Thermotropic Behavior of Dipalmitoylphosphatidylcholine Vesicles Reconstituted with the Glycoprotein of Vesicular Stomatitis Virus†

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ABSTRACT: The vesicular stomatitis virus glycoprotein reconstituted into dipalmitoylphosphatidylcholine (DPPC) vesicles exerts a profound effect upon the DPPC gel to liquid-crystalline phase transition. The glycoprotein was reconstituted into DPPC vesicles by octyl glucoside dialysis. The gel to liquid-crystalline phase transition of these vesicles was monitored by differential scanning calorimetry. Vesicles formed in the absence of glycoprotein (600–2100-Å diameter) underwent the phase transition at 41.0 °C and had an associated enthalpy change of 8.0 ± 1.6 kcal/mol. Increasing the mole ratio of glycoprotein to DPPC in the vesicles to 0.15 mol % reduced both the transition temperature and the transition enthalpy change. The enthalpy change as a function of the mole percent glycoprotein could be fit to a straight line by a least-squares procedure. Extrapolation of the results to the glycoprotein concentration where the enthalpy change was zero indicated one glycoprotein molecule bound 270 ± 150 molecules of DPPC.

Vesicular stomatitis (VS)[1] virus is an RNA-containing rhabdovirus which is enveloped by a membrane derived from the plasma membrane of the infected animal cell (Wagner, 1975). The study of the VS virus membrane has been greatly facilitated by the ease of obtaining large quantities of highly purified virus and by the simplicity of the membrane which contains only two virally coded proteins (Patzer et al., 1979). The virus matrix (M) protein is not accessible to lactoperoxidase labeling of intact virus (McSharry, 1977), but it can be cross-linked to phosphatidylethanolamine in the virus membrane with the bifunctional free amino cross-linking reagent dimethyl suberimidate (Pepinsky & Vogt, 1979), suggesting that the M protein is in close proximity to the polar head groups of the inner surface of the viral membrane. The M protein, however, does not penetrate far enough into the inner monolayer to be accessible to labeling with 16-azido-[9,10-$^3H_2$]palmitate metabolically incorporated into viral phospholipids (Stoffel et al., 1978).

The second viral membrane protein, the glycoprotein (G), can be labeled by the 16-azido[9,10-$^3H_2$]palmitate incorporated into viral phospholipids (Stoffel et al., 1978) and is also accessible on the external surface of the virus to proteases (Cartwright et al., 1969) and lactoperoxidase labeling (McSharry, 1977). The glycoprotein forms the spikes seen on the viral surface by negative-stain electron microscopy, and it has an estimated molecular weight of 69 000 (Wagner, 1975). Protease digestion of the virus removes the hydrophilic spike portion of the glycoprotein, leaving a 6000-dalton fragment in the membrane rich in hydrophobic amino acids (Mudd, 1974; Schloemer & Wagner, 1975). When the glycoprotein messenger RNA is translated in vitro in the presence of dog pancreatic microsomal vesicles, the nascent glycoprotein is inserted from its amino-terminal portion into the lumen of the vesicles, leaving 3000 daltons of the carboxy terminus of the G protein accessible to proteases on the outside of the vesicles (Katz et al., 1977; Rothman & Lodish, 1977; Toneguzzo & Ghosh, 1978). Further evidence of the transmembrane nature of the glycoprotein has come from the protease treatment of plasma membrane vesicles from infected cells where all but 3000 daltons of the glycoprotein is protected in the vesicle lumen (Morrison & McQuain, 1978) and from the chemical cross-linking of the G to the M protein of intact virus (Dubovi & Wagner, 1977).

Reconstitution of the VS viral glycoprotein with egg phosphatidylcholine into vesicles has recently been reported (Petri & Wagner, 1979). The vesicles formed by dialysis of octyl glucoside from a mixture of glycoprotein and phospholipid contain glycoprotein spikes protruding in the same external orientation as in the VS virus membrane. Proteolytic digestion of these vesicles with thermolysin leaves a hydrophobic glycoprotein tail fragment embedded in the membrane that migrates identically on polyacrylamide gels with the tail fragment from VS virus treated with thermolysin (Petri & Wagner, 1979).

In the present studies the VS viral glycoprotein has been reconstituted with dipalmitoylphosphatidylcholine (DPPC), a phospholipid for which the characteristics of the gel to liquid-crystalline phase transition have been well-defined in pure systems (Suurkuusk et al., 1976). The effect of the reconstituted glycoprotein on the DPPC phase transition was used to probe the nature of the glycoprotein–phospholipid interaction in the reconstituted membranes.

Experimental Procedures

Isolation of VS Viral Glycoprotein. VS virus of the Indiana serotype was purified by differential, rate zonal, and equilibrium centrifugation in sucrose and tartrate gradients 21 h after infection of baby hamster kidney-21 cells with 0.1 plaque-forming unit/cell of plaque-purified virus (McSharry & Wagner, 1974). Viral proteins were radioactively labeled by the addition of 5 μCi/mL [^{3}H]leucine to the infection medium (58 Ci/mmol).

The VS virus glycoprotein and phospholipids were released from the virus with 30 mM octyl glucoside (octyl β-D-glucopyranoside) (Calbiochem, La Jolla, CA) as previously described (Petri & Wagner, 1979). The glycoprotein was pu-

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[1] Abbreviations used: VS virus, vesicular stomatitis virus; M protein, viral matrix protein; G protein, viral glycoprotein; DPPC, dipalmitoylphosphatidylcholine.

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rified from the phospholipids by sedimentation into a 15–30% sucrose gradient containing 60 mM octyl glucoside, 0.5 M NaCl, and 50 mM Tris (pH 7.6). The pooled protein-containing fractions from the gradient were 97% pure as determined by polyacrylamide gel electrophoresis and contained no detectable cholesterol and approximately one molecule of residual phospholipid per molecule of glycoprotein (Petri & Wagner, 1979).

Reconstitution of VS Viral Glycoprotein into Vesicles. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) was synthesized and purified as described previously (Suurkuusk et al., 1976). The glycoprotein was reconstituted with the phospholipid by the detergent dialysis method (Petri & Wagner, 1979). The glycoprotein purified free of cholesterol and phospholipid was added to 2 mL of 2.5 mM DPPC in 60 mM octyl glucoside and 50 mM KCl. The detergent solution of glycoprotein and phospholipid was then extensively dialyzed against 50 mM KCl for 48 h at 41 °C.

Calorimetry. Scanning calorimetry was performed on an instrument of the heat-conduction type, with an absolute temperature determination of better than ±0.05 °C and a precision of better than 25 μcal/K as base-line noise (Suurkuusk et al., 1976). The temperature of the maximum of the heat capacity function was estimated to be the gel to liquid-crystalline phase transition temperature. A 20-point least-squares fit was used to smooth the heat capacity curves. The phospholipid concentrations were between 3 and 5 mM and the scan rate was ~15 °C/h. The enthalpy change (ΔH) associated with the transition was calculated as the integral of the heat capacity function of the sample minus that of the buffer over the temperature range that the heat capacity deviated significantly from the base line, as demonstrated for the vesicles reconstituted with 0.154 mol % glycoprotein in Figure 2. The enthalpy change was also determined by using a base line slightly higher and a base line slightly lower than the base line in Figure 2, and the average ΔH using the three base lines was calculated.

Lipid and Protein Analysis. The total phosphorus of the scanning calorimetry samples was measured by a modification of the Bartlett procedure (Marinetti, 1962). The total protein of the sample was determined by the method of Lowry et al. (1951) and by the specific radioactivity of [^{3}H]leucine glycoprotein. The mole percent of glycoprotein was calculated by assuming a molecular weight of 69000 for the glycoprotein.

Electron Microscopy. Negative-stain electron microscopy was performed on a Siemans 1A electron microscope at an initial magnification of 32000×. Samples of reconstituted vesicles in 50 mM KCl were placed on a Formvar-coated grid for 30 s, and the excess liquid was blotted off before adding 1–2% phosphotungstic acid for 15 s. The size range of the reconstituted vesicles was estimated by measuring the largest and smallest vesicles from two microscope fields.

Results

Reconstitution by octyl glucoside dialysis yielded large vesicles of heterogeneous size which did not appear to be multilamellar when examined by negative-stain electron microscopy (Figure 1). The size of the vesicles varied with the protein concentration used for the reconstitution: DPPC vesicles formed in the absence of protein were 600–2100 Å in diameter (Figure 1A); DPPC reconstituted with 0.135 mol % glycoprotein yielded vesicles of diameters 600–1000 Å (Figure 1C). The glycoprotein spikes could not be seen by negative staining on these vesicles. The spikes were clearly visible and indistinguishable from those of the intact virus when the glycoprotein was reconstituted at a much higher concentration of 1.5 mol % (Petri & Wagner, 1979). Despite the heterogeneity of the size of the reconstituted glycoprotein vesicles, the vesicles reconstituted with 0.1 mol % glycoprotein banded as a single density class on a sucrose density gradient (data not shown).

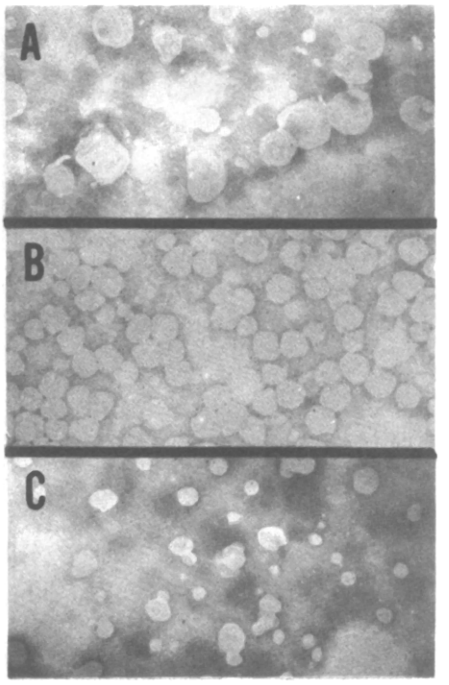

FIGURE 1: Negative-stain electron microscopy of dipalmitoylphosphatidylcholine vesicles containing (A) 0, (B) 0.063, and (C) 0.135 mol % VS viral glycoprotein. The glycoprotein was isolated from the virus and purified of viral lipid with the detergent octyl glucoside (Petri & Wagner, 1979). The glycoprotein was added at the appropriate concentration to 5 μmol of DPPC in 2 mL of 60 mM octyl glucoside and 50 mM KCl. The reconstituted vesicles formed as the detergent was removed by extensive dialysis at 41 °C against 50 mM KCl for 48 h. After the scanning calorimetry was completed, a drop of the vesicles was placed on a Formvar-coated grid for 30 s and the excess liquid was blotted off before adding 1–2% phosphotungstic acid for 15 s. After removal of excess phosphotungstic acid, the grids were examined with a Siemans 1A electron microscope. Magnification is 32000×.

The amount of residual detergent in the vesicles was measured with octyl [^{14}C]glucoside (octyl β-D-[U-^{14}C]glucopyranoside) (New England Nuclear, Boston, MA). Dialysis of the octyl [^{14}C]glucoside from DPPC was unaffected by the presence of the glycoprotein (data not shown). At the end of 48 h of dialysis at 41 °C, the octyl [^{14}C]glucoside was 99.97% removed, leaving ~1 residual detergent molecule for every 100 molecules of DPPC.

The gel to liquid-crystalline phase transition of DPPC vesicles reconstituted with the VS virus glycoprotein was determined by differential scanning calorimetry at a heating rate of 15 °C/h. The DPPC dialysis vesicles which did not contain glycoprotein and had a residual 1 mol of octyl glucoside underwent the gel to liquid-crystalline phase transition at 41.0 °C and had an associated enthalpy change of 8.0 ± 1.6 kcal/mol (Figure 2). This is in agreement with the data of Suurkuusk et al. (1976), who examined the thermotropic behavior of large DPPC vesicles formed when single-lamellar sonicated DPPC vesicles were incubated below the phase transition temperature. These workers found that the large vesicles had a transition temperature of 41.2 °C and calculated the transition enthalpy change to be 8.4 kcal/mol, while the small sonicated vesicles underwent the phase transition at 36.9 °C with a transition enthalpy change of 6.3 kcal/mol. The

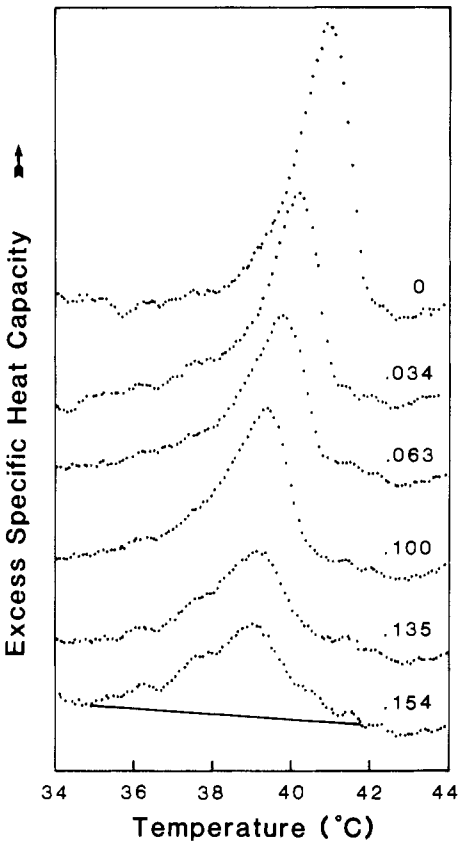

FIGURE 2: Calorimetric scans of large dipalmitoylphosphatidylcholine vesicles reconstituted with varying mole percentages of the glycoprotein of VS virus. Reconstitution was accomplished by using the detergent dialysis method as described in the text. Phospholipid concentrations for the calorimetry were between 3 and 5 mM, and the scanning rate was ~15 °C/h. Heat capacity was calculated per mole of phospholipid. A 20-point least-squares fit was used to smooth the curves.

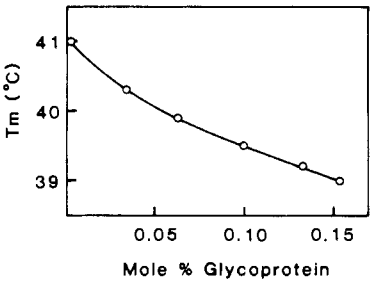

FIGURE 3: Temperature of the maximum of the excess heat capacity function of dipalmitoylphosphatidylcholine as a function of VS viral glycoprotein concentration in reconstituted vesicles.

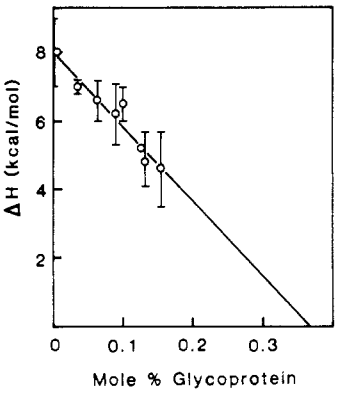

FIGURE 4: Effect of the VS viral glycoprotein on the total enthalpy change per mole of phospholipid of the gel to liquid-crystalline phase transition of dipalmitoylphosphatidylcholine. These data were calculated from the integral of heat capacity curves such as those of Figure 2. The extrapolation to the mole percent glycoprotein concentration where the enthalpy change equals 0 was by a least-squares fit, and the confidence limits for the intercept were determined as described by Johnson et al. (1976). The error bars represent the standard deviation about the mean of the integral of the heat capacity curve using three different base lines (see Experimental Procedures).

detergent dialysis vesicles and the large vesicles studied by Suurkuusk et al. seem to have nearly identical thermotropic properties. Repeated calorimetric scans of the DPPC dialysis vesicles reconstituted with or without the glycoprotein were superimposable with the initial scans, indicating that the vesicles were stable for the time course of the experiments. This is in contrast to the small sonicated DPPC vesicles which were transformed to the large vesicles after repeated calorimetric scans (Suurkuusk et al., 1976).

Bangham-type liposomes (Bangham et al., 1967) of DPPC containing 1 mol % of octyl glucoside had a transition temperature of 41.2 °C (data not shown), the same temperature as that reported for pure DPPC in the absence of octyl glucoside (Suurkuusk et al., 1976; Estep et al., 1978). The Bangham-type liposomes containing 1 mol % of octyl glucoside did not exhibit the pretransition normally seen for multilamellar dispersions of DPPC. Other work in this laboratory has shown that the pretransition of DPPC is sensitive to small amounts of impurities and is abolished with as little as 0.047 atm of halothane (Mountcastle et al., 1978) or in a mixed DPPC–cholesterol system with a cholesterol concentration of 3.6 mol % (Estep et al., 1978). It was not unexpected that the 1 mol % detergent behaved similarly.

The increasing mole percent of glycoprotein in the reconstituted vesicles had three observable effects on the DPPC phase transition: a decrease in the temperature of the transition; a broadening of the temperature range over which the transition occurred; a decrease in the transition enthalpy change (Figure 2). Illustrated in Figure 3 is the change in the transition temperature as a function of the mole percent glycoprotein. The transition temperature decreased from 41.0 °C with pure DPPC dialysis vesicles to 39.0 °C for vesicles containing 0.15 mol % glycoprotein. The enthalpy change of the transition as a function of the mole percent glycoprotein is depicted in Figure 4. The enthalpy changes were calculated from excess heat capacity curves such as those in Figure 2. The enthalpy change was determined as the integral of the excess heat capacity function of the sample minus that of the buffer over the temperature range that the excess heat capacity deviated significantly from the base line. The excess heat capacity curve for 0.15 mol % glycoprotein in Figure 2 shows a sample base line used for the calculation of the enthalpy change. The data in Figure 4 were fit to a straight line by a least-squares analysis, and the intercept of this line with the x axis was used to estimate the number of DPPC molecules that were removed from the phase transition by a single glycoprotein molecule. The molar ratio of protein to lipid at the point where there is no enthalpy change associated with the transition was 1 glycoprotein to 270 ± 150 molecules of DPPC.

The linear decrease in ΔH of the phase transition with increasing G protein concentration (Figure 4) indicated that the decrease in ΔH was primarily protein mediated and not a result of the decreased diameter of vesicles reconstituted with glycoprotein (Figure 2). Even if the vesicles with a ΔH of 4.6 kcal/mol containing 0.154 mol % glycoprotein had the same radius of curvature as small sonicated DPPC vesicles which have a ΔH of 6.3 kcal/mol (Suurkuusk et al., 1976), the number of DPPC molecules interacting with the G protein,

estimated by extrapolation of these two points to zero enthalpy, would be ~170.

Discussion

The 270 ± 150 molecules of DPPC removed from the phase transition by one G protein molecule represent a greater number of perturbed lipids than has been observed for other proteins which are known to span the bilayer and have been studied in reconstituted systems. The erythrocyte protein glycophorin removed 80–100 molecules of dimyristoylphosphatidylcholine from the phase transition monitored by scanning calorimetry (van Zoelen et al., 1978). The transition of dimyristoylphosphatidylcholine reconstituted with the bacteriophage M13 coat protein observed by paranaric acid fluorescence indicated that the protein removed 70–100 lipid molecules from the transition (Kimelman et al., 1979). The effect of the VS viral glycoprotein on the DPPC phase transition is most striking because only a 6000-dalton hydrophobic fragment of the glycoprotein is thought to anchor the protein in the viral membrane (Schloemer & Wagner, 1975). If the glycoprotein spans the membrane in an α-helical configuration, the 270 DPPC molecules removed from the phase transition would correspond to five to six concentric shells of DPPC molecules surrounding the glycoprotein on the inner and outer monolayers.[2] An alternative explanation may be that the 60 000-dalton hydrophilic part of the glycoprotein removes the DPPC molecules from the phase transition by interacting with the phospholipid head groups. Preliminary attempts to examine the thermotropic behavior of reconstituted vesicles which have had the hydrophilic portion of the G protein removed by protease have not been successful because of difficulties in purifying the vesicles after digestion from the large amounts of protease required to effectively hydrolyze the protein. Work is currently being carried out to answer this important question.

The effect of proteins on the enthalpy change and temperature of the transition has been used previously to characterize the interaction of proteins with the lipid bilayer (Papahadjopoulos et al., 1975). In the present case the apparent broadening of the transition curve caused by the G protein could be the result of several factors. First, the protein might in some manner be affecting the interaction energies between lipids at gel–liquid-crystalline interfaces. Second, the removal of some lipids from participating in the transition may reduce the maximum number of lipids which can participate in the cooperative melting (i.e., reduce the maximum cooperative unit). This latter effect could also account for the reduction in the transition temperature, but it should be kept in mind that the transition temperature change could also be caused by stabilization of the liquid-crystalline state by the protein. The exact molecular basis of these effects must await further experimentation, however.

[2] Assuming a radius of 3.6 Å for an α helix and a radius of 4.4 Å for a phosphatidylcholine molecule.

Acknowledgments

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