The Gaussian Curvature Elastic Modulus of N-Monomethylated Dioleoylphosphatidylethanolamine: Relevance to Membrane Fusion and Lipid Phase Behavior

D. P. Siegel* and M. M. Kozlov†
*Givaudan, Cincinnati, Ohio; and †Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel-Aviv University, Ramat Aviv, Tel Aviv, Israel

ABSTRACT The energy of intermediates in fusion of phospholipid bilayers is sensitive to $\bar{\kappa}_m$, the saddle splay (Gaussian curvature) elastic modulus of the lipid monolayers. The value $\bar{\kappa}_m$ is also important in understanding the stability of inverted cubic (Q_{II}) and rhombohedral (R) phases relative to the lamellar (L_α) and inverted hexagonal (H_{II}) phases in phospholipids. However, $\bar{\kappa}_m$ cannot be measured directly. It was previously measured by observing changes in Q_{II} phase lattice dimensions as a function of water content. Here we use observations of the phase behavior of N-mono-methylated dioleoylphosphatidylethanolamine (DOPE-Me) to determine $\bar{\kappa}_m$. At the temperature of the L_α/Q_{II} phase transition, T_Q, the partial energies of the two phases are equal, and we can express $\bar{\kappa}_m$ in terms of known lipid monolayer parameters: the spontaneous curvature of DOPE-Me, the monolayer bending modulus κ_m, and the distance of the monolayer neutral surface from the bilayer midplane, δ. The calculated ratio $\bar{\kappa}_m/\kappa_m$ is -0.83 ± 0.08 at $T_Q \approx 55°C$. The uncertainty is due primarily to uncertainty in the value of δ for the L_α phase. This value of $\bar{\kappa}_m/\kappa_m$ is in accord with theoretical expectations, including recent estimates of the value required to rationalize observations of rhombohedral (R) phase stability in phospholipids. The value $\bar{\kappa}_m$ substantially affects the free energy of formation of fusion intermediates: more energy (tens of k_BT) is required to form stalks and fusion pores (ILAs) than estimated solely on the basis of the bending elastic energy. In particular, ILAs are much higher in energy than previously estimated. This rationalizes the action of fusion-catalyzing proteins in stabilizing nascent fusion pores in biomembranes; a function inferred from recent experiments in viral systems. These results change predictions of earlier work on ILA and Q_{II} phase stability and L_α/Q_{II} phase transition mechanisms. To our knowledge, this is the first determination of the saddle splay (Gaussian) modulus in a lipid system consisting only of phospholipids.

INTRODUCTION

It is now fairly clear that fusion in lipid membranes occurs via the stalk mechanism (Kozlov and Markin, 1983; Siegel, 1993; Kuzmin et al., 2001; Kozlovsky and Kozlov, 2002; Kozlovsky et al., 2002; Markin and Albanesi, 2002; May, 2002), which is related to the mechanism of lamellar/inverted phase transitions in phospholipids (Siegel and Epand, 1997; Siegel, 1999; Kozlovsky et al., 2002; Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov, unpublished results). When stalks or fusion pores form between membrane vesicles, there is a change in topology that decreases the Gaussian curvature integrated over the surface. The same changes in the integrated Gaussian curvature occur when fusion pores form between flat lipid bilayers during the lamellar/inverted cubic (L_α–Q_{II}) phase transition of membrane-forming lipids. Fusion pores are also known as ''passages'' (Harbich et al., 1978) or interlamellar attachments (ILAs; Siegel, 1999). Related changes in the integrated Gaussian curvature must also accompany formation of stalks during formation of the recently discovered rhombohedral (R) phase (Yang and Huang, 2002, 2003; Yang et al., 2003) from the L_α phase.

Differences in free energy between such structures have usually been described in terms of the curvature elastic energy of membranes (Helfrich, 1973), with emphasis placed on changes in the bending (e.g., Siegel, 1999; Markin and Albanesi, 2002) or, more generally, splay elastic energy (Hamm and Kozlov, 1998, 2000; Kozlovsky and Kozlov, 2002; Kozlovsky et al., 2002). However, changes in the integrated Gaussian curvature also influence the free energy of fusion or phase transition intermediates. In treatments of intermediate energies, these contributions to the total curvature energy have usually been either neglected (Markin and Albanesi, 2002; Kozlovsky and Kozlov, 2002; Kozlovsky et al., 2002; May, 2002) or treated with simple models (Siegel, 1999). Recent work suggests that the Gaussian curvature elastic energy is one of the major contributions to the energy of fusion stalks for lipids that are near the L_α/inverted hexagonal (H_{II}) phase boundary (e.g., lipid compositions rich in phosphatidylethanolamine), and plays an important role in determining the stability of the R phase (Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov, unpublished results).

One reason that the Gaussian curvature elastic energy has been neglected is that it is proportional to an elastic modulus, $\bar{\kappa}$, that cannot be directly measured. Previously, this modulus was estimated by analysis of the dependence of Q_{II} phase unit cell dimensions on the water content (Turner et al., 1992; Chung and Caffrey, 1994; Templer et al., 1994, 1995,

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Address reprint requests to David Paul Siegel, Givaudan, Inc., 1199 Edison Dr., Cincinnati, OH 45216. Tel.: 513-948-4840; E-mail: david.siegel@givaudan.com.

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1998). This analysis is complex and inaccurate for Q_{II} phases with small unit cell constants (Templer et al., 1995), and can be hampered by the coexistence of different Q_{II} phases (Templer et al., 1998). Here, we use another approach, based on detailed observations of the L_α–Q_{II} phase transition in DOPE-Me (Siegel and Banschbach, 1990; Cherezov et al., 2003). We determine a value of the Gaussian curvature elastic modulus for DOPE-Me monolayers that is within the range expected by theory. To our knowledge, this is the first measurement of this modulus in a lipid system composed only of phospholipids, as opposed to mixtures of phospholipids with monoglycerides or fatty acids or in glycolipids. The results have a substantial impact on the theories of membrane fusion and lamellar/nonlamellar transition mechanisms.

THEORY

Criterion for L_α/Q_{II} phase transition

DOPE-Me has a fairly well-defined L_α–Q_{II} phase transition temperature, T_Q (Siegel and Banschbach, 1990; Cherezov et al., 2003). The chemical potentials for the L_α and Q_{II} phase must be equal at T_Q. The difference in chemical potential can be expressed in terms of the curvature energy of the bilayers in the two phases. We base our consideration on the Helfrich elastic model (Helfrich, 1973). The local membrane shape is determined at each point by two principal curvatures, c_1 and c_2, or alternatively, by the total curvature, $J = c_1 + c_2$ and Gaussian curvature, $K = c_1c_2$. The membrane structure is characterized by the spontaneous curvature, J_s, whereas the membrane elasticity is determined by the bending modulus, κ, and the Gaussian elastic modulus, $\bar{\kappa}$, which is also referred to as the saddle splay modulus. We do not include the effects of hydration energy and consider only systems in excess water.

The curvature elastic energy per unit area of a membrane is given by

$$f = (\kappa/2)(J - J_s)^2 + \bar{\kappa}K. \quad (1)$$

The total curvature energy is obtained by integration of Eq. 1 over the membrane area A.

The elastic model Eq. 1 can be used to calculate the elastic energy of a whole lipid bilayer. In this case the total and Gaussian curvatures describe the bilayer midsurface, whereas the elastic constants characterizing the bilayer are denoted by κ_b, $\bar{\kappa}_b$, and J_s^b. The same model determines the curvature energy of each of the two monolayers. Then it is convenient to relate the curvatures to the so-called neutral surface of the monolayer (Kozlov and Winterhalter, 1991), which is shifted by distance δ from the bilayer midplane toward the lipid-water interface. The elastic characteristics of a monolayer will be denoted by κ_m, $\bar{\kappa}_m$, and J_s^m.

Both the L_α and Q_{II} phases are composed of lipid bilayers, which are assumed to have the same lipid composition in both monolayers. Although each monolayer is characterized by a certain spontaneous curvature $J_s^m \neq 0$, the spontaneous curvature of the bilayer vanishes for symmetry reasons ($J_s^b = 0$). The bilayers of the L_α phases are flat and, consequently, characterized by $J = 0$ and $K = 0$. The Q_{II} phases formed by DOPE-Me (Gruner et al., 1988; Siegel and Banschbach, 1990; Cherezov et al., 2003) have the geometry of infinite periodic minimal surfaces (IPMS): the midplanes of the bilayers lie on surfaces which have, similarly to the L_α phase, a zero total curvature, $J = 0$, at each point. At the same time, and in contrast to the lamellar phase, the Gaussian curvature of the minimal surfaces is different from zero and negative at each point, $K < 0$. Hence, the difference in curvature energy between the two phases, ΔF, is due solely to the Gaussian curvature of the cubic phase and given by

$$\Delta F = F_{cubic} - F_{lamellar} = \bar{\kappa}_b \oint dAK = \bar{\kappa}_b \langle K \rangle A, \quad (2)$$

where $\langle K \rangle$ is the average of the bilayer Gaussian curvature K over the membrane area. There is an additional contribution to the free energy of the Q_{II} phase relative to the L_α phase due to the frustration energy (Anderson et al. 1988). We will neglect this contribution because it is small for the swollen Q_{II} phases, whereas the unit cell is large with respect to the monolayer thickness (Templer et al., 1998).

$\langle K \rangle$ is negative in the Q_{II} phase. Thus, according to Eq. 2, the Q_{II} phase is energetically more favorable than the L_α phase, $\Delta F < 0$, if the Gaussian curvature elastic modulus is positive, $\bar{\kappa}_b > 0$. For $\bar{\kappa}_b < 0$, the L_α phase is energetically more favorable than the Q_{II} phase, $\Delta F > 0$.

The Gaussian curvature modulus of the bilayer can be expressed in terms of the properties of the lipid monolayers. This is important because the monolayer spontaneous curvature and bending elastic modulus of many phospholipids have been measured by x-ray diffraction experiments. It can be shown (Helfrich and Rennschuh, 1990; Ljunggren and Erikkson, 1992; Templer et al., 1994; Schwarz and Gompper, 2001; see also Appendix A) that

$$\bar{\kappa}_b = 2\bar{\kappa}_m - 4\kappa_m J_s^m \delta. \quad (3)$$

The monolayer Gaussian curvature modulus should be negative, $\bar{\kappa}_m < 0$. This is predicted based on the inferred lateral stress profiles as a function of depth within lipid monolayers (Ben-Shaul, 1995) and on the measured positions of pivotal planes within phospholipid monolayers (Templer et al., 1998). Since the monolayer spontaneous curvature is a function of temperature, $J_s^m(T)$ (Tate and Gruner, 1989; Kozlov et al., 1994), the Gaussian curvature modulus of the bilayer, $\bar{\kappa}_b$, also becomes a function of temperature, $\bar{\kappa}_b(T)$. Thus, the temperature T_Q at which IPMS-based Q_{II} phases can form must be the temperature at which the monolayer spontaneous curvature reaches a value that makes the bilayer Gaussian curvature modulus equal to zero (Schwarz and Gompper, 2002),

$$\bar{\kappa}_b(T_Q) = 0. \quad (4)$$

Using Eqs. 3 and 4 and the experimentally found function $J_s^m(T)$, the Gaussian curvature modulus of the monolayers, $\bar{\kappa}_m$, can be determined at the temperature T_Q.

Free energy of fusion pores (ILAs)

An elementary step of L_α–Q_{II} transition must be formation of a bilayer passage between two flat membranes, which is also referred to as fusion pore or ILA (Siegel et al., 1989a; Siegel, 1986, 1999). The energy of these structures can be also determined by Eq. 1. Provided that fusion pores (ILAs) are minimal surface-based structures ($J \rightarrow 0$), with vanishing bending energy ($J_s^b = 0$), ILA formation is determined by the criterion Eq. 4. ILAs were formerly assumed to be circular toroids (Siegel, 1986, 1993, 1999; Nanavati et al., 1992; Chizmadzhev et al., 1995; Kuzmin et al., 2001; see Fig. 1 *A*). However, these structures have lower free energies if the midplanes of the bilayers of the pore lie on an axisymmetric surface with zero mean curvature at all locations (Harbich et al., 1978), referred to as catenoid (do Carmo, 1976). The infinite periodic minimal surface resembling an array of ILAs in a lamellar phase (Thomas et al.,1988) also fulfills this condition and represents one possible shape of a bicontinuous cubic phase. There must be a narrow region of membrane with nonvanishing curvature around the periphery of the catenoid-like ILA, to connect it to the flat bilayers of the L_α phase. It has been shown (Petrov and Kozlov, 1984; Kozlov and Chernomordik, 1998) that such regions can have negligibly small bending energies $\ll k_BT$. Markin and Albanesi (2002) modeled fusion pores as surfaces of constant curvature with J_b approaching zero, which can also have negligible bending energies $\ll k_BT$. An example is shown in Fig. 1

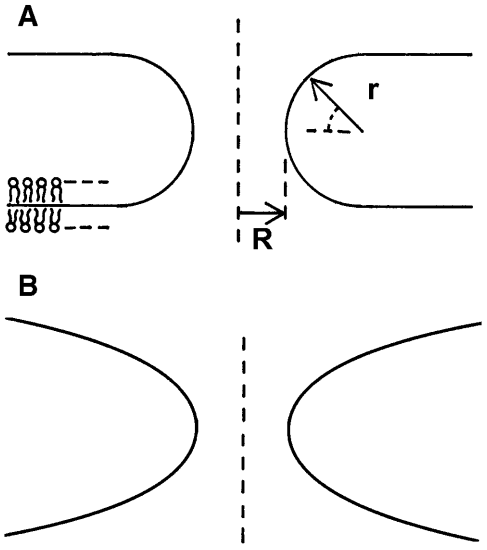

FIGURE 1 Fusion pore structures, seen in cross section in the plane of the cylindrical axes. The curves represent the midplane of the bilayers. (*A*) Fusion pores were formerly modeled as circular toroids (see text), defined by two radii, r and R. (*B*) However, fusion pores probably adopt shapes where the mean curvature of the bilayers is zero everywhere, and the bending energy is minimized. Shown here is a geometry with constant curvature J^b approaching zero, which has a bending energy $< k_BT$ (Markin and Albanesi, 2002).

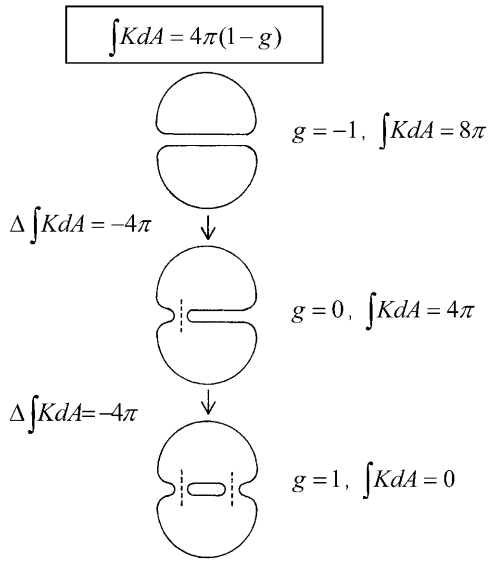

FIGURE 2 The change in the integrated Gaussian curvature accompanying ILA formation. The integrated Gaussian curvature of a closed bilayer structure is given by Eq. 5 (reproduced at the top of the figure). (*Top*) Two closed liposomes each have a genus $g = 0$ and $\int KdA = 4\pi$, for a total $\int KdA = 8\pi$ that is equivalent to genus $g = -1$. (*Middle*) Formation of a single ILA between the two liposomes (the ILAs are shown in cross section, with the axis indicated by *dashed line*). The result is a single liposome, with $g = 0$, and $\int KdA = 4\pi$. (*Bottom*) Formation of another ILA between the same two liposomes results in the topological equivalent of a sphere with one ''handle'' ($g = 1$), with $\int KdA = 0$. For formation of each ILA, the reduction in K of the total system is -4π. This would occur if the ILAs formed between liposomes, as indicated, or between concentric shells of a multilamellar structure (i.e., in MLVs of bulk L_α phase).

B. Since the bending energies of ILAs and the flat bilayers of the L_α phase are practically equal, the energy difference between the two structures is due to the Gaussian curvature.

ILAs have $\langle K\rangle < 0$ (the two principal radii of curvature have opposite signs at all points). According to the Gauss-Bonnet theorem, the integral of the Gaussian curvature of a closed surface over the area, dA, is

$$\int KdA = 4\pi(1-g) = 2\pi\chi, \tag{5}$$

where g is genus and χ is the Euler characteristic of the surface (do Carmo, 1976). The genus is zero, $g = 0$, for a sphere, whereas a torus, which is a sphere with one ''handle,'' has genus $g = 1$, and the genus increases by one for every additional handle (do Carmo, 1976). Formation of an ILA is equivalent to a handle formation ($\Delta g = 1$), so the change in the integrated Gaussian curvature on formation of an ILA is -4π (Eq. 5, Fig. 2). Thus the difference in energy between an ILA and an equivalent area of L_α phase lipid is

$$F_{ILA} = -4\pi\bar{\kappa}_b. \tag{6}$$

Eq. 6 shows that ILAs formation is also favored for $\bar{\kappa}_b > 0$, as is Q_{II} phase formation.

Lamellar-bicontinuous cubic-inverted hexagonal phase diagram

The criterion for Q_{II} phase formation (Eq. 4), accounting for Eq. 3, can be presented in the form

$$J_s^m < \frac{1}{2\delta}\left(\frac{\bar{\kappa}_m}{\kappa_m}\right). \tag{7}$$

At $T = T_Q$, where $F_{ILA} = 0$, and the monolayer spontaneous curvature is $J_s^m(T_Q)$, the ratio of the moduli can be presented as

$$\left.\frac{\bar{\kappa}_m}{\kappa_m}\right|_{T_Q} = 2J_s^m(T_Q)\delta. \tag{8}$$

By comparison, the free energy difference between the H_{II} phase and L_α phase per lipid molecule is (Hamm and Kozlov, 1998)

$$f_H = a[\kappa_t/18 - \kappa_m(J_s^m)^2/2], \tag{9}$$

where κ_t is the chain tilt modulus and a is the area/lipid molecules at the neutral plane. Since the free energy difference must vanish at the L_α–H_{II} phase transition temperature, $f_H|_{T=T_H} = 0$, we can use Eq. 9 to express κ_t through the spontaneous curvature at the transition point, $J_s^m(T_H)$, and present the energy of the H_{II} phase in the form

$$f_H = (a\kappa_m/2)[(J_s^m(T_H))^2 - (J_s^m)^2]. \tag{10}$$

Based on Eq. 10, the criterion of the L_α/H_{II} transition, $f_H \le 0$, is

$$J_s^m < -\frac{\sqrt{\kappa_t/\kappa_m}}{3} = J_s^m(T_H). \tag{11}$$

Thus, using Eqs. 7 and 11, a simple phase diagram can be presented in terms of the ratio $\bar{\kappa}_m/\kappa_m$ and $\delta \cdot J_s^m$ (Fig. 3). Systems with the ratio above the

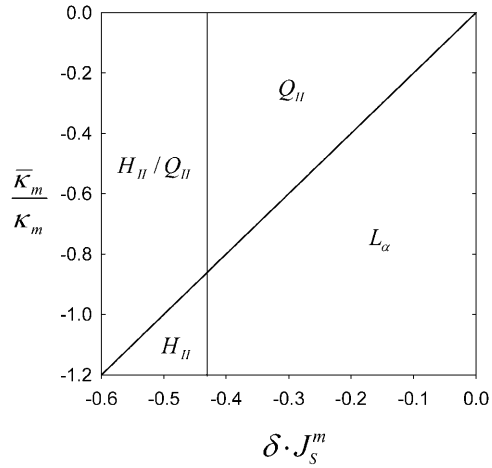

FIGURE 3 Phase diagram showing the phases expected to be most stable as a function of the product δJ_s^m and the ratio of the monolayer Gaussian curvature elastic and bending elastic moduli, $\bar{\kappa}_m/\kappa$. The present model cannot predict which phase will predominate in the H_{II}/Q_{II} region.

slanted line will form Q_{II} phases with decreasing δJ_s^m until the H_{II} phase boundary is encountered. At lower values of J_s^m, either a mixture of H_{II} and Q_{II} phases, or pure H_{II} phase, will be found. Whether or not Q_{II} phase will appear or predominate in the region indicated as H_{II}–Q_{II} (Fig. 3) will depend on the specific values of the unit cell parameters and the space group of the Q_{II} phases that form. More accurate models for ΔF are needed to predict the diagram in this region.

The tilt modulus appears in the expression for H_{II} lipid free energy (Eqs. 9 and 10), and not in the expression for the free energy of Q_{II} phase lipid (Eq. 2). The reason for that is tilt deformation of the hydrocarbon chains, which is generated within the nonbilayer structural defects forming unavoidably in the H_{II} phase and referred to as hydrophobic interstices (Hamm and Kozlov, 1998, 2000). In contrast, the bicontinuous cubic phases (Q_{II}) consist of intact lipid bilayers, which do not include nonbilayer structures and, hence, do not require tilt deformation.

Data from the phase behavior of DOPE-Me and related phospholipids

Several properties of the L_α/Q_{II} phase transition in DOPE-Me, and some properties of related lipids, are relevant to our calculations.

1. With increasing temperature, Q_{II} phase is first observed in a narrow band of temperature at ~60°C (Siegel and Banschbach, 1990; Cherezov et al., 2003). Careful observations indicate that it first forms in DOPE-Me at 55°C, although only after a delay of several hours (Cherezov et al., 2003). The transition is very slow in the heating direction, with considerable scan rate dependence, and is extremely hysteretic in the cooling direction. H_{II} phase first forms (after long incubations at constant temperature) at ~61°C.
2. ILAs have been observed in DOPE-Me at temperatures below T_Q, via electron microscopy and ^{31}P NMR; most of the time via NMR. This is often reported to start at ~40°C (Gagné et al., 1985; Ellens et al., 1989; Siegel et al., 1989a,b; Yeagle et al., 1991; Epand et al., 1994), although it is occasionally observed starting at temperatures of 50°C or above (van Gorkum et al., 1992; Epand and Epand, 1994; Davies et al., 1998). However, the isotropic resonance characteristic of ILAs and Q_{II} phase can be detected when it constitutes as little as 1% of the total resonance intensity (Ellens et al., 1989; Yeagle et al., 1991). Typically <10% of the intensity is present in the isotropic component when the temperature reaches 50°C (Yeagle et al., 1991). Therefore, the temperature at which the free energy of lipid in ILAs and flat bilayers is equal, where we would expect coexistence of the two types of structure, is probably close to the observed value of T_Q of 55°C.
3. The lattice parameter of the first Q_{II} phases to form in DOPE-Me is on the order of 30 nm (Cherezov et al., 2003), and the first Q_{II} phase to form is almost always Q_{II} (Pn3m). The large lattice parameter is significant because chain stretching contributions to the free energy of minimal surface-based ILAs and the Q_{II} phase should be negligible under these circumstances (Schwarz and Gompper, 2001), and higher-order Gaussian curvature effects are likely to be small. Therefore, the model we use here should give a reasonably accurate account of the threshold value of J_s^m for ILA and Q_{II} phase formation.
4. J_s is measured by x-ray diffraction experiments on H_{II} phases as a function of osmotic pressure. This has not been done for DOPE-Me, although J_s^m has been measured at one temperature for DOPE and a DOPE/DOPC mixture (Rand et al., 1990; Leikin et al., 1996; Chen and Rand, 1997). J_s^m values can be obtained for DOPE-Me if we assume that the distance between the neutral plane and the periphery of the H_{II} tube is the same for DOPE-Me as in these two lipid systems, which have similar lipid headgroups and the same acyl chains. Then J_s^m of DOPE-Me in excess water can be obtained as a function of temperature using measurements of the lattice parameter of the H_{II} phase of DOPE-Me in the presence of dodecane (Gruner et al., 1988). Values of J_s^m obtained in this manner are given in Table 1.
5. The value of κ_m has not been determined for DOPE-Me, but it has been measured for DOPE and for DOPC at 22°C (Chen and Rand, 1997) as 11 and 9 k_BT, respectively, where k_B is Boltzmann's constant. Since DOPC is triply methylated DOPE, we assume that the value for DOPE-Me is intermediate between the values for DOPE and DOPC, and use the value 10 k_BT.
6. We assume that the neutral plane is near the interface between the hydrophilic and hydrophobic regions of the monolayer. Using data from Rand and Parsegian (1989) for monomethylated egg PE, we estimate $\delta = 1.3$ nm. This value is uncertain to within ~10%.

The Gaussian curvature elastic modulus of DOPE-Me

With T_Q for DOPE-Me = 55°C, we find from Table 1 that $J_s^m(T_Q) = -0.322\,\text{nm}^{-1}$. With $\delta = 1.3$ nm, we find from Eq. 8 that

$$\left.\frac{\bar{\kappa}_m}{\kappa_m}\right|_{T_Q} = -0.64\delta = -0.83 \pm 0.08, \qquad (12)$$

where the uncertainty is due to the uncertainty in δ ($\pm$10%). In comparison, a 10° error in T_Q changes the ratio of the constants by only 4–5%. With $\kappa_m = 10\,k_BT$, $\bar{\kappa}_m = 8.3\,k_BT$. It should be noted that this value is measured

TABLE 1 Values of spontaneous curvature for DOPE-Me at relevant temperatures

Temperature (°C)	J_s^m (nm^{-1})
50	−0.316
55	−0.322
60	−0.328
65	−0.334
70	−0.341
75	−0.347

Temperatures were calculated using data from Gruner et al. (1988), as described in the text. J_s was calculated at temperatures between 20 and 75°C, and can be fitted with the equation $J_s^m(T) = -0.3583 - 0.001628\,(T-75) + 3.5632 \times 10^{-6}\,(T-20)^2$, where T is in °C. The error with respect to the derived values is 0.3% or less, which is within the error of the measurements themselves.

at $T = T_Q \approx 55°C$. The temperature dependence of $\bar{\kappa}_m/\kappa_m$ (i.e., the value at temperatures far from T_Q) is unknown.

Free energy of Q_{II} phase as a function of J_s and temperature

The free energy of Q_{II} phases is a complicated function of the unit cell parameter c, details of the geometry of the IPMS that is the basis of the particular Q_{II} phase, and the lipid volume fraction, as well as J_s^m and the values of the elastic constants (Schwarz and Gompper, 2001, 2002). A detailed analysis of the temperature-dependent free energy of the Q_{II} phase in DOPE-Me is beyond the scope of this work. However, we would like to estimate the free energy of lipid in the first Q_{II} phase to form as a function of decreasing J_s^m. The first Q_{II} phase to form in DOPE-Me during temperature-scan or constant-temperature incubation experiments is almost always the Pn3m phase, and c is initially 30 nm or more (Cherezov et al., 2003). The c values generally decrease rapidly after formation (e.g., in one case, c decreases from almost 35 nm to 28 nm in 20 min, and then to 26 nm over the next 1.5 h). The equilibrium values of c are smaller than the initial values observed at any temperature, and the equilibrium values fall rapidly with increasing temperature. For the large value of c corresponding to the initial lattice, the density of Gaussian curvature will be comparatively low. Accordingly, using Eqs. 2 and 5, we estimate the free energy f_Q per lipid molecule in this initial lattice relative to the L_α phase,

$$f_Q \approx (a/2)\bar{\kappa}_b\left(\frac{2\pi\chi_u}{A^{*}c^{2}}\right), \tag{13}$$

where a is the area per lipid molecules at the neutral plane; χ_u and A^* are the Euler characteristic of the unit cell, and the surface area of the unit cell (for unit lattice parameter), respectively, of the IPMS that is the structural basis of the Q_{II}. For Q_{II}–Pn3m, this is the D surface, for which $A^* = 3.838$ and $\chi_u = -16$ (Schwarz and Gompper, 2001). The term inside the brackets is the area average of the Gaussian curvature in the Q_{II}–Pn3m phase for which $\chi_u < 0$. Eq. 13 cannot describe the free energy of the equilibrium phase, because it indicates that c would shrink indefinitely. The rapid decrease of c after Q_{II} phase formation is as expected because, initially, $\bar{\kappa}_b$ must be positive. However, c stabilizes at an equilibrium value. The increase of the frustration elastic energy (Anderson et al., 1988) at small c is, probably, the factor that halts the collapse of the unit cell in practice (Schwarz and Gompper, 2002). Using $\kappa_m = 10\ k_BT$ and $\bar{\kappa}_m = 8.3\ k_BT$, the free energy of an ILA in DOPE-Me, F_{ILA} (Eq. 6), is plotted as a function of J_s in Fig. 4. The values of J_s^m corresponding to T_Q and $T_Q \pm 10°C$ are indicated, to emphasize the slow change in F_{ILA} with temperature in the lamellar/nonlamellar phase transition interval. The values f_Q (Eq. 13) and f_H (Eq. 10) are plotted as a function of J_s^m in Fig. 5. The rate of change with temperature of f_Q is smaller than for f_H.

DISCUSSION

The value of $\bar{\kappa}_m/\kappa_m$ derived here from T_Q is in accord with theoretical expectations. The value required for stability of lamellar and Q_{II} phases is $-2 < \bar{\kappa}_m/\kappa_m < 0$ (Schwarz and Gompper, 2001, 2002), and it has been argued that there is the further restriction $-1 < \bar{\kappa}_m/\kappa_m$ (Templer et al., 1998). The value of $\bar{\kappa}_m/\kappa_m$ determined here for DOPE-Me, -0.83 ± 0.08, is also comparable to the value for DOPE estimated from studies of R phase stability (Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov, unpublished results). It is also close to the value of -0.75 ± 0.08 calculated from a study of the swelling of a Q_{II} phase of monoolein/DOPC/DOPE = 58/38/4 (Templer et al., 1998). To our knowledge, the value determined in this study is the first measurement in a system consisting only of phospholipid.

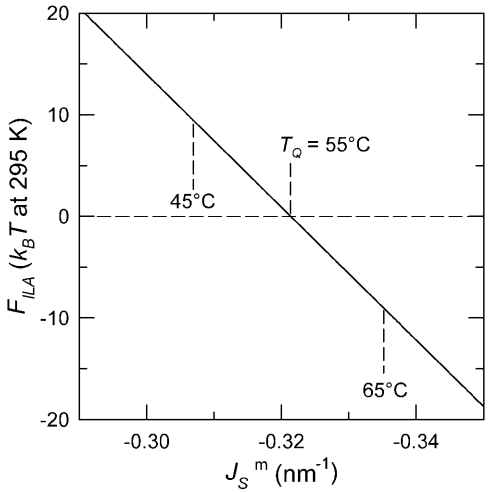

FIGURE 4 Plot of the free energy of an ILA with respect to an equivalent amount of L_α phase lipid as a function of J_s^m. The values of J_s^m corresponding to temperatures 10 K above and below T_Q are indicated by dashed vertical lines. The unit of energy is k_BT evaluated at 295 K.

The present analysis is possible because DOPE-Me, a single lipid component system, has a well-defined T_Q. To our knowledge, T_Q values have not been reported for other phospholipids. Multicomponent lipid systems that form Q_{II} phases would be difficult to analyze in this way because of possible differences in lipid composition of the two phases at the transition temperature.

Is the value of $\bar{\kappa}_m/\kappa_m$ determined here relevant to biomembrane lipids? PE is the most abundant inverted phase-forming lipid in mammalian cell membranes. The values of $\bar{\kappa}_m/\kappa_m$ for PEs, PE-rich mixtures of PE and PC, and PE/PC/

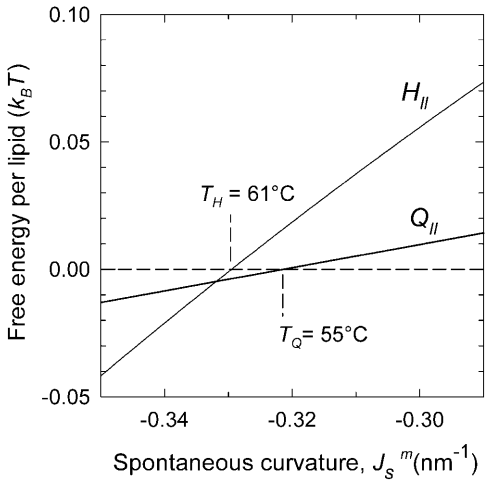

FIGURE 5 Comparison of the driving force for formation of the initial Q_{II} phase lattice and bulk H_{II} phase as a function of J_s^m. The J_s^m values corresponding to T_Q and T_H are indicated. The driving force for H_{II} phase formation increases with decreasing J_s^m (increasing T) much faster than the Q_{II} phase.

cholesterol mixtures should be similar to the value determined here for DOPE-Me. PE/PC mixtures form ILAs at temperatures 10–15° below T_H (e.g., up to a DOPC/DOPE ratio of 1:2 mol/mol; Ellens et al., 1989). Mixtures of DOPE, DOPC, and cholesterol form ILAs and/or Q_{II} phases below T_H across a wide range of PE/PC ratio (Tilcock et al., 1982). This is the same behavior observed in DOPE-Me. As with DOPE-Me, this means that $T_Q \approx T_H$. Hence $\bar{\kappa}_m/\kappa_m$ should have approximately the same value as for DOPE-Me. Pure PEs (DOPE, DiPoPE, and DEPE) do not form Q_{II} phase upon heating through T_H, but they do form Q_{II} phases when the temperature is cycled repeatedly between temperatures ~10°C below and above T_H (Shymasunder et al., 1988; Erbes et al., 1994; Tenchov et al., 1998). This implies that T_Q is greater than T_H, but not much greater. Using Eqs. 8, 10 and 11, the expected value for $\bar{\kappa}_m/\kappa_m$ when $T_Q = T_H$ is

$$\left.\frac{\bar{\kappa}_m}{\kappa_m}\right|_{T_Q=T_H} = 2\delta J_s(T_H) = -\frac{2\delta\sqrt{\kappa_t/\kappa_m}}{3}. \qquad (14)$$

Using $J_s^m(T_H) = \sim -1/3\,\mathrm{nm}^{-1}$ for PEs near T_H (e.g., Gruner et al., 1988; Tate and Gruner, 1989), or alternatively $\kappa_t = 0.04$ N/m (Hamm and Kozlov, 1998) and $\kappa_m = 4 \times 10^{-20}$ J, we estimate the value of the ratio for pure PEs to be

$$\left.\frac{\bar{\kappa}_m}{\kappa_m}\right|_{T_Q=T_H} \approx -0.9. \qquad (15)$$

Note that for any given PE, Eq. 15 is strictly valid only at $T_Q \approx T_H$. The value in Eq. 15 is more negative than the range −0.6 to −0.3 predicted by Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov (unpublished results) for DOPE at room temperature. This may be due partly to the temperature dependence of $\bar{\kappa}_m/\kappa_m$. The T_H of DOPE (3°C; Toombes et al., 2002) is substantially different from room temperature. It is not known how rapidly $\bar{\kappa}_m/\kappa_m$ changes with temperature away from T_Q. Also, the fact that κ_m has most often been measured near room temperature makes it difficult to estimate $\bar{\kappa}_m$ itself via Eq. 15 for lipids with T_Q far away from room temperature.

Eq. 15 shows that the stability of Q_{II} phases is in sensitive balance in PEs, their derivatives, and their mixtures with PC and PC/cholesterol. For example, if a change in system composition slightly increases $\bar{\kappa}_m/\kappa_m$ in a pure PE, Q_{II} phases may appear spontaneously where none did before. The value $\bar{\kappa}_m$ is the second moment of the lateral pressure profile in the lipid monolayer (Helfrich, 1990). As such, it might be very sensitive to small changes in the effective volume of, or interactions between, lipid (or lipid and peptide) moieties at different planes in the monolayer. This might explain the surprisingly large effect of hydrophobic or transmembrane peptides on Q_{II} phase stability (Keller et al., 1996; Morein et al., 2000; Liu et al., 2001; D. P. Siegel, V. Cherezov, D. V. Greathouse, R. E. Koeppe, J. A. Killian, and M. Caffrey, unpublished observations). It might also explain the effects of aqueous solutes on the kinetics of Q_{II} phase formation in temperature-cycled DEPE (Tenchov et al., 1998). The lipid compositions of some biomembranes may be close to the L_α–Q_{II} phase boundary. For example, cholesterol, a ubiquitous membrane constituent, induces Q_{II} phases in POPE (Wang and Quinn, 2002). Moreover, recently it has been found that mixtures of PC and cholesterol (i.e., with no inverted phase-preferring lipid) form numerous ILAs at ~40°C, as determined via ^{31}P NMR (Epand et al., 2003, 2004).

Implications for membrane fusion intermediates

The Gaussian curvature energy term in Eqs. A8 and A9 has usually been neglected in computing the energies of fusion intermediates on the basis of the monolayer curvature elastic energies (Siegel, 1999; Kozlovsky and Kozlov, 2002; Kozlovsky et al., 2002; Markin and Albanesi, 2002). However, recent work on the stability of the stalk phase has shown that Gaussian curvature elastic energies can make substantial contributions to stalk stability (Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov, unpublished results). The value measured here for a phospholipid is comparable to the value of bending modulus, κ_m, and confirms that this is true. The Gaussian curvature elastic energy has a very substantial affect on the stability of fusion pores. Previous authors (Siegel, 1993, 1999; Markin and Albanesi, 2002) calculated the energy of the fusion pore on a monolayer basis (Eqs. A8 and A9) with the assumption that $\bar{\kappa}_m = 0$. For a fusion pore with minimal curvature (zero bilayer curvature; $J^b = 0$), comparison with Eqs. 1, 3, A8, and A9 show that this approach underestimates the energy by $-8\pi\bar{\kappa}_m$, or 200 k_BT, if we use the value of $\bar{\kappa}_m$ for DOPE-Me. Thus, fusion pores are much higher in energy than estimated solely on the basis of the monolayer bending elastic energy. The data in Fig. 4 show that phospholipid fusion pores can be higher in energy than an equal amount of lamellar phase lipid at physiological temperatures, depending on the values of the elastic moduli and the monolayer spontaneous curvature J_s^m. An important function of fusion proteins in biomembranes may be to stabilize nascent fusion pores. This is an activity inferred by others from recent experimental studies of viral fusion proteins (Markosyan et al., 2000). It has been proposed that protein coats of viruses act cooperatively to stabilize an expanding fusion pore (Kozlov and Chernomordik, 2002). The transmembrane domains of fusion-catalyzing proteins may play a similar role, based on their ability to lower T_Q. The measurement of $\bar{\kappa}_m$ in the present work emphasizes the potential importance of such an effect.

Implications for the mechanism of transitions between L_α, Q_{II}, and H_{II} phases

Siegel and Epand (1997) and Siegel (1999) proposed a stalk-based mechanism for the transitions between lamellar and

nonlamellar phases. The theory neglected the effects of the Gaussian curvature energy, and hence estimated ILA energies that are far too low. This resulted in a prediction that ILAs should be thermodynamically stable in the temperature interval between the chain-melting (gel/liquid crystalline transition) temperature, T_m, and some temperature $>T_H$. Siegel (1999) did estimate the size of the effect of Gaussian curvature energy on ILA stability, but used a value estimated for monoglycerides by Chung and Caffrey (1994) from studies of Q_{II} phase swelling in water. The procedure used to calculate this value of $\bar{\kappa}_m$ has been questioned (Templer et al., 1995), and the value is much smaller than the present value for DOPE-Me. Siegel (1999) proposed that ILAs, and perhaps Q_{II} phase, did not form at temperatures far below T_H because some other factor, like the membrane rupture tension, inhibited ILA formation under these circumstances. In view of the large value of $\bar{\kappa}_m$ measure here, a better explanation is that ILAs do not become thermodynamically stable until the temperature reaches the vicinity of T_H in DOPE-Me. Presumably, small numbers of ILAs can form at temperatures as low as 40°C or 50°C because the ILA free energy is within 13 k_BT or 4 k_BT, respectively, of the free energy of an equivalent amount of L_α phase lipid (Fig. 4). This may also be another reason why temperature cycling through T_H has been observed to produce Q_{II} phases in PEs that do not form them during temperature ramp experiments (Shyamsunder et al.; 1988; Veiro et al., 1990; Tenchov et al., 1998). According to Eqs. 3 and 6, ILAs may not be stable at J_s^m values corresponding to temperatures lower than T_H, but a few accumulate each time the temperature exceeds T_H. These persist below T_H because ILAs are kinetically trapped, and are present unless the lipid composition is frozen below the main transition temperature T_m (Cullis et al., 1978; Ellens et al., 1986, 1989).

In DOPE-Me, T_Q and T_H are close together (Siegel and Banschbach, 1990; Cherezov et al., 2003). The rates of the L_α–H_{II} and L_α–Q_{II} phase transitions are in kinetic competition in the temperature interval above T_Q (Cherezov et al., 2003), with the rate of H_{II} phase formation predominating at higher temperatures. Cherezov et al. (2003) explained this in terms of the transition mechanism. The H_{II} phase nucleates from clusters of ILA precursors, which subsequently grow rapidly by diffusion. In contrast, the Q_{II} phase accrues by formation of individual ILAs and slow subsequent arrangement of these structures into arrays and then Q_{II} phase. This is still a reasonable explanation. The data in Fig. 5, however, provide an additional explanation for the preponderance of the H_{II} phase formation rate at higher temperatures: the thermodynamic driving force for H_{II} phase formation increases more rapidly than the driving force for formation of the initial Q_{II} lattice in this temperature interval. Note that the free energy f_Q (Eq. 13) plotted in Fig. 5 refers to the initial Q_{II} phase lattice. The free energy of the equilibrium Q_{II} phase, which has a smaller lattice constant (Cherezov et al., 2003), may be quite different. It remains to be seen why the Q_{II} lattice constant is initially so much larger than the equilibrium values.

Siegel and Epand (1997) and Siegel (1999) proposed that transitions between L_α, Q_{II}, and H_{II} phases occur via formation of stalks and transmonolayer contacts (TMCs). The latter structures are similar to the early stages of growth of the hemifusion diaphragms described by Kozlovsky et al. (2002). Siegel (1999) pointed out that TMCs appeared to have too high an energy to form in large enough numbers to mediate the lamellar/nonlamellar phase transitions. The work of Kozlovsky et al. (2002) shows that the bending and interstitial energies of such structures are much lower than calculated by Siegel (1999); so much so that they would have been thermodynamically stable under conditions where no such phases are observed. The large negative value of $\bar{\kappa}_m$ determined here increases the predicted energies of these structures to values roughly consistent with both the observations of rhombohedral phase formation in phospholipids (Y. Kozlovsky, D. P. Siegel, and M. M. Kozlov, unpublished results) and with observed rates of membrane fusion. We are approaching an understanding of the energetics of lipid intermediates in membrane fusion and lipid phase transitions.

APPENDIX A

Let us assume that the bilayer is symmetric, i.e., each monolayer has the same spontaneous curvature, J_s^m. The bending modulus of a monolayer is κ_m and the Gaussian modulus of a monolayer is $\bar{\kappa}_m$. We describe the bilayer in terms of the area A of its midplane. The mean and Gaussian curvatures of the bilayer midplane are denoted by J and K, respectively. The neutral surface of each of the monolayers (Kozlov and Winterhalter, 1991) is shifted by a distance δ from the bilayer midplane.

The areas of the neutral surfaces of the outer and inner monolayers corresponding to the area A of the bilayer midplane are (do Carmo, 1976)

$$A_{out} = A(1 + J\delta + K\delta^2) \tag{A1}$$

and

$$A_{in} = A(1 - J\delta + K\delta^2). \tag{A2}$$

The total and Gaussian curvatures of the two monolayers are (do Carmo, 1976)

$$J_{out} = \frac{J + 2K\delta}{1 + J\delta + K\delta^2} \tag{A3}$$

$$K_{out} = \frac{K}{1 + J\delta + K\delta^2} \tag{A4}$$

$$J_{in} = -\frac{J - 2K\delta}{1 - J\delta + K\delta^2} \tag{A5}$$

$$K_{in} = \frac{K}{1 - J\delta + K\delta^2}. \tag{A6}$$

The energy of the bilayer in terms of the bending energy of the constituent monolayers is

$$f_b A = f_{out}A_{out} + f_{in}A_{in}, \tag{A7}$$

where f_{out} and f_{in} are the curvature energies of the outer and inner monolayers, respectively, and

$$f_{out} = \frac{1}{2}\kappa_m(J_{out} - J_s^m)^2 + \bar{\kappa}_m K_{out} \quad \text{(A8)}$$

$$f_{in} = \frac{1}{2}\kappa_m(J_{in} - J_s^m)^2 + \bar{\kappa}_m K_{in} \quad \text{(A9)}$$

where κ_m and $\bar{\kappa}_m$ are the bending elastic modulus and Gaussian curvature modulus, respectively, of the lipid monolayers. We insert Eqs. A1–A6 into Eqs. A7–A9. If we make the approximation that

$$(1 + J\delta + K\delta^2) \approx (1 - J\delta + K\delta^2) \approx 1 \quad \text{(A10)}$$

and retain terms only up to the first power of δ, we obtain

$$f_b = \frac{1}{2}\kappa_b J^2 + \bar{\kappa}_b K, \quad \text{(A11)}$$

where the bilayer elastic coefficients are

$$\kappa_b = 2\kappa_m \quad \text{(A12)}$$

$$\bar{\kappa}_b = 2\bar{\kappa}_m - 4\kappa_m J_s^m \delta. \quad \text{(A13)}$$

In deriving Eqs. A11–A13, the bilayer spontaneous curvature vanishes, $J_s^b = 0$, because the spontaneous curvatures of the monolayers are equal. The bilayer bending modulus is twice that of the monolayer (assuming that the monolayers can slide with respect to each other). Retaining terms with δ^2 in Eq. A11 produces a term $= 4\kappa_m\ \delta^2 K^2$. Retaining this term has a negligible effect on the calculated value of $\bar{\kappa}_m$. Eq. A10 is correct to within 3% for the Q_{II} phases dealt with in the present work.

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