ELSEVIER

Thermochimica Acta 267 (1995) 365–372

thermochimica acta

Effect of Na^+ concentrations on both size and multiplicity of multilamellar vesicles composed of negatively charged phospholipid as revealed by differential scanning calorimetry and electron microscopy[1]

Michiko Kodama*, Takahiro Miyata

Department of Biochemistry, Faculty of Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700, Japan

Received 13 December 1994; accepted 23 January 1995

Abstract

Vesicles of anionic phospholipid, dimyristoylphosphatidylglycerol (DMPG) were prepared by suspending the lipid film in aqueous solutions of different Na^+ concentrations. These vesicles were revealed to show ionic-strength-dependent properties at Na^+ concentrations lower than approx. 100 mM. Thus, the mean diameters and multiplicities of the vesicles obtained by negative stain electron microscopy increased linearly with an increase in Na^+ concentration, similar to the transition enthalpy of the gel to liquid crystal phases of the vesicles by calorimetry. Furthermore, a marked broadening of the transition peak with a decrease in Na^+ concentration proceeded simultaneously with a gradual growth of shoulders at the high temperature side. These results were discussed from screening effects of Na cation interposed between the bilayers of DMPG on negatively charged, concave and convex surfaces of their inner and outer membranes, particularly focusing on the effective cross-sectional area occupied by the charged head group, related to the lipid packing.

Keywords: DSC; Electron microscopy; Phospholipid; Multilamellar vesicle

1. Introduction

Phosphatidylglycerol (PG) is a ubiquitous phospholipid in bacterial, mitochondrial and chloroplast membranes. It has a phosphate acidic group with pK_a value of 3 [1] and

* Corresponding author.

[1] Presented at the 30th Anniversary Conference of the Japan Society of Calorimetry and Thermal Analysis, Osaka, Japan, 31 October–2 November 1994.

SSDI 0040-6031 (95) 02493-X

is negatively charged at neutral pH. The bilayer structures of the acidic lipid have been the subject of many investigators from the viewpoint of a role in biomembrane functioning as they are greatly affected by a change in environmental conditions such as protons and ions in an aqueous medium [1–5]. That is, shielding effects of external cations on the negative charge of PG alter the lateral interactions and packings of the lipid head group at the bilayer surface [6,7]. In this connection, PG in the presence of sufficient counterion was found to form multilamellar structures characterized by the pretransition (T_p) and main transition (T_m) temperatures [2,3], similar to zwitterionic phosphatidylcholine (PC) [8]. Our previous paper also revealed that dimyristoyl (DM) PG forms spherical, multilamellar large vesicles (diameter 2–3 μm) with a defined interbilayer spacing when the lipid film was suspended in aqueous solution containing 1 M NaCl and the T_m transition peak of the vesicles were characterized by a high cooperative unit of ~160 [5]. This indicates that electrostatic repulsion operating between the PG head groups is fairly depressed by Na cation of 1 M concentration. In this study, we investigated how the size and multiplicity of the PG vesicles are varied by alteration of Na^+ concentration, focusing on the effective cross-sectional area occupied by the PG head group, which is related to the bilayer surface curvature and the lipid packing. Thermotropic properties of the PG vesicles at different Na^+ concentrations were studied by high-sensitivity differential scanning calorimetry and structural information was obtained by negative stain electron microscopy.

2. Experimental

2.1. Material

1,2-Dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG, sodium salt) was purchased from Sigma and used without further purification. The lipid yielded a single spot by thin-layer chromatography on a silica gel plate (E. Merck) using chloroform/methanol/7 M ammonia (230:90:15, by vol.) [9]. Lipid concentrations were estimated by a modified Bartlett phosphate assay [10].

2.2. Preparation of vesicles

A dispersion of vesicles composed of DMPG was prepared as follows [11,12]: the lipid film was first prepared by removing chloroform from a DMPG stock solution on a rotary evaporator, and then under high vacuum (10^{-4} Pa) to achieve complete removal of traces of the solvent. The dried lipid films were then suspended in aqueous solutions of NaCl at concentrations of 20,50, 80, 100, 250, 500 and 1000 mM, and gently vortexed at liquid crystal phase temperature of 45°C to ensure complete suspension of the lipid.

2.3. High sensitivity differential scanning calorimetry

All calorimetric experiments were performed with a Microcal MC 2 differential scanning calorimeter. The calorimeter was interfaced to an IBM PC microcomputer system using an A/D converter board (Data Translation DT-2801) for automatic data collection

and analysis. The lipid concentration in the calorimetric experiments was in the range of 1–2 μmol ml^{-1} with a calorimetric cell volume of 1.2 ml. A scanning rate of 45°C h^{-1} was used for heating scans.

2.4. Electron microscopy

Vesicle preparations were examined by negative stain electron microscopy [13–15]. A drop of vesicle dispersion (lipid concentration ~2 μmol ml^{-1}) was placed on copper grids covered with carbon-coated collodion films, allowed to remain for 5 min and then drained. A 2% solution of sodium phosphotungstate (pH approx. 7) was added and after 10 min the excess solution was drained. The preparation was examined immediately in a JEOL JEM-2000EX electron microscope operated at 200 kV. All operations were performed at around 20°C.

3. Results

3.1. High sensitivity differential scanning calorimetry of PG vesicles prepared at different Na^+ concentrations

Thermotropic behavior of all the PG vesicles of different Na^+ concentrations is characterized, as a rule, by the T_p and T_m transitions as shown in Fig. 1 [2,16]. At Na^+ concentrations higher than 250 mM (see Fig. 1A), the T_m transition peaks show sharp shapes although the transition temperatures decrease, little by little, with a decrease in Na^+ concentration. The cooperative T_m phase transition observed at the high Na^+ concentrations suggests that a shielding effect of Na cation on the negative phosphate group enables the PG molecules to pack as closely as PC molecules with large head group [7]. At Na^+ concentrations lower than 100 mM (see Fig. 1B), a broadening of the T_m transition peak with decreasing Na^+ concentration (d $\rightarrow$ g) proceeds simultaneously with a gradual growth of shoulder peak at the high-temperature side. In distilled water (g), the T_m transition peak greatly broadens, extending over the temperature range from 12 to 33°C (see enlarged curve in Fig. 1B) [6]. In Fig. 2, the T_m transition enthalpies of these vesicles are plotted against Na^+ concentration.

In accordance with the thermotropic behavior shown in Fig. 1, the transition enthalpies are nearly constant at Na^+ concentrations above 200 mM, below which they go down linearly with decreasing the concentration.

On the other hand, as is obvious from Fig. 1, all the vesicles, even at Na^+ concentration of 0 mM, are shown to preserve the pretransition, the temperature of which gradually decreases with decreasing Na^+ concentration. This indicates that the PG molecules adopt a tilted acyl chain packing in the bilayer structure, independently of Na^+ concentration [2,17].

3.2. Negative stain electron microscopy of PG vesicles prepared at different Na^+ concentrations

Fig. 3 shows typical electron micrographs of the PG vesicles at Na^+ concentrations

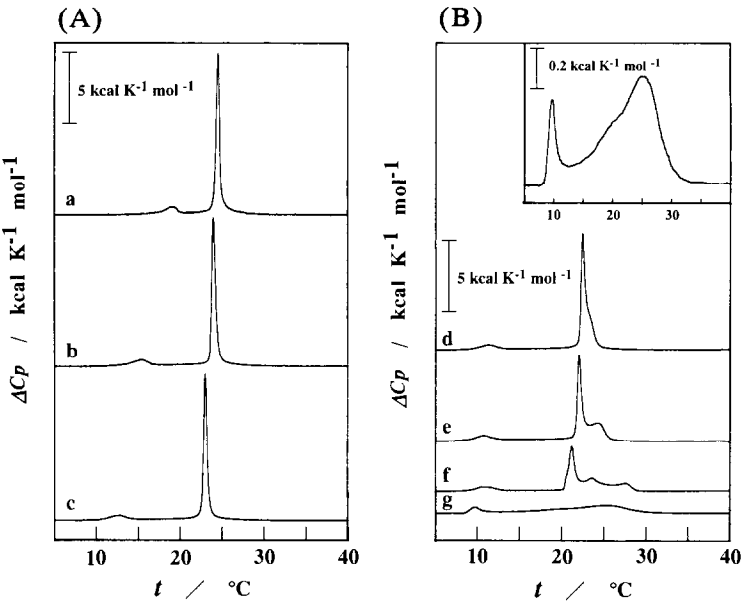

Fig. 1. Variation with decrease in Na^+ concentration of thermotropic behaviors of DMPG. Apparent, excess heat capacity (ΔC_p) is plotted as a function of temperature (t). In this figure, results at high ((A) >250 mM) and low ((B) <100 mM) Na^+ concentrations are shown separately. Enlarged curve in (B) corresponds to the result in distilled water shown by curve g in the same figure. Na^+ concentration (mM): (a) 1000; (b) 500; (c) 250; (d) 100; (e) 50; (f) 20; (g) 0.

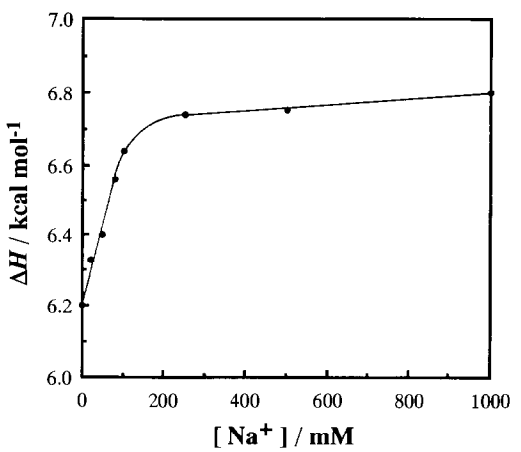

Fig. 2. Variation with increase in Na^+ concentration of enthalpy change (ΔH) associated with the T_m transition for vesicles of DMPG.

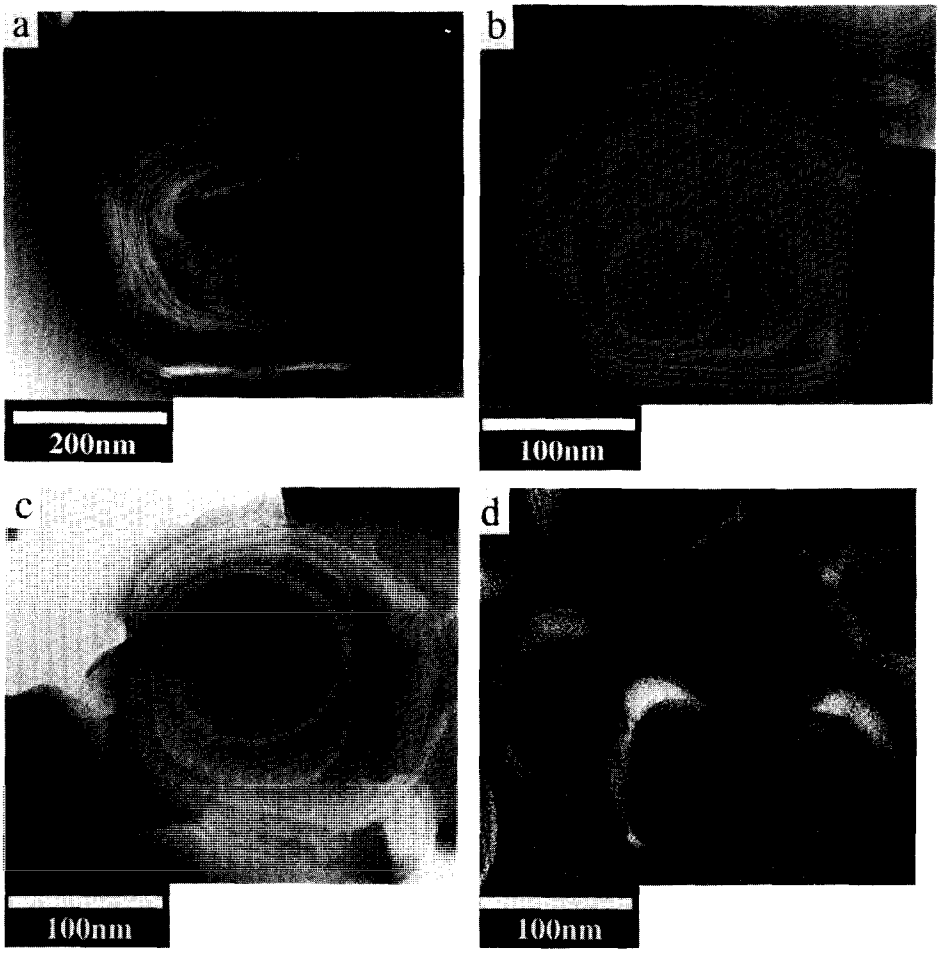

Fig. 3. Negative stain electron micrographs of DMPG vesicles prepared at different Na^+ concentrations. Na^+ concentration (mM): (a) 100; (b) 50; (c) 20; (d) 0.

lower than 100 mM. Above 100 mM, inner regions of the vesicles were not sufficiently stained. Presumably, a close packing of the PG at high Na^+ concentrations relates to this phenomenon. In this figure, except for an electron micrograph (a) at 100 mM Na^+ concentration, all electron micrographs at Na^+ concentrations of 50 (b), 20 (c) and 0 mM (d) are given with the same scale. A comparison of these electron micrographs indicates that both the size and multiplicity of the vesicles diminish with decreasing Na^+ concentration. Furthermore, the interlamellar spacings are not uniform, generally showing longer distances with approaching the center of vesicles. This tendency becomes more remarkable as the Na^+ concentration is lowered and in distilled water, only the vesicles wrapped by unilamellar are observed (see Fig. 3d) [18]. These observations are more clearly recognized in Fig. 4 where the number of bilayer lamellar (A) and approximate mean diameter

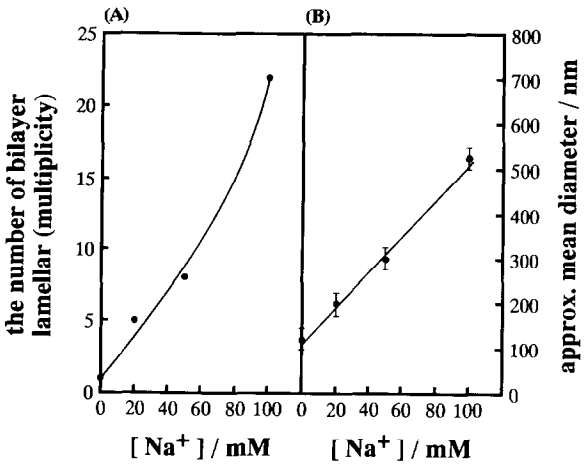

Fig. 4. Variation with increase in Na^+ concentration of the number of lamellar (A) and approx. mean diameter (B) of DMPG vesicles.

(B) of vesicles obtained from electron micrographs are plotted against Na^+ concentration. The approximate mean diameters were calculated from the size distributions composed of about 100 vesicles. As is obvious from Fig. 4, both the vesicle mean diameter and the lamellar number increase with an increase in Na^+ concentration up to 100 mM. However, the lamellar number plot shows an upward curve, in contrast to a linear increase of the vesicle diameter plot, indicating a narrowing of the average interlamellar distance with an increase in Na^+ concentration.

4. Discussion

The shape of lipids is an important determinant of their packing properties. Fig. 5 schematically illustrates lipid packing adaptations to differences in the geometrical cross-

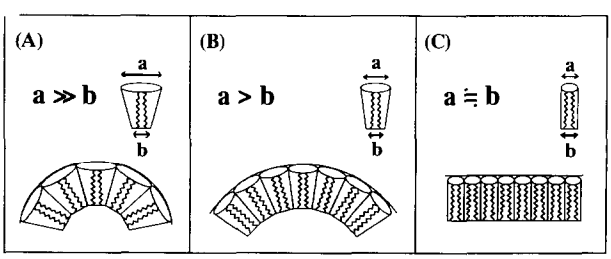

Fig. 5. Schematic illustration of lipid-packing adaptations to differences in cross-sectional areas of the polar head group and the acyl chains. The area of the head group (a) relative to the acyl chains (b = const.) increases in the order C < B < A.

sectional areas of the polar head group (a) and the acyl chains (b). In this figure, when the areas of the head group relative to the acyl chains become larger in the order C < B < A, the surface curvatures of lipid assemblies are shown to increase in the same order, indicating that their sizes decrease in the order C > B > A. For anionic lipids such as PG, the charged head group occupies a larger area than predicted by its geometrical size, because of electrostatic repulsion operating between neighboring head groups [6]. The electrostatic repulsion is reduced by bulky cations which exert a non-specific screening effect on the charged surface by forming a diffuse double layer [7]. Therefore, the effective area, that is, geometrical size plus repulsion space, of the charged head group is also diminished. This is illustrated graphically in Fig. 5; when Na^+ concentrations increase in the order A < B < C, the effective areas of the charged head group decrease in the order A > B > C. Therefore, the linear increase of the vesicle diameter with an increase in Na^+ concentration shown in Fig. 4B can be explained on the basis of the ionic strength dependence of the effective area of the charged head group.

Furthermore, the linearity of the transition enthalpy below 100 mM Na^+ concentration shown in Fig. 2 can be also interpreted from the same viewpoint; the gel phase at a higher Na^+ concentration is much more enthalpically stabilized as a consequence of a closer packing of the PG molecules, so that the gel phase is accompanied by a higher transition enthalpy to the liquid crystal phase. However, the linearities of the transition enthalpy and vesicle diameter plots are observed at Na^+ concentrations lower than ca. 100 mM. In fact, above 100 mM, the vesicle diameters (not shown in Fig. 4B) obtained by electron microscopy are considerably smaller than estimated by extrapolating the linear plot of Fig. 4B to higher Na^+ concentration; mean diameters at Na^+ concentrations of 500 and 1000 mM are approx. 1.5 and 2 μm, respectively. These results indicate that the screening effect of Na cation on the negatively charged PG surface is saturated at around 100 mM, at which the effective area of the PG head group reaches a limiting value.

For multilamellar spherical vesicles formed by a stacking of lipid bilayers in this study, the packing properties of the charged head group at the inner membrane surface are also of importance. As the surface curvature of the inner membrane is concave toward aqueous medium, the head groups at this membrane are required to pack closely, in contrast with their loose packing at the outer membrane having a convex surface. This tendency becomes more pronounced with a decrease in the curvature radius of bilayers. That is, as the bilayer becomes more and more close to the center of the vesicle, the head group of the inner membrane is more closely packed (an increase in T_m temperature) and, on the contrary, the head group of the outer membrane is more loosely packed (a decrease in T_m temperature). Presumably, the broadening of the T_m transition peak with a decrease in Na^+ concentration shown in Fig. 1B results from the decrease of vesicle diameters (see Fig. 4B), by which the head group packings of the inner and outer membranes are caused to differ greatly. Furthermore, it is considered that the greatly different packings of the head groups between these membranes is enabled by Na cations interposed between adjacent bilayers. Thus, this is attained by forming two diffuse double layers depending on each surface charge density of the inner and outer membranes of adjacent bilayers [19]. The preferred surface curvatures of these membranes could be achieved by mutually altering Na^+-concentration gradients of two diffuse layers. Such a flexibility of the diffuse layers causes the negatively charged PG to form multilamellar

vesicles surrounded by the bilayers of different curvature radii, resulting in the dependence of the multiplicity of vesicles on Na^+ concentration shown in Fig. 4A.

References

[1] A. Watts, K. Harlos, W. Maschke and D. Marsh, Biochim. Biophys. Acta, 510 (1978) 63.
[2] D.A. Wilkinson, D.A. Tirrell, A.B. Turek and T.J. McIntosh, Biochim. Biophys. Acta, 905 (1987) 447.
[3] I.S. Salonen, K.K. Eklund, J.A. Virtanen and P.K.J. Kinnunen, Biochim. Biophys. Acta, 982 (1989) 205.
[4] R.M. Epand, B. Gabel, R.F. Epand, A. Sen, S.W. Hui, A. Muga and W.K. Surewicz, Biophys. J., 63 (1992) 327.
[5] M. Kodama, T. Miyata and T. Yokoyama, Biochim. Biophys. Acta, 1168 (1993) 243.
[6] R.M. Epand and S.W. Hui, FEBS Lett., 209 (1986) 257.
[7] F. Jähnig, K. Harlos, H. Vogel and H. Eibl, Biochemistry, 18 (1979) 1459.
[8] M. Kodama, T. Miyata and Y. Takaichi, Biochim. Biophys. Acta, 1169 (1993) 90.
[9] H.O. Hauser, Biochem. Biophys. Res. Commun., 45 (1971) 1049.
[10] G.V. Marinetti, J. Lipid Res., 3 (1962) 1.
[11] A.D. Bangham, M.W. Hill and N.G. Miller, Methods Membr. Biol., 1 (1974) 1.
[12] F.Jr. Szoka and D. Papahadjopoulos, Annu. Rev. Biophys. Bioeng., 9 (1980) 467.
[13] C. Huang, Biochemistry, 8 (1969) 344.
[14] S.M. Johnson, A.D. Bangham, M.W. Hill and E.D. Korn, Biochim. Biophys. Acta, 233 (1971) 820.
[15] S.E. Schullery, C.F. Schmidt, P. Felgner, T.W. Tillack and T.E. Thompson, Biochemistry, 19 (1980) 3919.
[16] K. Jacobson and D. Papahadjopoulos, Biochemistry, 14 (1975) 152.
[17] T.J. McIntosh, Biophys. J., 29 (1980) 237.
[18] H. Hauser, Biochim. Biophys. Acta, 772 (1984) 37.
[19] H. Träuble, M. Teubner, P. Woolley and H. Eibl, Biophys. Chem., 4 (1976) 319.