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Tris buffer causes acyl chain interdigitation in phosphatidylglycerol

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The structure of the gel phase and the properties of the acyl chain disordering transition of dipalmitoyl phosphatidylglycerol (DPPG) have been studied using differential scanning calorimetry, differential scanning dilatometry, and X-ray diffraction. In the presence of small, monovalent cations, DPPG at 22°C exists in a lamellar phase in which the hydrocarbon chains are tilted from the perpendicular to the bilayer surface. Around 34°C, there is a small pretransition ($\Delta H < 1$ kcal/mol) followed by the main transition at 40.4°C ($\Delta H = 8.3$ kcal/mol; $\Delta V = 0.0381$ ml/g). If DPPG is suspended in Tris-HCl buffer in the absence of other monovalent cations, X-ray diffraction data show that at 22°C, the gel phase consists of interdigitated acyl chains perpendicular to the plane of the bilayer. No pretransition is observed and the main transition occurs at 41.3°C with $\Delta H = 9.1$ kcal/mol and $\Delta V = 0.0514$ ml/g. If sufficient Na^+ or K^+ ions are added to the Tris-buffered DPPG, the phase behavior reverts to what is observed in the absence of Tris. Analysis of the energetics of the main transition shows that the increase in van der Waals interaction energy resulting from the larger ΔV in Tris can be compensated by the favorable energetics of removing terminal methyl groups from the bilayer surface. The amount of disordering, i.e. formation of *gauche* rotamers, is likely to be the same in Tris as it is in buffers without amphiphilic cations.

Introduction

Phosphatidylglycerol is one of the major phospholipids in green leaves, algae, and many bacterial species [1]. At neutral pH, it is negatively charged and in the absence of sufficient counterion, phosphatidylglycerol, like other lipids, will swell continuously in excess water due to electrostatic effects [2]. However, in the presence of counterions, multilamellar structures with a defined interbilayer spacing are formed [3]. Previously, we studied multilamellar vesicles of saturated phosphatidylcholine and phosphatidylethanolamine (both of which are zwitterions at neutral pH) using calorimetry and density measurements [4,5], and showed that the energetics of the main phase transition (gel → liquid crystalline) depends almost entirely on alkyl chain interactions. In this work, we extend these measurements and the analysis of transition energetics to dipalmitoylphosphatidylglycerol (DPPG). Data are presented

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DMPG(DPPG), L-α-dimyristoyl(dipalmitoyl)-phosphatidylglycerol; DMPA, L-α-dimyristoylphosphatidic acid; DMPS, L-α-dimyristoylphosphatidylserine.

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which show that the phase behavior of this phospholipid below and during the main chain melting transition is different in Tris buffer compared to other buffers or neutral salt solutions.

The effect of cations on the structure of anionic phospholipids has been the subject of several earlier investigations. They can be summarized as follows.

Small monovalent cation. Li^+ when present in large excess raises the chain melting point of DMPS [6] and of DPPG (unpublished data from this laboratory) apparently by dehydration of the headgroup.

Small divalent cation. Ca^{2+}, Mg^{2+}, and Mn^{2+} are able to form complexes with DMPG, DPPG, DMPS, and DMPA resulting in cochleate cylinders or lamellar sheets exhibiting higher melting points that the lipid plus monovalent cation [7–13].

Large cation. Cations formed from amphiphilic molecules such as choline and acetylcholine as well as natural (polymyxin B) and synthetic (ionene-6,6) cationic polymers cause interdigitation of acyl chains from opposing halves of DPPG bilayers [14–16]. Evidence that gel phase DPPG exists in an interdigitated form in the presence of Tris buffer is presented in this paper.

Materials and Methods

L-α-Dipalmitoylphosphatidylglycerol (ammonium salt) was obtained from Sigma Chemical Co. and was judged sufficiently pure on the basis of the narrowness of the main transition (a half-width of 0.3 K). Tris-HCl and Hepes buffers were obtained from Sigmal Chemical Co. Sodium phosphate and sodium chloride were obtained from the J.T. Baker Chemical Co. All buffers and salts were reagent grade. Lipid samples for calorimetry and dilatometry were hydrated in aqueous buffer at pH 7.4 by heating them to 70 °C for several minutes and dispersing the lipid by vortexing (5 times, 15 s per time). Lipid concentrations were 1 mg/ml for the differential scanning calorimetry (DSC) and 5.7 mg/ml for differential scanning dilatometry (DSD). The apparent heat capacity was measured using a Microcal MC-1 DSC (Amherst, MA) at a scanning rate of 12 K/h. The change in volume was determined by a differential dilatometer [17] and scanning rates of 5 K/h were used. The actual specific volume at one temperature was determined using a standard 25 ml Weld picnometer the capillary tube of which had been modified to increase precision.

For X-ray diffraction experiments, DPPG was suspended in two buffer systems, 50 mM sodium phosphate (pH 7.4) and 50 mM Tris-HCl (pH 7.4), at a concentration of 5 mg/ml. Both suspensions were prepared and analyzed by the following procedure. The specimens were equilibrated for at least two hours at 60 °C with periodic vortexing and then concentrated, at room temperature, by centrifugation at $13000 \times g$ for 10 min. The pellets were then sealed with excess buffer in 1.0 mm quartz X-ray capillary tubes and mounted in a pinhole collimation X-ray camera equipped with a flat plate film casette. X-ray patterns were recorded at room temperature (20 ± 2 °C) on Kodak DEF X-ray film and scanned with a Joyce Loebl microdensitometer, model MK III C, as described previously [18]. Specimen to film distance was 10 cm and exposure times were about 10 h.

Results and Discussion

Gel phase structure

X-ray diffraction results

X-ray diffraction patterns from DPPG suspended in 50 mM sodium phosphate buffer and 50 mM Tris-HCl buffer are shown in Fig. 1. The pattern in phosphate buffer contains broad bands centered at about 48 and 24 Å, and two wide-angle reflections, a sharp reflection at 4.20 Å surrounded by a broad band centered at about 4.07 Å. An additional broad and very weak band centered at about 15 Å is observable on the original film. The pattern in Tris-HCl buffer contains a broad reflection centered at about 34 Å and a single sharp wide-angle reflection at 4.10 Å. The same patterns as shown in Fig. 1 were obtained with DPPG from either Sigma (ammonium salt) or Avanti Polar Lipids (sodium salt).

Since DPPG has a net charge at pH 7.4, the multilayer structure swells in aqueous media so that the fluid spaces between adjacent bilayers are large and irregular in width. Thus, the low-angle diffraction from these dispersions consists of broad bands, which are related to the square of the

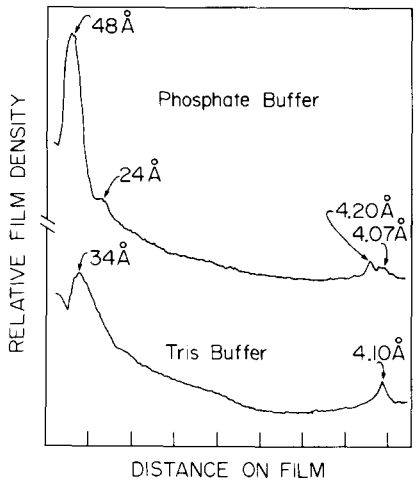

Fig. 1. X-ray diffraction patterns from DPPG suspended in 50 mM phosphate buffer and 50 mM Tris buffer at 22° C.

continuous Fourier transform of the bilayer [19]. The wide-angle reflections, in the region of 4.0 to 4.2 Å, are due to the packing of the lipid hydrocarbon chains [20]. The X-ray patterns of Fig. 1 show that the structure of the DPPG bilayer is quite different in phosphate and Tris buffers. The pattern in phosphate buffer is completely consistent with a bilayer in the $L_{\beta'}$ phase, which consists of a gel phase lipid with the hydrocarbon chains tilted relative to the normal to the plane of the bilayer [20]. The broad bands observed centered at about 48, 24, and 15 Å correspond to broad intensity maxima in the continuous transform of the $L_{\beta'}$ phase of DPPG [21]. The double wide-angle reflection, consisting of a sharp reflection at 4.20 Å surrounded by a broad band at 4.07 Å, is characteristic of the tilted hydrocarbon chain packing of the $L_{\beta'}$ phase as shown by Tardieu et al. [20]. In contrast, both the low-angle and wide-angle reflections from DPPG in Tris buffer are consistent with lipids in the interdigitated phase, where the lipid hydrocarbon chains interpenetrate or interdigitate [22,23]. The broad band centered at 34 Å corresponds to the first peak in the continuous transform of the interdigitated phase [23], and the single sharp reflection at 4.10 Å is similar to the wide-angle patterns which have been observed for other examples of the interdigitated phase [22–24]. The position of this spacing shows that the lipid hydrocarbon chains are packed closer together than they in a normal $L_{\beta'}$ gel phase, and the sharpness of the reflection indicates that the hydrocarbon chains are oriented approximately perpendicular to the plane of the bilayer [20,22].

Lower transition

The presence of a pretransition occurring a few degrees below the main transition has been reported previously for DPPG [9,11]. We also observe a pretransition when DPPG is suspended in 50 mM phosphate buffer, 50 mM Hepes buffer, or 100 mM NaCl (Fig. 2b, c and d). All three of these salts supply a small cation as the counterion for the negatively charged lipid bilayer. In contrast, no lower transition is observable in DSC heating scans of DPPG in 50 mM Tris buffer, pH 7.4 (Fig. 2a). McDaniel et al. [24] reported that interdigitation of DPPC by the addition of glycerol eliminated the pretransition in that lipid. The lack of such a transition in the interdigitated gel phase and in the fully protonated DPPG [25,3] is consistent with the idea that the pretransition occurs only in lipids with tilted chains [18]. In the interdigitated phase and the fully protonated phase, the acyl chains are perpendicular to the bilayer surface.

From the work of Watts et al. [3], it is clear that the normal $L_{\beta'}$ gel phase exists in a buffer consisting of both Tris and potassium ions. We therefore

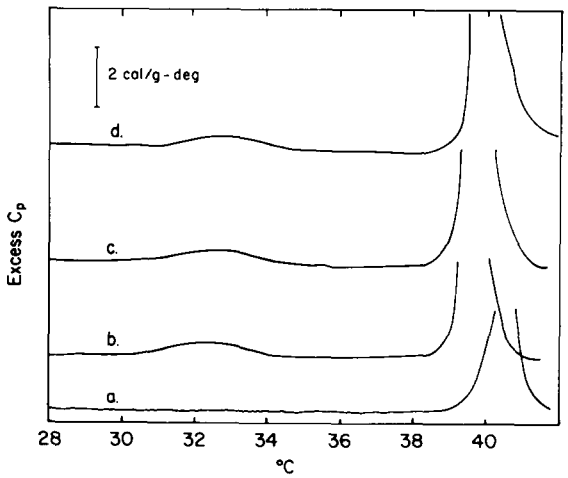

Fig. 2. Excess heat capacity versus temperature for DPPG (2.5 mg/ml) at pH 7.4 in (a) 50 mM Tris buffer, (b) 50 mM phosphate buffer, (c) 50 mM Hepes buffer, and (d) 100 mM NaCl.

thought it would be of interest to see if the lower transition could be restored in Tris-buffered DPPG by the addition of a small cation such as Na^+ (as NaCl). An example of what was found is shown in Fig. 3. In curve 3a (DPPG in 5 mM Tris) the pretransition is absent, whereas in curve 3b (same + 150 mM NaCl) it is now present. In a series of experiments we studied how the pretransition was affected by ionic strength, pH, and the ratios $[Na^+]/[Tris]$ and $[Na^+]/[Tris^+]$. The results are summarized in Table I. Several conclusions may be drawn from these data. First, the absence of the pretransition in Tris-buffered DPPG is not due to an ionic strength effect since at comparable values of Z, phosphate-buffered DPPG shows this transition. Second, addition of sodium ions in sufficient quantity to Tris-buffered DPPG causes changes in the gel phase such that a pretransition is observed. In this regard, it is the ratio of sodium ions to Tris cations that is important rather than the ratio of sodium ions to the total amount of Tris present in the suspension. (As seen in Table I, a pretransition is induced when R is changed from 2.4 to 24 while R' remains constant.) This implies that, as one would expect, it is the Tris cation rather than the uncharged Tris moiety that is effective in interacting with DPPG so as to eliminate the pretransition. In examining Table I it is also clear that the ratio of Na^+ to $Tris^+$ must be more than 2.4 in order to establish conditions

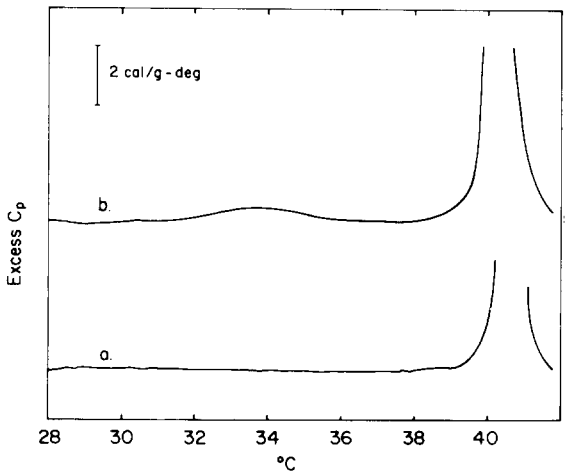

fig. 3. Excess heat capacity versus temperature for DPPG (2.5 mg/ml) at pH 7.4 in (a) 5 mM Tris buffer and (b) 5 mM Tris buffer + 150 mM NaCl.

TABLE I

EFFECT OF IONIC STRENGTH (Z) [a], pH AND RATIOS [a] OF SODIUM TO Tris ON THE PRETRANSITION IN DPPG

$R = [Na^+]/[Tris^+]$; $R' = [Na^+]/[Tris]$.

Z(M)	pH	Buffer	R	R'	T_p(°C)
0.041	7.4	Tris	0	0	–
0.141	7.4	Tris	2.4	2.0	–
0.154	7.4	Tris	37	30	34.6
0.541	7.4	Tris	12	10	35.4
0.004	9.1	Tris	0	0	–
0.104	9.1	Tris	24	2.0	32.0
0.504	9.1	Tris	115	10	36.4
0.011	7.4	phosphate	∞	∞	34.5
0.109	7.4	phosphate	∞	∞	32.0

[a] Calculated for 37 °C.

leading to the pretransition. When that ratio is 12 or more the lower transition can be observed. It is significant also that when the ratio of K^+ to $Tris^+$ is large (approx. 30) the interdigitated gel phase is not observed [3].

A simple picture serves to summarize these ionic effects. In the presence of a small cation, the headgroup spacing of DPPG in the gel phase is appropriate for tilted, non-interdigitated chains. Substitution of a large cation increases the area per molecule and leads to interdigitation and the absence of the pretransition. The structure of the bilayer in the presence of both small and large cations may depend on the relative binding constants of each to the lipid. In all our experiments involving $Tris^+$ plus Na^+ ions where a lower transition was observed, the main transition displayed only the characteristics of the melting of the non-interdigitated form (i.e. $T_m = 40$ °C; $\Delta H = 8$ to 8.5 kcal/mol). What was never seen was either a double peaked endotherm characteristic of the melting of two separate gel phase structures or a broadened endotherm occurring between the T_m values of Tris- or Na-DPPG. This implies that most, if not all, the DPPG was in the $L_{\beta'}$ form.

Main transition

The specific heat and the specific volume of DPPG suspended in 50 mM Tris-HCl buffer and in 50 mM phosphate buffer at pH 7.4 as a function of temperature near the main transition are

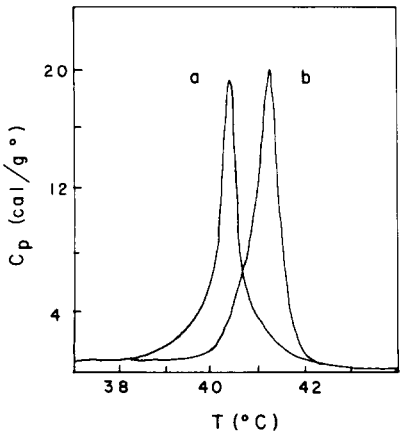

Fig. 4. The specific heat as a function of temperature near the main transition in (a) 50 mM phosphate buffer and (b) 50 mM Tris buffer.

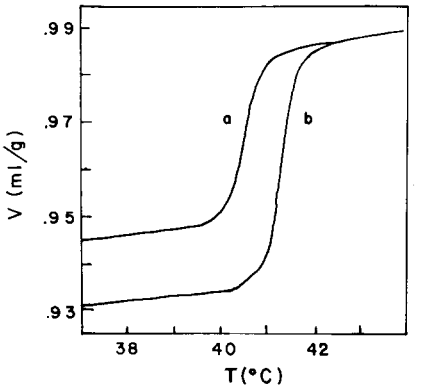

Fig. 5. The specific volume as a function of temperature near the main transition in (a) 50 mM phosphate buffer and (b) 50 mM Tris buffer.

shown in Figs. 4 and 5. The specific volume at 37 °C (0.946 ml/g) for the $L_{\beta'}$ phase is incompatible with the one reported by Watts et al. [3] at 20 °C. The accuracy of our picnometry was checked by measuring the density of a standard NaCl solution to within 4 parts in 10^4 of literature values. In addition to picnometry, we performed centrifugation experiments on DPPG in phosphate buffer of known density. The observed precipitation of the lipid provided upper bounds on the specific volume of DPPG at several temperatures. The results we obtained by this method at 22 °C are inconsistent with the low density determined by Watts et al. We have no satisfactory explanation for these differences. The coefficient of thermal expansion, $\alpha = dV/dT$ for DPPG in the gel phase was found to be $105 \cdot 10^{-5}$ ml/g per K, a value only slightly higher than that observed for the neutral lipids [4]. Our values for the thermodynamic parameters that characterize the main transition are listed in Table II.

It is readily apparent from Figs. 4 and 5 that the transition midpoint (T_m) of the interdigitated DPPG is about 0.8 K higher than that of the $L_{\beta'}$ bilayer. A similar increase in T_m for glycerol-induced interdigitation of DPPC bilayers was observed by McDaniel et al. [24]. The value for ΔH in Na^+ or K^+ is the same as reported by Boggs and Rangaraj [12] for DPPG in Hepes buffer.

The volumetric data can be used to calculate the change in the van der Waals interaction energy (ΔU_{vdW}) associated with the main phase transition [4]. If repulsive energies are ignored then the change in the van der Waals energy at the transition is given by

$$\Delta U_{vdW} = 2.3[(r_0/r_b)^5 - (r_0/r_a)^5] \text{ kcal/mol}$$

where r is the nearest neighbor separation between chains. At 0 K, $r = r_0$; just below the transition, $r = r_b$, and just above the transition, $r = r_a$. The X-ray spacings at 20 °C are used to compute the cross-sectional area per chain, f_0, in the manner described by Watts et al. [3]. The volume per methylene, $v_{CH_2} = 1.27 f_0$, is calculated and then extrapolated to 41 °C using the measured

TABLE II

ENERGETICS OF THE MAIN TRANSITION OF $DPPG^-$

Cation	ΔV (ml/g)	ΔH (kcal/mol)	r_a (Å)	ΔU_{vdW} (kcal/mol)	$\Delta H - \Delta U_{vdW}$ (kcal/mol)
Na^+ or K^+	0.0381 ± 0.0005	8.3 ± 0.1	5.01	6.0	2.3
$Tris^+$	0.0514 ± 0.0011	9.1 ± 0.1	5.00	8.5	0.6

value for α ($105 \cdot 10^{-5}$ ml/g per K). In accord with our earlier work [4], we are assigning all of the expansion to the hydrocarbon chain region of the lipid. One then obtains r_b from the volume per methylene, $v_{CH_2} = 1.27 r_b^2 3^{1/2}/2$. The chain separation above the transition, r_a, is then computed from r_b plus the measured volume change. Substitution of these values into the above equation leads to the numbers listed in the last two columns of Table II. For DPPG in the presence of either Na^+ or K^+, the difference between the value of ΔH obtained from calorimetry and the value of ΔU_{vdW} provides an estimate of the energy required for the *trans-gauche* isomerization characteristic of acyl chain melting of phospholipids. (Actually, $\Delta U_r = \Delta H - \Delta U_{vdW} - \Delta U_{other} - P\Delta V$. The last term is negligible at atmospheric pressure. A discussion of this energy balance can be found in Ref. 4.)

Since the average energy difference between the *trans* and *gauche* states is 500 to 600 cal/mol [26], the disordering of the DPPG chains consists of the formation of 4 to 4.5 *gauche* rotamers per molecule. This is smaller by one than estimates for DPPC [4]. While changes in headgroup interactions could also contribute to ΔH, they are likely to be small. For example, Watts et al. [3] calculated that the Gouy-Chapman electrostatic surface energy for DPPG in the charged gel phase is about 1 kcal/mol. Changes as large as 25% in surface area (and surface charge density) would in fact produce energy differences less than 0.5 kcal/mol.

While the chain melting of DPPG in Tris is somewhat more endothermic than it is in phosphate ($\Delta H = 9.1$ kcal/mol vs. 8.3 kcal/mol), the much large volume changes in this buffer translates into a value for ΔU_{vdW} which accounts for more than 90% of ΔH. Thus, the energy remaining for rotameric disordering ($\Delta U_r = \Delta H - \Delta U_{vdW}$) appears to be much less than for DPPG in phosphate. However, let us assume instead that ΔU_r has the same value for DPPG in both buffers. This is consistent with the observation that the parameter (r_a) we use to characterize the melted phase has the same value in both buffers (Table II) and the intuitive idea that the interdigitated state is no more disordered than the normal gel state. Using this assumption, the sum of ΔU_{vdW} and ΔU_r for Tris-DPPG exceeds the measured ΔH by 1.7 kcal/mol. Thus an additional energy term equalling -1.7 kcal/mol must be derived. As Simon and McIntosh [27] have discussed in their work on DPPC in ethanol solutions, interdigitation exposes terminal methyl groups to water, an energetically unfavorable configuration. During the conversion of interdigitated gel phase DPPG into the L_α phase, the reverse process occurs. That is, terminal methyl groups go from a hydrophilic environment to a more favorable lipophilic one. Simon and McIntosh made estimates of 2.8 to 4.6 kcal/mol for the change in free energy of this process, depending on the particular value for the surface tension chosen. Nagle [28] in a similar type of calculation has a lower estimate (1.4–2.3) based on a smaller effective exposed area. Both the magnitude and sign of the free energy change are appropriate for balancing the energies. However, it is the change in enthalpy, ΔH, rather than free energy, ΔG, that must be considered. Without direct calorimetric data, we can make some estimates of ΔH by considering the entropic contribution to the free energy change. Tanford [29] has presented arguments that the exposure of hydrocarbon to bulk water causes an increase in the ordering of the water molecules (i.e. $\Delta S < 0$). However, the water to which chain interdigitation exposes terminal methyl groups is already highly structured by the polar headgroups in the interbilayer region and so it is more likely that introduction of hydrocarbon will produce a disordering effect ($\Delta S > 0$). If this is the case, then ΔH is equal to or larger than ΔG and this mechanism will account for the differences in the energy balance seen between DPPG in the two buffers studied.

Acknowledgments

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