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Calorimetric and spectroscopic studies of the phase behavior and organization of lipid bilayer model membranes composed of binary mixtures of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol

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Abstract

The thermotropic phase behavior of hydrated bilayers derived from binary mixtures of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) was investigated by differential scanning calorimetry, Fourier-transform infrared spectroscopy and ^{31}P-nuclear magnetic resonance spectroscopy. Binary mixtures of DMPC and DMPG that have not been annealed at low temperatures exhibit broad, weakly energetic pretransitions (~11–15 °C) and highly cooperative, strongly energetic gel/liquid-crystalline phase transitions (~23–25 °C). After low temperature incubation, these mixtures also exhibit a thermotropic transition form a lamellar-crystalline to a lamellar gel phase at temperatures below the onset of the gel/liquid-crystalline phase transition. The midpoint temperatures of the pretransitions and gel/liquid–crystalline phase transitions of these lipid mixtures are both maximal in mixtures containing ~30 mol% DMPG but the widths and enthalpies of the same thermotropic events exhibit no discernable composition dependence. In contrast, thermotropic transitions involving the L_c phase exhibit a very strong composition dependence, and the midpoint temperatures and transition enthalpies are both maximal with mixtures containing equimolar amounts of the two lipids. Our spectroscopic studies indicate that the L_c phases formed are structurally similar as regards their modes of hydrocarbon chain packing, interfacial hydration and hydrogen–bonding interactions, as well as the range and amplitudes of the reorientational motions of their phosphate headgroups. Our results indicate that although DMPC and DMPG are highly miscible, their mixtures do not exhibit ideal mixing. We attribute the non-ideality in their mixing behavior to the formation of preferential PC/PG contacts in the L_c phase due to the combined effects of steric crowding of the DMPC headgroups and charge repulsion between the negatively charged DMPG molecules.

Keywords: Phosphatidylcholine; Phosphatidylglycerol; Phospholipid bilayer; Model membrane; Phospholipid miscibility; Differential scanning calorimetry; Infrared spectroscopy; Nuclear magnetic resonance spectroscopy

1. Introduction

The structure and organization of lipid bilayer model membranes derived from mixtures of two or more lipids are of special biological importance because the lipid components of all cell membranes are heterogeneous mixtures of different classes and molecular species of lipids. This fact has provided the impetus for numerous studies of the thermotropic phase behavior and mixing properties of a wide range of lipid mixtures (see [1]).

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; FTIR, Fourier-transform infrared; ΔH_{cal}, calorimetric enthalpy; L_α, lamellar liquid/crystalline; P'_β, rippled lamellar gel phase; L_β, lamellar gel; L'_β, lamellar gel phase with tilted chains; L_c, lamellar crystalline; T_m, gel/liquid–crystalline phase transition temperature; T_p, pretransition temperature

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The data obtained from such studies are usually used to construct temperature-composition pseudophase diagrams from which important information about the miscibility of specific lipid components can be obtained. Recently, the importance of such studies has come into sharper focus because of suggestions that cell membranes may contain laterally segregated, compositionally distinct domains that may be essential for the normal functioning of membrane proteins and transmembrane signaling phenomena (see [2–6]).

Of the limited number of studies of mixtures of different classes of phospholipids that have been performed, only a few have been on phosphatidylglycerol (PG)-containing systems, probably because PG is not a major component of most mammalian cell membranes. However, PG is an important component of mitochondrial and chloroplast inner membranes [7] and of mammalian pulmonary surfactant [8,9]. PG is also a major structural component of bacterial membranes, where it is often the predominant anionic lipid component [7] and may perform a number of specialized functions [10] aside from its role in regulating membrane lipid surface charge density [11–13]. The properties of PG-containing lipid membranes are therefore of direct relevance to the general field of cell membrane structure and function and to the more specialized field of pulmonary function and lung disease. The study of PG-containing lipid membranes is also important in the field of membrane-targeted antimicrobial peptides, where considerable effort is currently being expended to develop therapeutically useful antibiotics which can lyse bacterial membranes in preference to animal cell membranes (see [14–16]). Indeed, interest in antimicrobial peptide–phospholipid bilayer interactions has provided considerable impetus to the study of PG-containing lipid vesicles as models of bacterial cell membranes (see [17,18]).

The relatively few studies that have been performed so far indicate that PG is highly miscible with most naturally occurring membrane lipid classes [1] and that its presence in membranes can have a significant effect on the behavior of the other lipids present [19,20]. However, the focus of virtually all previous work has been on the mixing properties of binary phospholipids mixtures as revealed by the hydrocarbon chain–melting phase transition with very little attention being given to other aspects of lipid thermotropic phase behavior. Moreover, it has been demonstrated that complex polymorphic lipid phase behavior can occur in the natural PG-containing *Acholeplasma laidlawi* membrane [21], which raises fundamental issues about overall lipid miscibility in bacterial membranes. We present here the results of a detailed study of binary mixtures of dimyristolyphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) using a combination of high-sensitivity differential scanning calorimetry (DSC), and ^{31}P-nuclear magnetic resonance (^{31}P NMR) and Fourier-transform infrared (FTIR) spectroscopy. Although this system has been studied previously, the primary focus of these studies has been a characterization of the gel/liquid-crystalline phase transition [22,23] and aspects of the mobility and orientation of the polar headgroups in the liquid-crystalline phase [24,25]. This study encompasses all aspects of the polymorphic phase behavior exhibited by the mixtures and addresses a number of fundamental issues related to the overall miscibility of these two lipids and the general principles underlying lipid/lipid interactions in all of the polymorphic phases formed.

2. Materials and methods

Unlabeled samples of the phospholipids DMPC and DMPG were obtained from Avanti Polar Lipids Inc (Alabaster, AL) and were used without further purification. Doubly ^{13}C═O-labeled DMPC was synthesized in this laboratory using the methodology described by Lewis and McElhaney [26]. Binary mixtures were prepared by mixing chloroform solutions of the lipids in the amounts required to obtain the desired composition. The solvent was then slowly removed in a stream of nitrogen such that the lipid mixture was cast as a thin film on the sides of a clean glass tube whilst maintaining a temperature near 50 °C. The lipid film was then dried in vacuo overnight to ensure the removal of the last traces of solvent. Hydration of the lipid film was achieved by placing some wet cotton wool into the tube (without contacting the lipid film) and allowing the sample to absorb water from the water vapor-saturated air by warming the sample to temperatures near 50 °C. Subsequently, the cotton wool was removed and the hydrated sample was quickly dispersed in an appropriate pre-warmed buffer and mixed by vigorous vortexing at temperatures near 50 °C.

Samples were prepared for DSC as follows. Mixtures containing 4–5 mg of lipid were hydrated as described above and dispersed in 600 μl of a buffer composed of 100 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, 1 mM NaN_3, pH 7.4, and a 500 μl aliquot was introduced into the hastelloy capsule of a Calorimetry Sciences Corporation (Spanish Fork, UT) Multi-Cell DSC heat conduction instrument. Prior to initial data acquisition, the sample was preconditioned by heating to 50 °C followed by cooling to −7 °C at scanning rates near 60 °C h^{-1}. Data acquisition scans were performed at scanning rates near 10 °C h^{-1}. Typically, the process first involved three cycles of heating and cooling between −7 °C and 50 °C. Subsequently the samples were cooled to −30 °C and incubated at that temperature for 12 h, then heated to 2 °C and incubated at that temperature to ensure that all the ice had completely melted. This low-temperature incubation protocol was repeated twice and the sample was then cooled to −7 °C and a further set of data acquisition runs

consisting of two cycles of heating and cooling between −7 °C and 50 °C was performed. At the end of these data acquisition runs, the sample was removed from the hastelloy capsules and introduced into a clean tube and thoroughly mixed by vigorous vortexing at temperatures near 50 °C. A 100 μl aliquot of this sample was then diluted with 1.5 ml of buffer, mixed thoroughly and a 323 μl aliquot introduced into a Calorimetry Sciences Corporation Nano-DSC power compensation instrument, wherein three cycles of data acquisition runs were performed between 5 °C and 45 °C at scan rates near 10 °C h^{-1}. The protocol used in the Multi-Cell DSC enabled an accurate mapping of the gel phase polymorphic behavior of these samples, whereas the Nano-DSC measurements enabled a more accurate mapping of the boundaries of the pretransitions and the gel/liquid-crystalline phase transitions of the various lipid mixtures examined. The data acquired were analyzed and plotted with the Origin software package (OriginLab Corporation, Northampton, MA).

FTIR spectroscopy was performed on samples containing 2–3 mg of lipid. Samples were hydrated as described above and dispersed in 100 μl of a D_2O-based buffer containing 100 mM sodium phosphate, 150 mM NaCl, pD 7.4. The dispersion was squeezed between the CaF_2 windows of a heatable, demountable liquid cell (NSG Precision Cells, Farmingdale, NY) equipped with a 25 μM teflon spacer. Once mounted in the sample holder of the spectrometer, the sample could be heated between −20 °C and 90 °C by an external, computer-controlled water bath. Infrared spectra were acquired as a function of temperature with a Digilab FTS-40 Fourier-transform spectrometer (Biorad, Digilab division, Cambridge, MA) using data acquisition parameters similar to those described by Mantsch et al. [27]. The experiment involved a sequential series of 2 °C temperature ramps with a 20 min interramp delay for thermal equilibriation, and was equivalent to a scanning rate of 4 °C per hour. The data obtained were analyzed using computer programs obtained from the instrument manufacturer and from the National Research Council of Canada, and plotted with the Origin software package. In cases where absorption bands appeared to be a summation of components, a combination of Fourier deconvolution and curve fitting procedures were used to obtain estimates of the position of the component bands and to reconstruct the contours of the original band envelope.

For the ^{31}P NMR spectroscopic measurements, 10–15 mg of dried lipid were hydrated and dispersed in 0.6 ml of a buffer composed of 100 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM NaN_3, pH 7.4. Spectra were acquired as a function of temperature with a Varian Unity-300 spectrometer operating at 121.41 MHz for ^{31}P. Spectra were acquired using the single-pulse data acquisition techniques and other data acquisition parameters described by Lewis et al. [28] and plotted with the Origin software package.

3. Results

3.1. Differential scanning calorimetric studies

Illustrated in Fig. 1 are DSC thermograms which exemplify the pattern of thermotropic phase behavior exhibited by the aqueous dispersions of DMPC, DMPG and binary mixtures composed of these two lipids. Detailed descriptions of the thermotropic phase behavior of both DMPC and DMPG have been published elsewhere [29,30], but are summarized here as references against which the behavior of binary mixtures of the two lipids can be compared. Freshly prepared, fully hydrated aqueous dispersions of DMPC and DMPG both exhibit phenomenologically similar patterns of thermotropic phase behavior. Upon heating, unannealed samples of both lipids exhibit both pretransitions (L'_β/P'_β transitions) and gel/liquid-crystalline (P'_β/L_α) phase transitions. The pretransitions are weakly energetic, solid-state rearrangements of the lipid molecules and are observed at temperatures near 13.4 °C (DMPC) and 12.5 °C (DMPG), some 11–12 °C below the onset of the gel/liquid-crystalline phase transition. The gel/

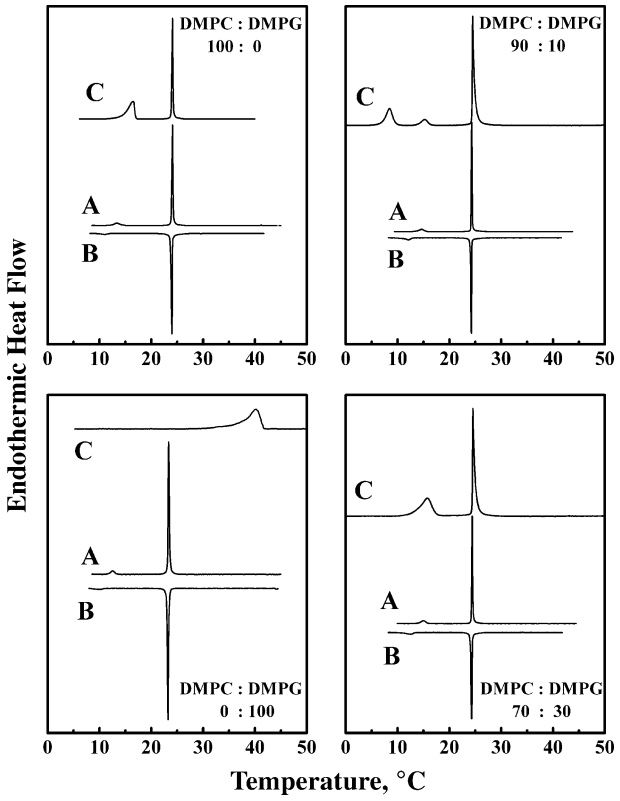

Fig. 1. DSC thermograms illustrating the polymorphic phase behavior exhibited by aqueous dispersions of DMPC, DMPG and binary mixtures of the two lipids. The thermograms shown exemplify the behavior of aqueous dispersions of the lipids and mixtures before (A and B) and after (C) extensive equilibriation at low temperature. The thermograms labeled A and B were recorded with the Nano-DSC from the same sample, whereas thermograms labeled C were obtained from a different sample using the Multi-Cell DSC. The thermograms have all been normalized with respect to the mass of the sample used. The DMPC:DMPG ratios are as indicated.

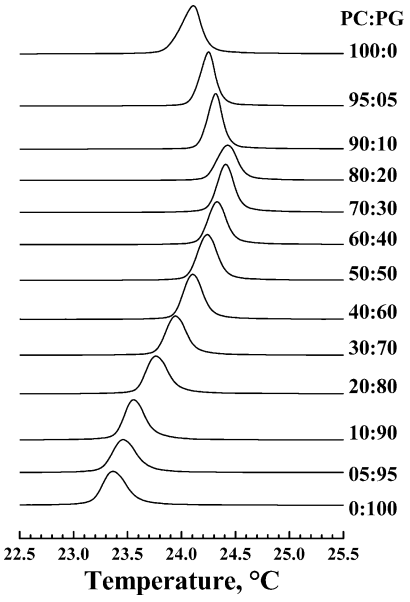

Fig. 2. DSC thermograms illustrating the composition-dependent changes in the gel/liquid-crystalline phase transitions exhibited by the DMPC/DMPG mixtures. The thermograms shown were acquired with the high-sensitivity power-compensation calorimeter operating at scan rates of 10 °C h^{-1} and are normalized with respect to the mass of the sample used. The DMPC:DMPG ratios are as indicated.

liquid-crystalline phase transitions of these lipids are highly energetic thermotropic events in which the cooperative melting of the lipid hydocarbon chains occur at temperatures near 24.1 °C (DMPC) and 23.3 °C (DMPG). Upon cooling, the L'_β/P'_β and P'_β/L_α phase transitions of these lipids are both fully reversible, albeit with some hysteresis in the case of the L'_β/P'_β phase transitions (Fig. 1, thermograms A and B). Fig. 1 also shows that the heating thermotropic phase behavior of DMPC and DMPG both change after prolonged equilibration at low temperature. With DMPC, the change is manifest by the suppression of the pretransition endotherm near 13 °C and the appearance of a considerably more energetic endotherm (ΔH_{cal} ~6.2 Kcal/mol) centered near 16.5 °C (Fig. 1, thermogram C), whereas with DMPG prolonged low-temperature equilibriation results in the suppression of both the L'_β/P'_β and P'_β/L_α endotherms and the appearance of a broader and considerably more energetic endotherm (ΔH_{cal} ~14.2 Kcal/mol) centered near 40 °C (Fig. 1, thermogram C). These changes are attributable to the formation of the lamellar crystalline (L_c) phases of these lipids which convert to either the P'_β phase (DMPC) or the L_α phase (DMPG) upon heating. Fig. 1 also shows that binary mixtures of DMPC and DMPG also exhibit a complex pattern of polymorphic phase behavior which, in some instances, may differ from that exhibited by either of the pure lipids. The compositional dependence of the thermotropic phase behavior of the various binary mixtures are presented below.

DSC thermograms exhibited by freshly prepared aqueous dispersions of binary mixtures of DMPC and DMPG all exhibit thermotropic events analogous to the pretransitions and gel/liquid-crystalline phase transitions of the pure lipids (see Figs. 1–3). Interestingly, however, the incorporation of small amounts of DMPG into DMPC results in small but significant increases in both the pretransition temperature (T_p) and gel/liquid-crystalline phase transition temperature (T_m) of the lipid mixture, despite the fact that the T_p and T_m values of DMPG are both lower that those of DMPC. Thus, as illustrated in Figs. 2 and 3, increases in DMPG content up to 30 mol% are accompanied by progressive increases in both the T_p and T_m values of the mixtures, and further increases in DMPG content are accompanied with progressive decreases in the magnitudes of T_p and T_m. Thus, DMPC:DMPG mixtures containing 5–50 mol% DMPG exhibit T_p and T_m values that are higher than those of either of the pure lipid components. Interestingly, however, this unusual behavior is not accompanied by significant composition-dependent changes in the transition widths or the enthalpy changes associated with the L'_β/P'_β and P'_β/L_α phase transitions of the DMPC/DMPG mixtures (see Figs. 2, 3 and 5). We therefore conclude that although DMPC and DMPG seem to be miscible in all proportions, the two lipids do not form ideal mixtures. The possible basis of such behavior will be explored in the Discussion.

As noted above, low-temperature equilibriation of aqueous dispersions of binary mixtures of DMPC and DMPG result in the appearance of additional thermotropic events attributable to the formation of lamellar-crystalline (L_c) phases and, as illustrated in Fig. 4, this aspect of the thermotropic phase behavior of the DMPC:DMPG mixtures also varies significantly with the composition of the

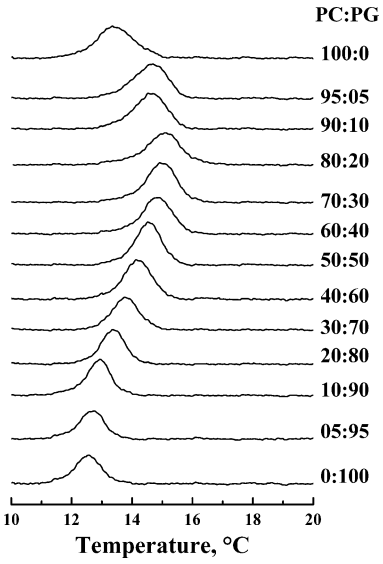

Fig. 3. DSC thermograms illustrating the composition-dependent changes in the pretransitions exhibited by the DMPC/DMPG mixtures. The data shown are a 30 fold expansion of the 10 °C–20 °C range of the same DSC scans used for Fig. 2. DMPC:DMPG ratios are as indicated. The thermograms shown were acquired with the high-sensitivity heat-conduction calorimeter operating at scan rates of 10 °C h^{-1} and are normalized with respect to the mass of the sample used. The DMPC:DMPG ratios are as indicated.

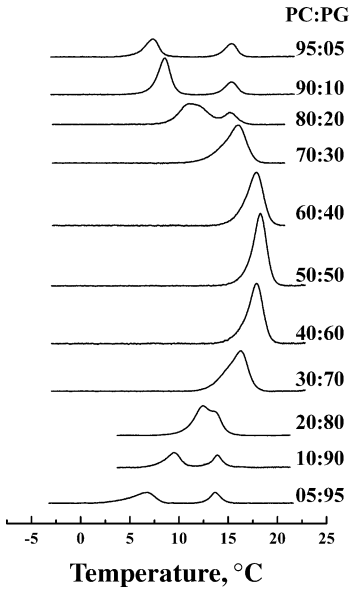

Fig. 4. DSC thermograms illustrating the composition-dependent changes in the gel-phase polymorphism exhibited by binary DMPC:DMPG mixtures after extensive annealing at low temperatures. The thermograms have all been normalized with respect to the mass of the sample used. The DMPC:DMPG ratios are as indicated.

mixture. Mixtures containing ≤20 mol% PC and/or ≥80 mol% PC exhibit two solid-phase thermotropic transitions, one of which (the higher-temperature event) corresponds to the pretransition exhibited by freshly prepared samples as described above. The other thermotropic transition is broader and considerably more energetic than the pretransition, and is attributed to a thermotropic phase transition from the lamellar crystalline phase. For such mixtures, the L_c phase transition occurs at temperatures below their respective T_p values and discrete L_c/L'_β and L'_β/P'_β transitions are observed (see Fig. 4). All of the other mixtures examined (i.e. those containing 30–70 mol% PC) exhibit a single, broad and fairly energetic solid-phase thermotropic transition at temperatures between those of the pretransition and gel/liquid–crystalline phase transitions exhibited by their corresponding freshly prepared samples (see Fig. 4). Our FTIR and ^{31}P NMR spectroscopic data (see below) indicate that the structural transformations accompanying the single solid-phase transition observed in the composition range 30–70 mol% PC is, in effect, a summation of the L_c/L'_β and L'_β/P'_β transitions exhibited by the other mixtures (i.e. a L_c/P'_β transition).

Fig. 4 also shows that the temperatures at which the L_c phase of the various mixtures converts to either a L'_β or P'_β gel phase are maximal at compositions approaching equimolar amounts of DMPC and DMPG and decrease smoothly as the content of either component increases (see Figs 4 and 5). Moreover, the enthalpy change associated with the L_c/L'_β or L_c/P'_β phase transitions approaches a maximum at near equimolar proportions of the two lipids and decreases monotonically as the content of either lipid increases (Fig. 5). This differs sharply from what is observed with the enthalpy changes accompanying the corresponding pretransitions and gel/liquid–crystalline phase transitions, for which there is at best only a small monotonic change over the entire composition range studied (see Fig. 5). The possible structural basis for the different dependencies of the L_c/L'_β or L_c/P'_β phase transitions, and the pretransition and main transitions, on the DMPC:DMPG ratio observed by DSC was probed by a combination of ^{31}P NMR and FTIR spectroscopy.

3.2. ^{31}P-nuclear magnetic resonance spectroscopic studies

Illustrated in Fig. 6 are ^{31}P NMR powder patterns which typify those exhibited by the L_c phases of the binary mixtures examined. To facilitate comparison with the pure lipid components, the ^{31}P NMR powder patterns of the stable L_c phase of DMPC and the metastable L_c phase of DMPG are also shown. The ^{31}P NMR spectra exhibited by the gel and liquid-crystalline phases of the various DMPC:DMPG mixtures (not shown) are very similar to those of the gel and liquid-crystalline phases of DMPC and DMPG alone and are not considered here. The stable L_c phase of DMPC exhibits a broad ^{31}P NMR powder pattern (basal line width ~250 ppm, see Fig. 6), the contours of which approach those of the so-called rigid-limit spectra of crystalline forms of these lipids [31,32]. DMPG also forms a stable L_c phase but its phosphate groups are immobilized into lamellar structures with long-lived hydrogen bonds, which make the recording of its ^{31}P NMR powder pattern impractical under our experimental conditions [30]. However, ^{31}P NMR powder patterns of the L_c phases of binary mixtures of DMPC and DMPG are easily recorded and, as illustrated in Fig. 6, the overall line shapes and line widths of their powder patterns are remarkably similar over the entire compositional range examined. These powder pat-

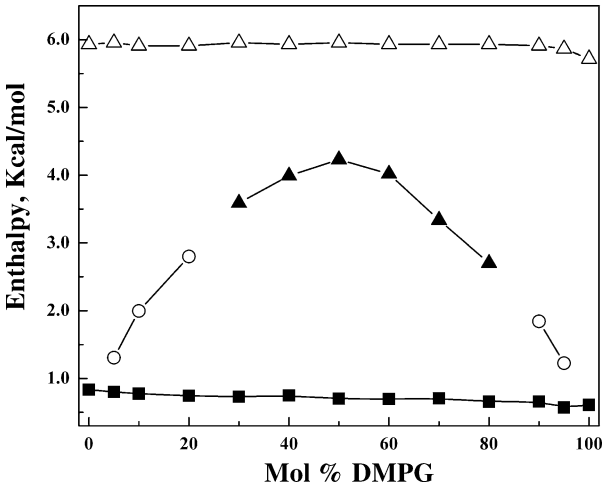

Fig. 5. Effect of lipid composition on the enthalpy changes associated with the thermotropic phase transitions exhibited by binary mixtures of DMPC and DMPG. The data presented represent the enthalpy changes observed at: -△- P'_β/L_α phase transitions. -■- L'_β/P'_β phase transitions; -○- L_c/L'_β phase transitions; -▲- L_c/P'_β phase transitions.

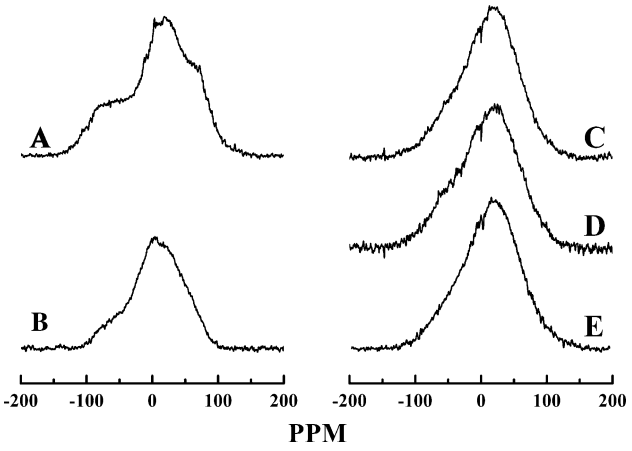

Fig. 6. Proton-decoupled ^{31}P NMR spectra exhibited by the L_c phases formed by: (A) DMPC; (B) DMPG; (C) DMPC:DMPG (3:7); (D) DMPC:DMPG (1:1); (E) DMPC:DMPG (7:3). The ^{31}P NMR powder patterns illustrated in A, C, D and E were obtained with the most stable of the L_c phases formed by these mixtures whereas B was obtained with the metastable intermediate formed *en route* to the stable L_c phase of DMPG. For technical reasons, ^{31}P NMR powder patterns of the stable L_c phase of DMPG cannot be recorded under our conditions [30].

terns (basal linewidths ~200 ppm) are all narrower than those formed by the stable L_c phase of DMPC, indicating that in the L_c phases of the binary mixtures, the lipid phosphate headgroups are all capable of restricted motions that are nevertheless faster and of higher amplitude than in the stable L_c phase of DMPC and presumably of DMPG as well. However, the line widths and line shapes of the ^{31}P NMR spectra of the mixtures more closely resemble the metastable L_c phases of DMPG (see Fig. 6). This observation suggests that the structures of the L_c phases formed by these binary mixtures may also be similar to the metastable L_c phase of DMPG, at least with respect to constraints on the motion and orientation of the phosphate headgroups. However, this suggestion does seem inconsistent with the composition-dependent changes in the temperature and enthalpy of the L_c phase transitions of these binary lipid mixtures. This issue will be explored further in the Discussion.

3.3. Fourier-transform infrared spectroscopic studies

Illustrated in Fig. 7 are the C═O stretching and CH_2 bending regions of FTIR spectra obtained after aqueous dispersions of DMPC, DMPG and representative binary mixtures of these two lipids are incubated under conditions favoring to the formation of their L_c phases. These spectroscopic observations are interpretable in terms of hydration and hydrogen-bonding interactions of the ester carbonyl groups in the polar/apolar interfacial regions of those bilayers, and the lateral packing interactions between the all-*trans* hydrocarbon chains (see [33,34]). The FTIR spectroscopic signatures exhibited by the gel and liquid–crystalline phases of the binary lipid mixtures (not shown) are similar to those of the comparable phases of DMPC and DMPG which have been described and interpreted elsewhere [26,30,35]. However, the stable L_c phases of DMPC and DMPG exhibit markedly different IR spectroscopic signatures. Detailed structural interpretations of the IR spectroscopic signatures of the L_c phases of both DMPC and DMPG have been presented elsewhere [26,30,35] and are summarized in Table 1. A comparison of the FTIR spectroscopic features exhibited by the L_c phases of the binary lipid mixtures studied here with those listed in Table 1 reveals a number of structurally significant differences. First, the L_c phases of all of the lipid mixtures examined exhibit ester C═O stretching bands which are composed of a relatively sharp, higher frequency band centered near 1738 cm^{-1} and a considerably broader, lower-frequency component centered near 1726 cm^{-1} (see Fig. 7). These spectroscopic features differ markedly from those exhibited by the L_c phases of the pure lipids (for details of the latter, see Refs. [29,30]) and, despite some composition-dependent variations in the relative sizes of the two absorption bands, are consistent features of the L_c phases of all of the DMPC:DMPG mixtures examined. With most hydrated diacylglycerolipid bilayers, the low- and high-frequency components of the ester C═O stretching absorption band arise from the stretching vibrations of populations of hydrogen-bonded and nonhydrogen-bonded ester carbonyl groups, respectively [36,37]. Thus, our observations suggest that the L_c phases of these lipid mixtures are composed of populations of free and H-bonded ester C═O groups and, because of the greater line width of the lower-frequency band, we can also suggest the H-bonded population is involved in a dynamic association with H-bonding donor groups on the PG headgroup and/or interfacially located solvent molecules. We also note that the consistency of these spectroscopic features across the range of compositions examined suggests that there may not be major

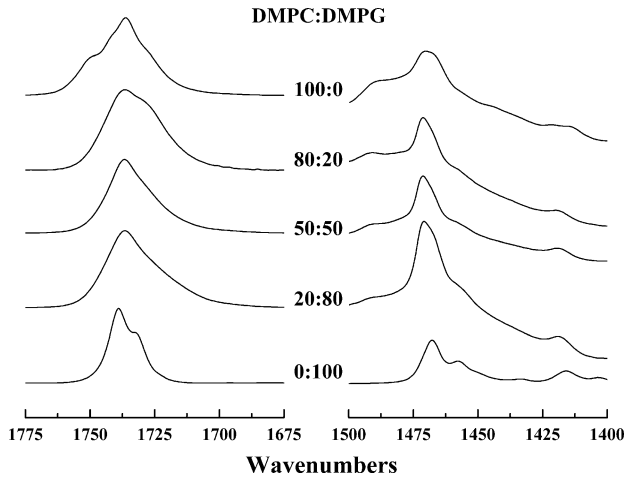

Fig. 7. The C═O stretching (left) and CH_2 bending (right) regions of FTIR spectra exhibited by the L_c phases formed by DMPC and DMPG, and by binary DMPC:DMPG mixtures over the entire compositional range examined. The absorbance spectra shown were acquired at 0 °C for the compositions indicated.

Table 1
Structural interpretation of the spectroscopic signatures of DMPC, DMPG and their binary mixtures

Lipid	C═O stretch (cm^{-1})	CH_2 scissoring (cm^{-1})	Comments
DMPC	1750, 1742, 1736, 1728	1472, 1466	Distinct populations of "free" and H-bonded ester C═O groups from two inequivalent molecules per unit cell Fairly dehydrated polar/apolar interface with a small population of H-bonded ester C═O groups Perpendicularly packed hydrocarbon chains forming near ideal orthorhombic ⊥ subcells. Rigid, weakly H-bonded headgroup phosphate
DMPG	1738, 1732, 1722	1467	Fairly dehydrated polar/apolar interface with a very small population of H-bonded ester C═O groups. Orientationally disordered all-*trans* hydrocarbon chains Rigid, strongly H-bonded headgroup phosphate
Binary mixtures	1738, 1726	1470, 1466[a]	Partially dehydrated polar/apolar interface containing sizeable populations of H-bonded ester C═O groups Perpendicularly packed hydrocarbon chains forming in distorted orthorhombic ⊥ subcells. Weakly H-bonded headgroup phosphate capable of slow reorientational fluctuations.

[a] Shoulder on the higher frequency peak.

composition-dependent structural variations as regards hydration and hydrogen-bonding interactions in the polar/apolar interfaces of the L_c phases formed by these lipid mixtures.

The CH_2 bending region of the IR spectra (~1400–1500 cm^{-1}) exhibited by the L_c phases of these lipid mixtures all contain a main CH_2 scissoring band (~1468–1470 cm^{-1}), which is composed of a main peak centered near 1470 cm^{-1} and a shoulder centered near 1466 cm^{-1} (Fig. 7, right panel). The appearance of these two CH_2 scissoring band components is indicative of so-called factor group splitting, a spectroscopic feature consistent with the formation of an extended array of all-*trans* hydrocarbon chains in which the hydrocarbon chains are arranged in subcells with their zigzag planes perpendicular to each other (see [33,34]). This form of hydrocarbon chain packing is commonly observed when the chains are arranged in an orthorhombic ⊥ subcell [38,39]. However, the fact that the two components of the CH_2 scissoring band are of unequal intensity also suggests that the subcellular packing is distorted from the ideal format, most probably by some rotation of the hydrocarbon chains within the unit cell (see [38,40]). As illustrated in Fig. 7, these spectroscopic features differ markedly from those exhibited by the L_c phases of either DMPC (near ideal orthorhombic ⊥ packing) or DMPG (hexagonal packing), but are consistent features of the L_c phases formed by DMPC:DMPG mixtures over the entire compositional range examined. We therefore conclude that there is probably little or no composition-dependent variation in the structures of the L_c phases of these mixtures with respect to the lateral packing interactions between their hydrocarbon chains.

The picture which emerges from the ^{31}P NMR and FTIR spectroscopic data described above is one in which the L_c phases formed by the binary mixtures of DMPC and DMPG are structurally similar over the entire compositional range examined. However, this picture seems incongruous with the composition-dependent changes in the transition temperatures and enthalpy values illustrated in Figs. 4 and 5. This aspect of the behavior of these compounds was further investigated by an FTIR spectroscopic study of mixtures of unlabeled DMPG and ^{13}C═O-labeled DMPC.

FTIR spectra of mixtures of binary mixtures of DMPG and ^{13}C═O-labeled DMPC were acquired in their respective L_c, L_β and L_α phases and the results are summarized in Fig. 8. Because of the greater reduced mass of the ^{13}C═O oscillator, C═O stretching bands emanating from the unlabeled DMPG (~1735 cm^{-1}) and ^{13}C═O-labeled DMPC (~1695 cm^{-1}) are sufficiently well resolved that

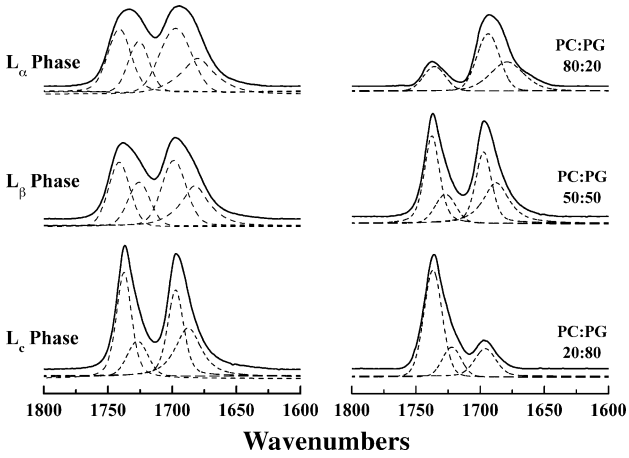

Fig. 8. The C═O stretching regions of the FTIR spectra exhibited by binary mixtures of unlabeled DMPG and ^{13}C═O-labeled DMPC. The left panel shows the ester carbonyl absorption bands exhibited by the L_c, L_β and L_α phases of an equimolar mixture of DMPG and ^{13}C═O-labeled DMPC. The right panel shows spectra exhibited by the L_c phases of representative binary mixtures of DMPG and ^{13}C═O-labeled DMPC (T=0 °C). Spectra are shown in the absorbance mode with the solid lines representing the spectral contours actually acquired and the dashed lines representing component bands resolved by a combination of Fourier self-deconvolution and band-fitting procedures.

fairly accurate measurements of the contours of the C═O stretching bands of the labeled and unlabeled lipid species could be made. The data shown in Fig. 8 (left panel) indicate that in each of the lipid phase states examined, the contours of the absorption bands arising from DMPC (^{13}C═O labeled) and DMPG (unlabeled) components of any given mixture are remarkably similar, aside from the frequency differences attributable to the isotopic labeling. Thus, the local environments of the ester carbonyl groups of the DMPC and DMPG components of the mixture are very similar. The data also show that the C═O stretching bands emanating from the unlabeled DMPG and ^{13}C═O labeled DMPC components of the various binary mixtures are each resolvable into subcomponents, centered near 1738 cm^{-1} and 1728 cm^{-1} and near 1695 cm^{-1} and 1685 cm^{-1}, respectively. The components centered near 1738 cm^{-1} and 1728 cm^{-1} can be assigned to populations of nonhydrogen-bonded and hydrogen-bonded DMPG ester carbonyl groups, whereas those near 1695 cm^{-1} and 1685 cm^{-1} can be attributed to the corresponding populations of DMPC ester carbonyl groups, respectively. Thus, the DMPC and DMPG components of these binary mixtures both exhibit spectra consistent with the existence of subpopulations of non-hydrogen-bonded and hydrogen-bonded ester carbonyl groups when the lipids are in either the gel or liquid-crystalline state, and that the relative sizes of the two hydrogen-bonded populations increase at the lipid gel/liquid-crystalline phase transition (data not shown). Moreover, with all of the mixtures examined, the formation of the L_c phase is accompanied by a significant decrease in the relative areas of the components centered near 1725 and 1685 cm^{-1}, suggesting that L_c phase formation involves a marked decline in the populations of hydrogen-bonded DMPC and DMPG ester carbonyl groups. Interestingly, however, the L_c phase-induced decline in the populations of hydrogen-bonded carbonyl groups of any component of the mixture seems inversely related to the amount of that component present. Thus, for example, mixtures containing about 80 mol% of DMPC or DMPG form L_c phases in which there is no discernable population of H-bonded C═O groups emanating from the minor component of the mixture (see Fig. 8, right panel), whereas for mixtures in which there are comparable amounts of DMPC and DMPG, sizeable populations of hydrogen-bonded ester C═O carbonyls from both DMPC and DMPG persist (Fig. 8, right panel). This observation suggests preferential DMPC:DMPG interactions in the L_c phase, which may explain the composition-dependent variations in the thermodynamic characteristics of the L_c phases of these mixtures. The implications of this and other observations are examined below.

4. Discussion

In this study, high-sensitivity DSC at very slow scan rates (~10 °C h^{-1}) was used in conjunction with FTIR- and ^{31}P NMR spectroscopy to characterize the boundaries and structural characteristics of the L_c, L'_β, P'_β and L_α phases formed by binary mixtures of DMPC and DMPG (5–95 mol% DMPG). Our results are summarized as a temperature-composition pseudophase diagram in Fig. 9. Consistent with the results of previously published work [22,23], our calorimetric and spectroscopic data both indicate that the two lipids are highly miscible in all proportions, as manifested primarily by the highly cooperative, single-component hydrocarbon chain-melting transition endotherms (transition half-widths ~0.25 °C, this work; ~0. 5 °C, Ref. [23]), by hydrocarbon chain-melting half-widths and enthalpy values that are essentially composition-independent (this work, [23]), and by the overall similarity in the structural characteristics of the lamellar gel and lamellar crystalline phases observed over the entire composition range (this work). Such behavior is consistent with the fact that the temperatures and enthalpy changes associated with the gel/liquid-crystalline phase transitions of the two lipids are very similar [29,30], and with previous work suggesting that DMPC and DMPG exhibit near ideal mixing behavior [22,23].

Our data also indicate that the midpoint temperatures of the pretransitions and gel/liquid-crystalline phase transitions of mixtures containing 5–50 mol% DMPG are all higher than those of bilayers composed of either of the two pure lipids. The latter experimental observation is inconsistent with DMPC and DMPG forming ideal mixtures and, to our knowledge, such behavior has not been reported before. However, Garidel et al. [23] have reported that the midpoint temperatures and enthalpy changes associated with the pretransitions of binary DMPC:DMPG mixtures are highest with mixtures containing equimolar amounts of the two lipids. They also reported pretransition enthalpy values as high as 3.0 Kcal/mol for mixtures containing ~50 mol% DMPG. Those observations contrast sharply with the results of this study, wherein we demonstrate that the pretransition temperatures are highest with mixtures containing ~30 mol% DMPG and that the pretransition enthalpies (~500–700 cal/mol) are essentially composition independent. It should be noted, however, that the composition dependence of the pretransition reported by Garidel et al. [23] is remarkably similar to that which we report here for L_c phases of these mixtures. Given this, and the fact that the pretransition enthalpy values reported by Garidel et al. [23] are also considerably higher than normally observed for the pretransitions of PC and PG bilayers [29,30], we suggest that this discrepancy between our observations and those of Garidel and coworkers may have arisen because of unrecognized L_c phase formation under their conditions (see below).

Currently it is not clear why DMPC:DMPG mixtures containing small to modest amounts of an anionic lipid like DMPG should exhibit L'_β/P'_β and P'_β/L_α transition temperatures that are higher that those of DMPC alone. These experimental observations seem incongruous with the fact

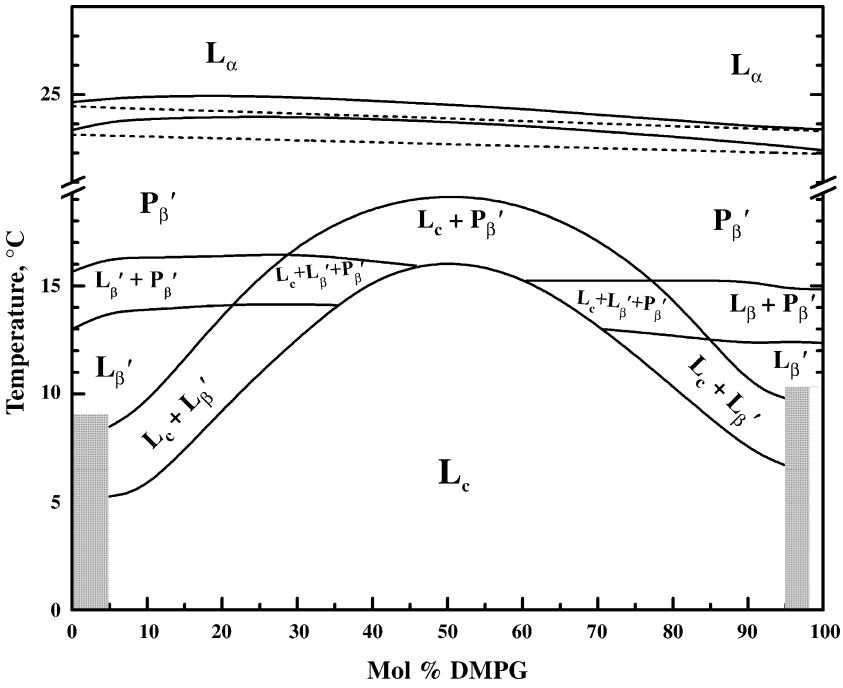

Fig. 9. Temperature-composition pseudo-phase diagrams of binary mixtures of DMPC and DMPG. The phase boundaries shown represent the temperature range which encompasses 2% to 98% of the of the total area under the DSC heating endotherm. Phase assignments were made by a combination of FTIR and ^{31}P NMR spectroscopic measurements. The dashed lines indicate the boundaries of the calculated ideal phase diagram of the gel/liquid-crystalline phase transition. The idealized phase diagram was calculated from transition temperature and transition enthalpy values of the pure components as described by Mabrey and Sturtevant [43]. The solidus and liquidus curves were also adjusted to account for the finite widths of the transitions of the pure components as described by Mabrey and Sturtevant [43].

that the L_β'/P_β' and P_β'/L_α transition temperatures of DMPG are both lower than those of corresponding transition temperatures of DMPC, and the fact that the incorporation of anionic molecules like DMPG into a zwitterionic DMPC matrix increases bilayer negative surface charge density and should therefore weaken lipid/lipid packing interactions through charge repulsion effects. However, we can rationalize our experimental observations as follows. First, it is well established that the mean cross-sectional area occupied by the polar headgroup of PC molecules is larger than the area required for optimal packing of the lipid hydrocarbon chains [41]. It thus follows that pure PC bilayers are subject to a destabilizing stress arising from the steric crowding of the polar headgroups. Second, the polar headgroups of PG molecules are smaller than those of PC molecules [42]. Consequently, the inclusion of small amounts of PG into a PC matrix will probably relieve some of the stress arising from the steric crowding of the PC headgroups. In the absence of other considerations, the relief of steric crowding stress should promote stronger lipid–lipid interactions and result in an elevation of the transition temperature. However, because of its anionic properties, the inclusion of DMPG in a bilayer matrix of zwitterionic DMPC molecules will increase the concentration of negative charges at the bilayer surface. Thus, any stabilizing influences arising from the relief of steric crowding stress will be progressively attenuated with increases in DMPG content because of a combination of charge repulsion-induced weakening of lipid/lipid packing interactions and the overall bulk effect of the intrinsically lower transition temperature of DMPG. At the other end of the scale, the inclusion of DMPC into a DMPG bilayer will also have a stabilizing effect on bilayer packing interactions by lowering the overall density of negative charge at the bilayer surface though, as DMPC levels increase, this effect would eventually be attenuated because of increased DMPC-induced steric crowding stress. If this explanation is correct, DMPC:DMPG mixtures should actually behave non-ideally and the phase coexistence region of the temperature-composition phase diagram should be upward shifted relative to the "ideal mixing line" at all finite compositions. Our observations of the pretransitions and the gel/liquid-crystalline phase transitions of these mixtures are both compatible with this prediction (see Fig. 9).

The composition-dependent changes in the midpoint temperatures and enthalpy values recorded for the thermotropic transitions involving the L_c phases are quite large and they describe a symmetrical curve centered at equimolar amounts of the two lipids. This contrasts sharply with comparable data on the pretransition and the gel/liquid-crystalline phase transition, for which comparable composition-dependent changes are maximal with mixtures containing ~30 mol% DMPG and are considerably smaller in magnitude. In principle, these differences could be indicating that the physical basis of the composition-dependent behavior of the L_c phase is different from that underlying

the composition-dependent properties of the L'_β and P'_β phases. However, it is difficult to reconcile such a suggestion with considerations raised in the paragraph above, because issues such as steric crowding stress and charged group repulsion should be more applicable to the close-contact intermolecular interactions which occur in condensed structures such as lipid L_c phases. However, the shape of the curves describing the composition dependence of the midpoint temperatures and enthalpy values of the L_c phase can be approximated by a simple probability function, based on the assumption that the magnitude of the measured parameter is directly proportional to the probability of forming PC:PG contacts (see Fig. 10). The suggestion that the stability of the L_c phase formed by these DMPC:DMPG mixtures is directly related to the number of PC:PG contacts implies that these phospholipids do not mix ideally in the L_c phase and that DMPC:DMPG contacts are preferred over DMPC:DMPC and DMPG:DMPG contacts in this phase. The latter suggestion is consistent with our observations of the temperature dependence of the properties of the L_c phase, the results of our FTIR spectroscopic studies of mixtures of unlabeled DMPG and $^{13}C{=}O$-labeled DMPC, and the fact that there does not appear to be any major differences in the spectroscopic signatures of the L_c phase across the range of compositions examined.

Our studies also show that that the incorporation of relatively small (~5 mol%) amounts of DMPC into a DMPG matrix suppresses the formation of the native L_c phase of DMPG and vice versa, observations which seem incongruous with the fact that DMPC and especially DMPG are each capable of forming stable L_c phases with transition temperatures and enthalpies which are considerably higher than those of the L_c phases formed by many of the mixtures examined in this study [29,30]. Given this, and the fact that the nucleation and growth of the L_c phases DMPC and DMPG are very slow processes [29,30], the preferential formation of the "mixed-species" L_c phase in mixtures containing a large excess of one component is almost certainly a reflection of more favorable kinetics. It is therefore possible that preferential DMPC:DMPG contacts in the gel phase may also be providing a more favorable kinetic path for the nucleation and growth of the L_c phases of the mixed species, a suggestion consistent with our observation that the formation of the "mixed species" L_c phase proceeds more rapidly than with the pure lipids alone. The latter further implies that as the quantities of DMPC and DMPG in the mixture become more comparable, the formation of the "mixed-species" L_c phase would also become more kinetically and thermodynamically favored, as observed experimentally.

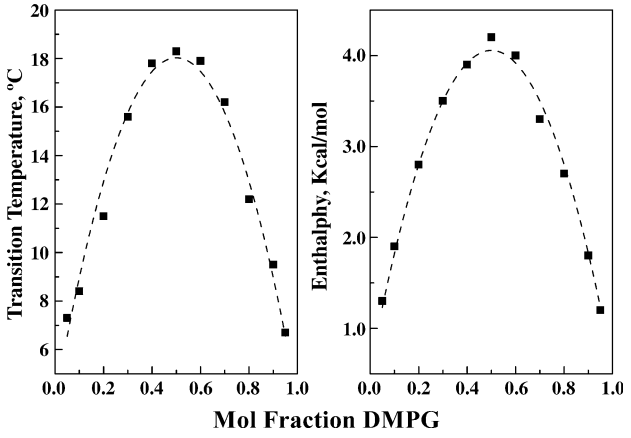

Fig. 10. Composition-dependent changes in the midpoint temperatures and enthalpy changes associated with the thermotropic phase transitions of the L_c phases formed by binary mixtures of DMPC and DMPG. The data points show the experimentally determined values whereas the dashed lines show a fitted curve based on the assumption that the magnitude of the measured property is proportional to the probability of PC:PG contacts. The fitted curve is described by an equation of the form:

$$E_{obs} = E_0 + 2\alpha(1-\alpha)(E_{max} - E_0)$$

where E_{obs}=measured value, α=mol fraction of PG, and E_{max} and E_0 are constants.

Finally, we note that the suggestion that the L_c phase is stabilized through preferential PC:PG contacts implies that PC:PG contacts make a positive contribution to the stabilization of the system. Moreover, since the destabilizing effects attributable to steric crowding stress and charged group repulsion (see above) are also applicable to the L_c phase, it follows that the contribution arising from PC:PG contacts must be quite substantial and most probably dominant. This conclusion differs markedly from that which we have proposed to explain the composition dependence of the midpoint temperatures of the pretransitions and gel/liquid-crystalline phase transitions of these mixtures. Implicit to the latter is the idea that these bilayers are subject to destabilizing stress through unfavorable PC:PC contacts (steric crowding) and unfavorable PG:PG contacts (charged group repulsion). The behavior of the system is thus driven by a strong tendency to minimize these unfavorable contacts and there is no requirement for PC:PG contacts to make any positive contribution to the stabilization of the system per se. The overall stability of such a system is thus determined by the relative weights of the two concurrent destabilizing influences and the free energy minimum will occur at compositions where the cumulative weights of the two destabilizing influences are balanced. With the L'_β/P'_β and the P'_β/L_α phase transitions of these DMPC:DMPG mixtures, our data indicate that the free energy minimum occurs at compositions containing ~30 mol% DMPG, suggesting that steric crowding of the DMPC headgroups exerts a greater destabilizing effect on the L'_β and P'_β phases of these mixtures than does charge repulsion between negatively charged DMPG molecules.

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