over a Whatman P11 phosphocellulose column, as previously described (Zakowski et al., 1981). The M protein fractions obtained by elution at 0.65 M NaCl in 10 mM Tricine (pH 7.5) containing 10% glycerol were pooled and stored at 4 °C. M protein isolated in this manner is >98% pure as determined by SDS–polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Lowry et al. (1951) and by the specific radioactivity of the M protein labeled with ^{3}H-amino acids. The mole percentage of the matrix protein was calculated by assuming a molecular weight of 29 000.

Methods

Preparation of Large DPPC/DPPG Fused Unilamellar Vesicles (FUV). Small unilamellar vesicles were prepared by the method of Barenholz et al. (1977). Equimolar amounts of DPPC and DPPG containing 0.08 μCi of [^{14}C]DPPC (New England Nuclear, Boston, MA; 100 mCi/mmol) per mol of lipid were dried under a stream of nitrogen and subsequently lyophilized for 12 h. The dried lipid mixture was then resuspended in 7.5 mL of 10 mM Tricine (pH 7.5) containing 0.65 M NaCl, bubbled with N_2, and gently vortexed at 46 °C to assure complete resuspension of the lipids. The samples were sonicated with a Branson sonifier under N_2 at 46 °C in 4-min bursts until they became clear. Total sonication time did not exceed 20 min. The sonicated lipid dispersions were then centrifuged at 16000*g* for 185 min at 47 °C to pellet any residual multilamellar vesicles. The upper third of the supernatant was removed and incubated for at least 35 days under nitrogen at 4 °C in order to promote and assure complete fusion of the small unilamellar vesicles (SUV, ~400 Å in diameter) into larger fused unilamellar vesicles (FUV) of ~1100-Å diameter.

Reconstitution of Viral M Protein with Preformed DPPC/DPPG Vesicles. Reconstitution of VSV M protein with preformed fused unilamellar vesicles was performed by a modification of the method of Petri & Wagner (1980). Matrix protein was added to a known quantity of fused vesicles (concentration determined by scintillation spectrometry) to give the desired M/phospholipid molar ratio. The mixture was then dialyzed at 46 °C for 24 h vs. 5 L of 10 mM Tricine (pH 7.5) containing decreasing molar concentrations of salt. Finally, the reconstituted vesicles were dialyzed for 24 h vs. 5 L of buffer containing no salt. Protein/vesicle reconstitutions prepared in this manner give a single peak on sucrose density gradients with essentially all the protein bound to the membrane.

High-Sensitivity Scanning Calorimetry. All calorimetric experiments were performed with a Microcal MC1 differential scanning calorimeter. The sensitivity and precision of the basic calorimetric unit have been improved by the use of two separate Keithley amplifiers connected to the heat capacity and temperature outputs of the instrument and interfaced to a TEC86 microcomputer system for automatic data collection and processing. The calorimetric data are digitized by a TM-AD212 A/D converter operating at a 40-kHz conversion rate and stored on floppy disks at 0.05 °C intervals for subsequent analysis. In this way, it is possible to perform experiments by using very dilute biological materials. With pure lipid dispersions, concentrations lower than 0.5 mg/mL can be used with a total sample volume of 0.7 mL. With the protein/lipid reconstitutions, the sample concentrations were 3 mg/mL of lipid. All the experiments in this paper were performed at a scanning rate of 20 °C/h.

Fluorescence Spectroscopy. Steady-state fluorescence depolarization experiments were performed as described by

Barenholz et al. (1976) using a modified Perkin-Elmer MPF3 fluorescence spectrophotometer with polarizers in the excitation and emission beams. The cuvette temperature was controlled with a Lauda water bath, and the temperature was monitored within ±0.1 °C by means of a Yellow Springs Instrument thermistor probe connected to a digital ohmmeter. For the measurement of fluorescence depolarization as a function of temperature, the sample was heated to 60 °C and cooled at a rate of 25 °C/h. 1,6-Diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Junction City, OR) was dissolved in tetrahydrofuran and added to the vesicle suspensions at a ratio of 1 probe molecule per 400 phospholipid molecules. The quantity of tetrahydrofuran present in the suspension did not exceed 0.1% relative to the buffer. Probe incorporation was allowed to continue for 1 h under a nitrogen atmosphere at 41 °C prior to the experiments. Light scattering of the vesicle preparations in the absence of probe did not exceed 2% of the fluorescence intensity perpendicular to the plane of the excitation beam. Fluorescence was performed by excitation of DPH at 360 nm. Emission was monitored at 430 nm parallel and perpendicular to the plane of excitation, using the MPF3 390-nm cutoff filter to reduce scattering. Anisotropy was calculated as described elsewhere (Barenholz et al., 1976).

Additional Procedures. Multilamellar liposomes (MLV) were prepared by dissolution of dried phospholipid films containing equimolar quantities of DPPG and DPPC in Tricine buffer containing 0.65 M NaCl in the presence or absence of the M protein and dialyzed as described above. Preliminary experiments using rate zonal centrifugation in sucrose gradients indicated that the M protein bound quantitatively to the liposomes. Protease digestion of M protein was performed by incubation of reconstituted vesicle preparations with thermolysin (Sigma, St. Louis, MO), 50 units/mg of protein, for 30 min at 37 °C. Sucrose density centrifugation was performed exactly as described previously (Wiener et al., 1983). Vesicle preparations were examined by transmission electron microscopy; fresh vesicle suspensions, prepared as described above, were placed on Formvar/carbon-coated grids, stained with 2% ammonium molybdate, and viewed immediately for size estimation.

Results

Vesicle Characterization. The sonication of equimolar mixtures of DPPC and DPPG in buffer containing 0.6 M NaCl results in the formation of small unilamellar vesicles (SUV) of ~400-Å diameter as determined by quasi-elastic light scattering (Wiener et al., 1983) and gel filtration chromatography (data not shown). These vesicles undergo a gel to liquid-crystalline lipid phase transition similar to the one observed with sonicated dispersions of pure DPPC. As shown in Figure 1, the calorimetric scans of these preparations are characterized by a single peak in the heat capacity function centered at 38.5 °C and an enthalpy change (ΔH) of 5.0 kcal/mol of lipid. At low ionic strengths, the DPPC/DPPG vesicles are stable below the phase transition temperature (T_m) and do not give rise to a population of larger fused vesicles as in the case of pure DPPC vesicles. Under these conditions, the surface charge of the vesicles presumably prevents aggregation, an obligatory step for fusion (Wong et al., 1982). At higher ionic strengths (0.6 M NaCl), however, these vesicles behave very much like pure DPPC vesicles, giving rise to a second calorimetric peak centered at 41.5 °C that grows at the expense of the 38.5 °C peak. After 35 days at 4 °C, only the high-temperature peak representing fused unilamellar visicles (FUV) can be detected by the calorimeter (see Figure 1).

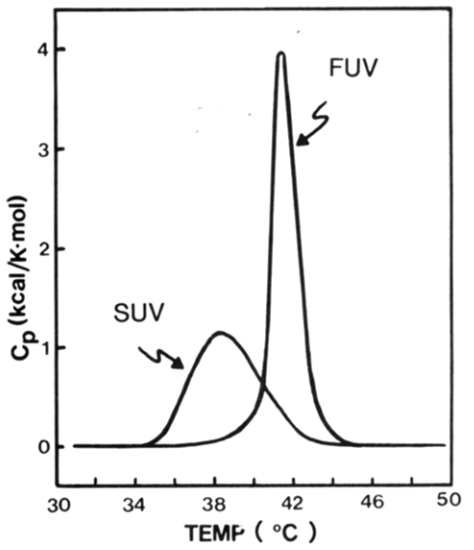

FIGURE 1: Scanning calorimetry plotted as excess heat capacity (C_p) vs. temperature for small sonicated unilamellar vesicles (SUV) and fused unilamellar vesicles (FUV) prepared from equimolar mixtures of DPPC and DPPG. The scans were performed in 10 mM Tricine (pH 7.5).

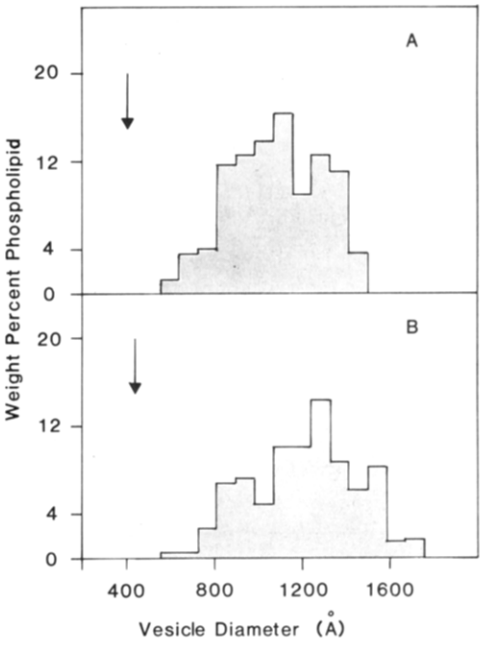

FIGURE 2: Size/frequency distribution function measured by negative-stain electron microscopy of fused unilamellar vesicles (FUV) in the absence (A) or presence (B) of M protein. Vesicles were prepared in 10 mM Tricine (pH 7.5) containing 0.65 M NaCl and dialyzed in the absence or presence of M protein as described under Experimental Procedures. Sample vesicle preparations were examined by electron microscopy at a magnification of 115000×. Representative photographs were analyzed for size distribution and weight percent phospholipid. The arrows show the vesicle diameters (~400 and ~440 Å, respectively) for SUV preparations in the absence (A) and presence (B) of M protein for comparison.

The resulting vesicle population was characterized with regard to its size distribution by negative-staining electron microscopy. Representative samples containing ~300 vesicles were sized and counted to obtain the size/frequency distribution function. The results of this analysis are shown in Figure 2 and indicate that the resulting unilamellar fused vesicles had an average diameter of 1100 ± 200 Å. Reconstitution of the M protein with these vesicles resulted in a population of slightly larger vesicles (1280 ± 300 Å in diam-

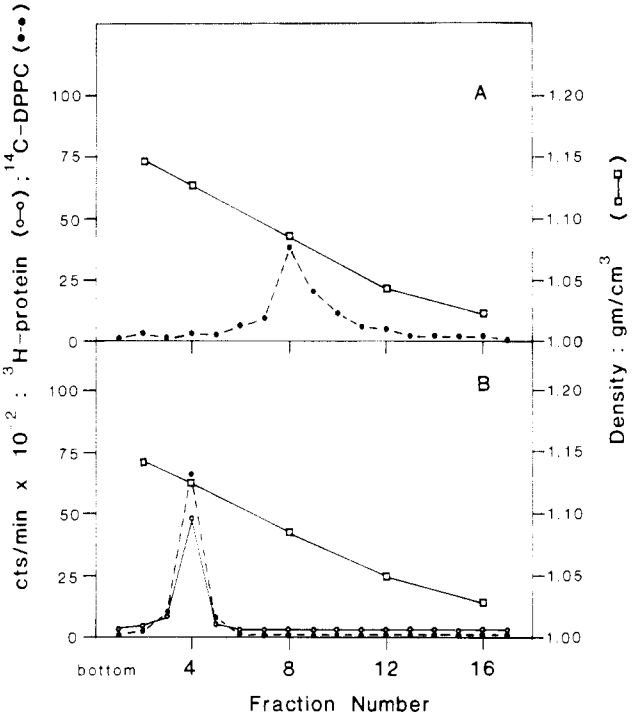

FIGURE 3: Comparative buoyant density of DPPG/DPPC (1:1) FUV dialyzed in the absence (A) or presence (B) of 0.5 mol % M protein. Vesicle preparations contained a trace quantity of [^{14}C]DPPC to track the phospholipid. The M protein was labeled metabolically with ^{3}H-amino acids. Buoyant density was determined by flotation upward through 0–30% linear sucrose gradients containing 0.15 M NaCl by centrifugation at 200000g at 46 °C for 20 h. Gradients were fractionated and aliquots subjected to scintillation spectrometry. Densities were determined by refractometry. (●) [^{14}C]DPPC; (○) ^{3}H-labeled protein; (□) density in grams per cubic centimeter.

eter) as shown in panel B of Figure 2. The homogeneity of these preparations was also demonstrated by sucrose density gradient centrifugation as shown in Figure 3. Under the conditions of this reconstitution protocol, all the M protein is located on the outer leaflet of these vesicles. Reconstitution of the M protein with fused unilamellar vesicles at varying M:phospholipid molar ratios indicated that protein bound to the FUV mixed-lipid bilayers with an efficiency equivalent to that with SUV (data not shown). The capacity of the large FUV to bind M protein reached apparent saturation at an M/phospholipid molar ratio of ~1/75. As shown in Figure 3, binding of 0.5 mol % M protein to the bilayer resulted in an increase in vesicle density of ~0.05 g/cm^3. Similar gradients run with a range of protein/phospholipid molar ratios indicated that the increase in density is proportional to the quantity of protein bound (data not shown).

Thermotropic Behavior. The thermotropic behavior of fused DPPC/DPPG vesicles containing increasing mole fractions of M protein was studied by high-sensitivity differential scanning calorimetry. As shown in Figure 4, in the absence of M protein the calorimetric profile is characterized by a single peak at 41.5 °C associated with the gel to liquid-crystalline transition of the phospholipid vesicles. The enthalpy change (area under the heat capacity curve) for this transition is 7.1 kcal/mol, similar to the value reported for pure DPPC fused unilamellar vesicles (Wong et al., 1982; Freire et al., 1983). The half-weight width for the mixture ($\Delta T_{1/2}$) is 1.7 °C, while that of pure DPPC fused vesicles is 0.4 °C. Such a broadening of the heat capacity profile is characteristic for mixtures and indicates a reduced cooperative behavior; the calculated cooperative unit for the mixture is only 60 lipid molecules compared to 300 lipid molecules for pure DPPC.

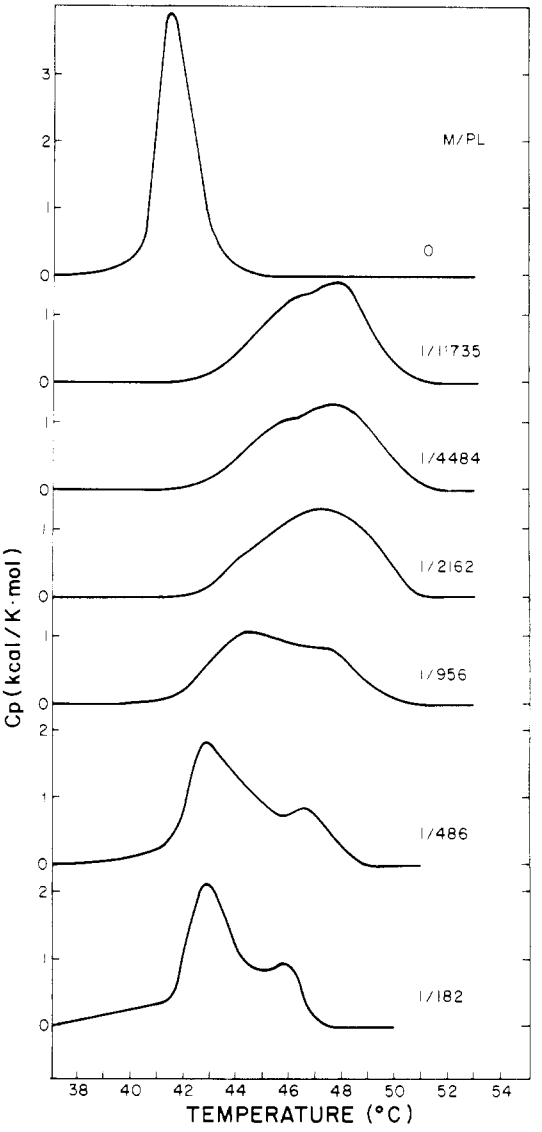

FIGURE 4: Scanning calorimetry plotted as excess heat capacity (C_p) vs. temperature for fused unilamellar vesicles (FUV) prepared from equimolar mixtures (1:1) of DPPC and DPPG at varying M protein/phospholipid (M/PL) molar ratios. All the scans were performed in 10 mM Tricine (pH 7.5).

Under the conditions of these experiments and in the absence of M protein, no evidence for phase separation between DPPC and DPPG was observed.

The addition of M protein, even at very low protein/phospholipid ratios, had a dramatic effect on the thermotropic behavior of the DPPC/DPPG vesicles. As shown in Figure 4, protein/phospholipid molar ratios as low as 1/12000 were capable of producing a 6 °C shift in T_m to higher temperatures and a considerable broadening of the heat capacity profile. The total disappearance of the peak at 41.5 °C indicates that the M protein, even though it is located only in the outer surface of the membrane, also perturbs the thermotropic behavior of the inner layer. Despite the dramatic changes in T_m and in the shape of the calorimetric peak, the enthalpy change for the lipid phase transition remained constant and was independent of the amount of M protein present (see Figure 5). This behavior should be contrasted to that observed with integral membrane proteins, including the G protein from vesicular stomatitis virus, which usually decrease the magnitude of the enthalpy change associated with the lipid phase transition (Petri et al., 1980; Paphadjopoulos et al., 1975; van Zoelen et al., 1978; Freire et al., 1983).

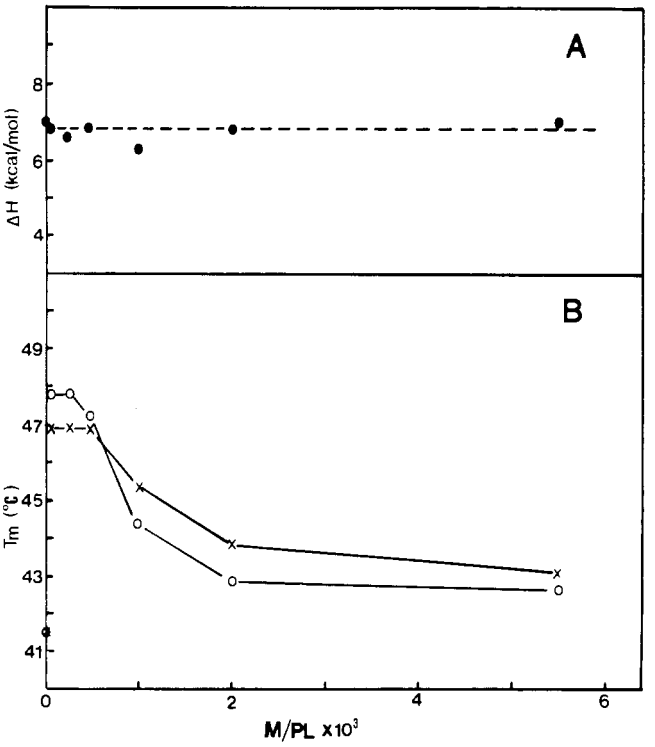

FIGURE 5: Dependence of the (A) enthalpy change (ΔH) and (B) transition temperature (T_m) associated with the phospholipid phase transition at varying M protein/phospholipid (M/PL) molar ratios. The transition temperature was calculated both as the position of the peak maximum in the heat capacity function (O) and as the temperature at which half of the transition has occurred (×).

As the concentration of M protein is increased beyond a molar ratio of 1/2000, the transition temperature of the reconstituted vesicles decreased and the heat capacity function clearly showed the presence of two superimposed peaks (Figure 4), indicating that phase separation takes place at high protein/phospholipid molar ratios. Repeated scans of the same protein/lipid reconstitutions gave identical heat capacity profiles, indicating that the structures formed were at equilibrium and did not represent time-dependent metastable configurations (data not shown).

Calorimetric experiments were also performed on multilamellar DPPC/DPPG vesicles containing M protein on the inner as well as the outer faces of the membrane. These experiments were designed to test whether the appearance of two peaks in the calorimetric scans represented a lateral phase separation process or a different thermotropic behavior of the inner and outer monolayers due to the asymmetric localization of the M protein in the unilamellar vesicles. As shown in Figure 6, the multilamellar vesicles (MLV) also induced the appearance of two peaks, suggesting that the phase separation process observed at high protein/phospholipid ratios is perhaps due to lateral separation of phospholipid domains. However, exposure to thermolysin of MLV/M protein complexes revealed that ~90% of the M protein was accessible to the protease and that only 10% of the protein not hydrolyzed by thermolysin was available for binding to the inner layers of the vesicles (data not shown). Nevertheless, all the lipid was affected by the M protein, judging by the total disappearance of the peak at 41.5 °C.

Fluorescence Anisotropy. The above calorimetric experiments, as well as previous fluorescence experiments performed on small unilamellar vesicles (Wiener et al., 1983), indicated that the M protein of VSV induced a dramatic increase in the transition temperature of the phospholipid molecules. This

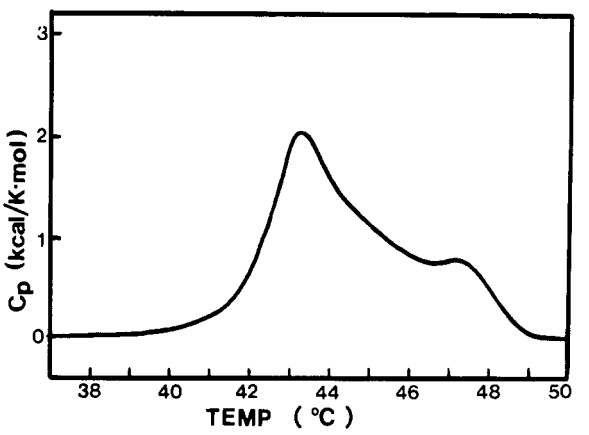

FIGURE 6: Excess heat capacity (C_p) function vs. temperature for multilamellar vesicles (MLV) prepared from an equimolar mixture of DPPC and DPPG in the presence of 0.33 mol % M protein.

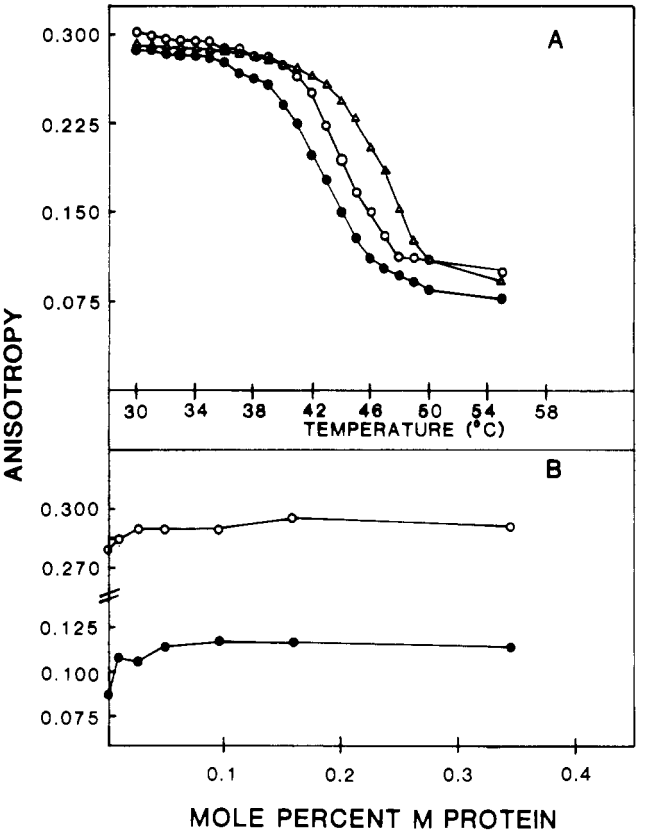

FIGURE 7: Steady-state DPH fluorescence anisotropy of FUV in the absence or presence of M protein. (A) Anisotropy of DPH vs. temperature of vesicles containing no protein (●), 0.05 mol % M (△), or 0.34 mol % M (O). (B) Anisotropy of DPH vs. mole percent M protein. Values for anisotropy were obtained from the experiment depicted in panel A and other experiments and plotted for two representative temperatures: (O) 35 °C; (●) 50 °C. FUV were prepared as described under Experimental Procedures. Incorporation of DPH was performed at a ratio of 1 probe molecule per 400 phospholipid molecules. Anisotropy was calculated as described previously (Barenholz et al., 1976).

effect should be contrasted to that of most membrane proteins, including the G protein of VSV, which usually induces a decrease in the transition temperature of the phospholipid (Petri et al., 1981; Pal et al., 1983). In order to investigate the origin of this effect, the steady-state emission anisotropy of the fluorescence probe DPH was measured as a function of temperature at different M protein concentrations. The results of these experiments are shown in Figure 7. As in the case of the calorimetric experiments, a large increase in T_m

was observed with a protein/lipid molar ratio as low as 1/2000 followed by a decrease in T_m at higher protein/lipid ratios. As shown in Figure 7B, the presence of the M protein, even at a low protein/phospholipid molar ratio, induced a relatively large increase in the transition temperature and increased the DPH anisotropy both below and above the lipid phase transition temperature, indicating that the M protein increased the apparent order in both the gel and liquid-crystalline phases of the membrane.

Discussion

The effects of membrane proteins on the thermotropic behavior of phospholipid bilayer vesicles have been studied in several laboratories by using differential scanning calorimetry (Curatolo et al., 1977; Chapman et al., 1979; Freire et al., 1983). These studies have shown that the primary effect of integral membrane proteins is to decrease the enthalpy change of the lipid phase transition with only a small effect on the transition temperature. This effect of integral membrane proteins has been attributed to a direct interaction between the hydrophobic domains of these proteins and the hydrocarbon chain of the lipid molecules; in fact, for most integral proteins studied, the decrease in ΔH can be accounted for by a single layer of lipid withdrawn from participating in the phase transition per protein molecule. The local nature of this perturbation has also been demonstrated by ESR measurements on several integral membrane proteins (Marsh et al., 1982).

As demonstrated by the experimental results presented in this paper, the effects of the peripherally associated matrix protein of vesicular stomatitis virus on the thermotropic behavior of the lipid bilayer are quite different from those induced by integral membrane proteins. First, the enthalpy change associated with the lipid phase transition remains constant even at saturating M protein concentrations; second, the transition temperature increases significantly even at very low M protein concentrations; and third, high M protein/lipid ratios induce lipid phase separation within the bilayer matrix. This unusual behavior of the M protein can be explained in part in terms of the known structural parameters of the protein. The M protein is primarily a peripheral protein whose association with the lipid bilayer is presumably initiated by an electrostatic attraction between the lysine residues of the protein and the acidic phospholipid head groups in the bilayer. This electrostatic attraction is, however, not the only force involved in the association since, once bound, the protein cannot be dissociated from the membrane by an increase in the ionic strength (Zakowski et al., 1981). Presumably, after the initial binding events take place, the M protein is capable of some penetration into the hydrophobic core of the bilayer despite the basence of long stretches of hydrophobic amino acids in the sequence of the M protein (Rose & Gallione, 1981). This penetration is, however, not deep enough to establish a direct interaction with the lipid acyl chains as demonstrated by the fact that the M protein cannot be labeled by lipophilic cross-linking reagents (Zakowski & Wagner, 1980). In addition, thermolysin protease treatment of M protein reconstituted unilamellar bilayers completely removes all of the protein from the membrane surface (Zakowski et al., 1981). The absence of a direct interaction of the M protein with the phospholipid acyl chains may explain the lack of effect of the M protein on the transition enthalpy.

The strong association of M protein with the acidic phospholipid components of the membrane most likely restricts the mobility of the lipid molecules located underneath the protein. This results in an apparent increase in the order of the phospholipid molecules as reflected by the increased anisotropy values obtained with the fluorescence probe DPH, both below and above the transition temperature. This strong association of the M protein with the membrane surface may result in an increase in the thermodynamic work required for the expansion of the phospholipid molecules at the phase transition. If this is so, the presence of the M protein will increase the free-energy change of the system due to an extra $P\Delta A$ term (P, lateral pressure; ΔA, increase in cross-sectional area per lipid molecule at the phase transition) with a concomitant increase in the phase transition temperature. Thus, the additional lateral pressure introduced by the protein may explain the observed increase in the transition temperature. Another possible factor contributing to the observed increase in T_m may be a preferential binding affinity of the protein for phospholipids in the gel state. However, the nature of this effect appears to be complex, probably involving competing influences, as suggested by the fact that at low protein/lipid ratios the M protein increases T_m whereas at high protein/lipid ratios the effect is reversed. The exact nature of the large increase in T_m observed at low M/PL ratios remains unclear.

At protein/phospholipid ratios higher than 1/1000, the M protein induces phase separation of the phospholipid components of the membrane, judging by the appearance of two well-defined maxima in the heat capacity function. This phase separation phenomenon is probably triggered by the strong association of the M protein with the acidic lipid components of the bilayer; this association would have the net effect of increasing the local concentration of acidic phospholipid in M protein rich areas of the membrane, thereby increasing the relative concentration of neutral phospholipid in those areas of the membrane devoid of M protein. This interpretation is consistent with the observation that the phase-separated peak approaches the melting temperature of pure DPPC when the M/phospholipid ratio is increased.

The data presented here strongly suggest that the interaction of the highly basic VSV M protein (p$I \simeq 9.1$; Carroll & Wagner, 1979) with mixed phospholipid bilayers containing equimolar quantities of neutral and acidic phospholipid results in the lateral redistribution of the individual lipid species into stable clustered domains. It is not yet clear if the domains represent mosaics of the lipid moieties or are composed exclusively of either acidic or neutral phospholipids. It is interesting to compare the effects observed here with studies of cytochrome c induced lateral phase separation in phosphatidylglycerol-containing model membranes. In a manner similar to M protein, the extrinsic cytochrome c protein (p$I \simeq 10.6$) electrostatically binds acidic phospholipids and induces the formation of patches enriched in these molecules (Birrell & Griffith, 1976). Similar findings have been observed in membranes reconstituted with human myelin basic protein (Boggs et al., 1977). Lateral, and possibly transversal, redistribution of phospholipids caused by the specific interaction of membrane proteins with particular lipid species may result in the formation and maintenance of bilayer asymmetry. In this context, we note that analysis of the phospholipid composition of the VSV envelope reveals that phosphatidylserine is the sole acidic species present in significant quantity ($\sim$18% of total phospholipid) and is highly asymmetrically oriented, the bulk ($\sim$85%) occupying the membrane inner leaflet (Patzer et al., 1979). The absolute requirement for VSV M protein in the viral budding process (Schnitzer & Lodish, 1979) and the apparent location of the protein in the virus on the phosphatidylserine-rich interior surface of the envelope (Zakowski & Wagner, 1980) suggest that M protein/acidic

phospholipid interactions may play a key role in viral maturation and budding from the cytoplasmic membrane of the infected host cell.

Acknowledgments

We are grateful to Nancy Salomonsky for electron microscopy and to Paige Hackney for excellent preparation of the manuscript.

Registry No. DPPC, 2644-64-6; DPPG, 4537-77-3.

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Photoaffinity Labeling of a Synaptic Vesicle Specific Nucleotide Transport System from *Torpedo marmorata*†

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ABSTRACT: We have employed azido derivatives of ATP and AMP to identify the ATP translocase of synaptic vesicles. Azido-AMP inhibits transport of both ATP and AMP in vitro. The affinity of the translocase for the azido derivatives is similar to that of the native ligands. Upon UV irradiation of vesicles incubated with radiolabeled azido-AMP or -ATP, a molecular weight (M_r) 34 000 polypeptide is selectively modified. On two-dimensional gel electrophoresis, the single radiolabeled polypeptide has a p*I* of ~7.7. Analysis of the fractions obtained when vesicles were purified on linear sucrose density gradients reveals that the M_r 34 000 polypeptide is highly enriched in the vesicle-containing fractions. The findings support the notion that this polypeptide is identical with a previously described vesicle-specific component of the same molecular size [Stadler, H., & Tashiro, T. (1979) *Eur. J. Biochem. 101*, 171–178], and we conclude on the basis of uptake inhibition and photoaffinity labeling results that this protein is directly involved in ATP translocation of synaptic vesicles.

Synaptic vesicles and vesicles from endocrine glands which act as storage sites and vehicles for the release of neurotransmitters and hormones often contain high concentrations of nucleotides (Winkler, 1977; de Potter et al., 1970). For example, cholinergic synaptic vesicles from the electromotor tissue of *Torpedo marmorata* have been shown to contain molar quantities of adenosine 5′-triphosphate (ATP) (Dowdall et al., 1974), which is released upon stimulation along with acetylcholine. Subsequent reuptake of the ATP into the newly formed vesicle population parallels that of acetylcholine (Zimmermann, 1979). Although the function of this large store of vesicular ATP is not known, modifications of transmitter response by adenosine nucleotides have been documented (Israel & Meunier, 1978; McAfee & Greengard, 1972).

The transport of ATP into synaptic vesicles has been shown

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0006-2960/83/0422-6123$01.50/0