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Thermodynamics of Phospholipid Self-Assembly

Derek Marsh*
Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany

ABSTRACT Negatively charged phospholipids are an important component of biological membranes. The thermodynamic parameters governing self-assembly of anionic phospholipids are deduced here from isothermal titration calorimetry. Heats of demicellization were determined for dioctanoyl phosphatidylglycerol (PG) and phosphatidylserine (PS) at different ionic strengths, and for dioctanoyl phosphatidic acid at different pH values. The large heat capacity ($\Delta C^o_P \sim -400$ J.mol^{-1} K^{-1} for PG and PS), and zero enthalpy at a characteristic temperature near the physiological range ($T^* \sim 300$ K for PG and PS), demonstrate that the driving force for self-assembly is the hydrophobic effect. The pH and ionic-strength dependences indicate that the principal electrostatic contribution to self-assembly comes from the entropy associated with the electrostatic double layer, in agreement with theoretical predictions. These measurements help define the thermodynamic effects of anionic lipids on biomembrane stability.

INTRODUCTION

The driving force for self-assembly of all biological membranes is the hydrophobic effect, which is contributed by the aliphatic lipid chains. The polar headgroups of diacyl phospholipids (or other membrane-forming lipids) are then responsible for the stability of the bilayer membrane as a self-assembled nanostructure. A zwitterionic amphiphilic lipid, such as phosphatidylcholine or phosphatidylethanolamine, is sufficient to satisfy the principle of opposing forces (1) that is needed for stable membrane equilibrium. Indeed, such lipids constitute the basic building blocks of many biological membranes. Nonetheless, biomembranes are known to contain a significant proportion of negatively charged lipids, which can contribute in several different ways to membrane function.

Fundamental biophysical characterization of membrane stability requires measuring the thermodynamic variables that govern the transfer of lipids from water to their membrane or micellar assembly. The equilibrium constant, or so-called critical micelle concentration (CMC), provides the standard free energy of transfer, ΔG^o_{tr} (see, e.g., Cevc and Marsh (2)). However, it is difficult to determine the enthalpy of transfer, ΔH^o_{tr}, from the van't Hoff relation because of the anomalous temperature dependence associated with the hydrophobic effect (3,4). Fortunately, isothermal titration calorimetry (ITC) can be used to determine ΔH^o_{tr} directly from the heat of demicellization (5,6). ITC has already been used for a thorough investigation of the chain-length dependence and hydrophobic contributions to self-assembly with both diacyl and lyso phosphatidylcholines (7). So far, however, such studies have not been made with negatively charged phospholipids.

The CMCs of spin-labeled anionic phospholipids determined by electron paramagnetic resonance techniques depend strongly on ionic strength and, in the case of phosphatidic acid, also on pH (4). Electrostatic interactions therefore can play an important role in the thermodynamics of self-assembly for charged phospholipids. Here, I use ITC measurements to determine the thermodynamic parameters, enthalpy, free energy, entropy, and heat capacity, for the transfer of negatively charged phospholipids from water to micellar aggregates. Ionic strength dependence and pH titration are used to dissect out the contributions from electrostatics to the thermodynamics of self-assembly. Calculations with diffuse double-layer theory serve to identify the predominantly entropy-driven electrostatic contribution with the distribution of ions in the charged double layer.

MATERIALS AND METHODS

Phospholipids

1,2-Dioctanoyl-*sn*-glycero-3-phosphoglycerol ($(8:0)_2$PG), 1,2-dioctanoyl-*sn*-glycero-3-phosphoserine ($(8:0)_2$PS), and 1,2-dioctanoyl-*sn*-glycero-3-phosphoric acid ($(8:0)_2$PA) were obtained from Avanti Polar Lipids (Alabaster, AL). Isothermal titration calorimetry (ITC) was undertaken using an iTC$_{200}$ calorimeter with a cell volume of $V_c = 207.8$ μL (MicroCal, Piscataway, NJ). Demicellization titrations were performed by injecting aliquots of concentrated phospholipid dispersion into the cell containing only buffer. Concentrations and aliquot volumes were chosen so as to remain within the calibrated power range of the calorimeter. The total concentration of lipid in the cell, C_i, after i injections of volume Δv is

$$C_i = \left(\frac{V_C}{V_C + \Delta v}\right)^i C_o + \left(\frac{c_S \Delta v}{V_C + \Delta v}\right) \cdot \frac{1 - \left(\frac{V_C}{V_C + \Delta v}\right)^i}{1 + \left(\frac{V_C}{V_C + \Delta v}\right)}, \quad (1)$$

where c_S is the concentration of lipid in the injecting syringe, and C_o is the concentration of lipid in the cell before the first full injection. The latter is

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*Correspondence: dmarsh@gwdg.de

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included to correct for any initial injections that do not register the full enthalpy of micellization; i is then counted from the first injection that achieves the full enthalpy (see Results). Except for phosphatidic acid, the buffer used for dispersing the lipid was 20 mM HEPES, 1 mM EDTA, pH 7.4. For phosphatidic acid, 20 mM acetate or HEPES buffers were used for pH 5.0 and pH 8.2, respectively.

THEORETICAL BACKGROUND

Demicellization titrations

The shape of the titration curve in an ITC experiment is determined by the rate of change of the monomer concentration, dc_{mon}/dc_T (or equivalently of the micelle concentration), with total lipid concentration, c_T, in the measurement cell.

At the initial injection, all micelles dissociate on dilution, and the full enthalpy of demicellization, ΔH_o, per mole of injected lipid is detected. As the titration proceeds, only part of the injected micelle population dissociates and a lower enthalpy, ΔH, per injection is recorded. This is given by

$$\Delta H = \Delta H_o\left(\frac{dc_{mon}}{dc_T}\right). \quad (2)$$

The heat of dilution, ΔH_{dil}, of the micelles is neglected, because ΔH tends to a limiting value of zero at the end of the titration when none of the injected micelles dissociate. The quantity in brackets is the fraction of injected micelles that dissociates into monomers. Integration of Eq. 2 yields the concentration of monomers, c_{mon}, in the measurement cell during the course of the titration:

$$c_{mon} = \int_0^{c_T}\left(\frac{\Delta H}{\Delta H_o}\right)\cdot dc_T. \quad (3)$$

Correspondingly, the concentration of lipid in micellar form at total lipid concentration c_T is given by

$$c_{mic} = c_T - c_{mon}, \quad (4)$$

which is obtained immediately from Eq. 3.

A complication arises because a fraction, CMC/c_s, of the total lipid in the syringe is already in the monomer form before injection, where CMC is the critical micelle concentration and c_s is the total lipid concentration in the syringe. Therefore, the intrinsic enthalpy of demicellization is related to the initially measured quantity, ΔH_o, by

$$\Delta H_{demic} = \frac{\Delta H_o - \Delta H_{dil}}{(1 - CMC/c_s)}. \quad (5)$$

At the beginning of the titration, when micelles are not present in the measurement cell, this monomer contribution also should be allowed for in Eqs. 2 and 3. But this is not the case toward the end of the titration, when micelles are present in the measurement cell and the monomer concentration in the cell remains almost constant. Equations 2 and 3 are then valid without correction. In practice, this effect can be allowed for approximately by optimizing the value of ΔH_o during the fitting of the titration curve.

Self-assembly

The standard free energy of transfer of a lipid monomer from water into a micelle of size m is given by (see, e.g., Cevc and Marsh (2))

$$\Delta G^o_{tr,m} = \mu^o_{mic,m} - \mu^o_w = RT\ln X_w - \frac{RT}{m}\ln\left(\frac{X_m}{m}\right), \quad (6)$$

where X_w and X_m are the mole fractions of lipid (with respect to water) in the monomer and micellar states, respectively. The mole fraction in the micellar form is therefore given by

$$X_m = mX_w^m \exp\left(\frac{-m\left(\mu^o_{mic,m} - \mu^o_w\right)}{RT}\right) = m(K_mX_w)^m, \quad (7)$$

where $\mu^o_{mic,m} - \mu^o_w \equiv -RT\ln K_m$. The total mole fraction of lipid that is in the micellar form is given by

$$X_{mic} = \sum_m X_m = \sum_m m(K_mX_w)^m. \quad (8)$$

The distribution of micelle sizes is determined by the m-dependence of the standard chemical potential, $\mu^o_{mic,m}$ (or equivalently K_m), and is expected to have a maximum that corresponds to the optimum area per molecule at the micelle surface, subject to molecular packing considerations (2).

The simplest assumption is that the micelles have a unique size, m, in which case the mole fraction of micelles is given by Eq. 7, and the total mole fraction of lipid can be expressed in terms of the mole fraction of monomers,

$$X_T = X_{mic} + X_w = m(KX_w)^m + X_w, \quad (9)$$

where K is then the unique value of the equilibrium constant, K_m, corresponding to a fixed micelle size. Equation 9 is used for nonlinear least-squares fitting of integrated demicellization curves that are obtained from Eq. 3, where X_w (or equivalently, the monomer concentration, c_{mon}) is viewed as the independent variable.

Temperature dependence

The enthalpy of transfer, ΔH^o_{tr}, of a lipid monomer into the micellar assembly is associated with a large heat capacity, ΔC^o_P, which is characteristic of the hydrophobic effect, and passes through zero at temperature T^*:

$$\Delta H^o_{tr}(T) = \Delta C^o_P(T - T^*). \quad (10)$$

Typically T^* is in the accessible range, and at this temperature the free energy of transfer is purely entropic. With the same reference temperature, the entropy of transfer ($\mathrm{d}\Delta S^o_{tr} = \Delta C^o_P \mathrm{d}T/T$) is

$$\Delta S^o_{tr}(T) = \Delta S^* + \Delta C^o_P \ln\left(\frac{T}{T^*}\right), \quad (11)$$

where ΔS^* is the standard entropy of transfer at temperature T^*. From Eqs. 10 and 11, the free energy of transfer of a monomer into the micelle is then

$$\Delta G^o_{tr}(T) = \Delta C^o_P(T - T^*) - T\Delta S^* - \Delta C^o_P T \ln\left(\frac{T}{T^*}\right). \quad (12)$$

For large micelles, or extended bilayers (i.e., $m \to \infty$), the free energy of transfer is related to the critical micellar concentration, CMC, by

$$\Delta G^o_{tr} = RT \ln X_{CMC}, \quad (13)$$

where X_{CMC} is the critical micelle concentration of the lipid monomer, in mole fraction units with respect to water (compare with Eq. 6).

The heat of demicellization, per monomer, (ΔH_{demic}, Eq. 5) that is measured in an ITC experiment is $-\Delta H^o_{tr}$. The entropy of transfer, ΔS^o_{tr}, is then obtained from the standard expression for the Gibbs free energy:

$$\Delta G^o_{tr}(T) = \Delta H^o_{tr}(T) - T\Delta S^o_{tr}(T). \quad (14)$$

RESULTS

Thermodynamics of demicellization

The upper panel of Fig. 1 shows the raw data from an ITC experiment with phosphatidylglycerol micelles. Aliquots of 1 μL are injected from a syringe containing 10 mM $(8:0)_2$PG in 20 mM HEPES, 0.15 M NaCl, 1 mM EDTA, pH 7.4 into the measurement cell, which contains the same buffer without lipid. The full enthalpy of demicellization is not achieved in the first injection because of possible surface adsorption of the lipid, and/or dilution effects in the syringe needle. This is allowed for in analyzing the titration by scaling the amount of lipid added in the initial injections by the ratio of the reduced ΔH to the full value ΔH_o that is established subsequently (see Eq. 1).

The lower panel of Fig. 1 gives the integrated heat, ΔH, per mole of injectant for each injection, as a function of the total lipid concentration, c_T, in the measurement cell. At 50°C, heat is evolved and the demicellization process is exothermic, whereas the process is endothermic at the lower temperature of 5°C. This behavior, and the large associated heat capacity, characterizes the hydrophobic nature

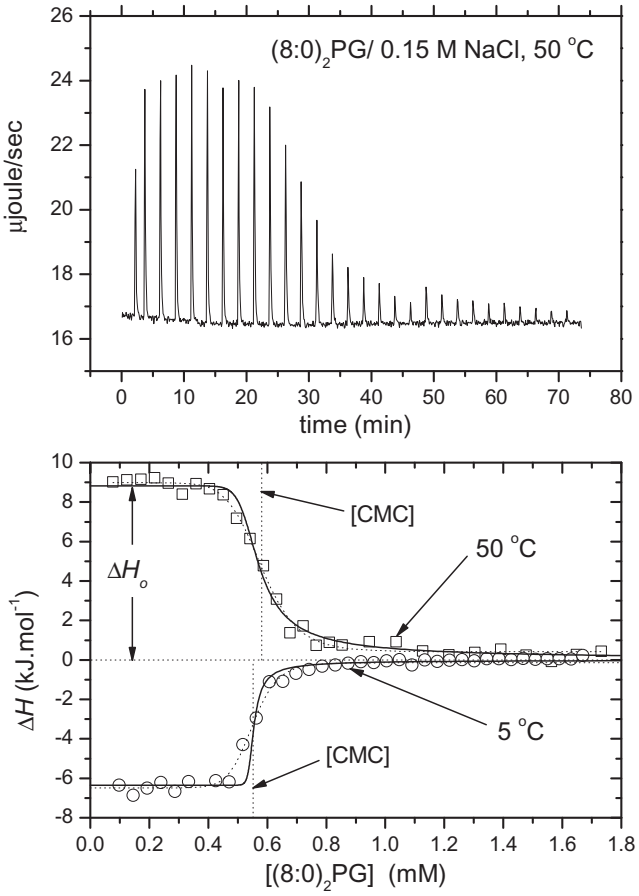

FIGURE 1 (*Top*) Raw data from an ITC demicellization titration of dioctanoyl phosphatidylglycerol, $(8:0)_2$PG, in 0.15 M NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.4, at 50°C. One-microliter injections of 10 mM lipid are followed by 2-μL injections at later times (cell volume = 207.8 μL). (*Bottom*) Integrated heat per mole of injectant for the top titration at 50°C (*squares*) and for the corresponding titration at 5°C (*circles*). The uncorrected heat of micellization is ΔH_o (cf. Eq. 5). (*Dotted lines*) Empirical fits to the titrations with a logistic sigmoid. (*Solid lines*) Numerical derivatives, according to Eq. 2, of the corresponding fits in Fig. 2 for a monodisperse distribution of micelle sizes (see Eq. 7).

of micelle assembly (see Gill and Wadsö (3) and Gill et al. (8)).

In the initial parts of the titration, ΔH remains constant at the value ΔH_o, which corresponds to complete dissociation of the injected micelles. Then, ΔH decreases steeply as the concentration of lipid in the measurement cell reaches the CMC. The critical micelle concentration is given by the value of c_T at the point of maximum slope in this region of the titration (5,9). (This is usually determined by fitting a logistic sigmoid, as indicated by the *dotted curves* in the *lower panel* of Fig. 1, which also yield the heats of demicellization.) Finally the value of ΔH tends to zero, indicating that the heat of dilution of undissociated micelles is rather small.

Values of the critical micelle concentration, and enthalpy of micelle formation ($-\Delta H_{demic}$) for the various

phospholipids studied are summarized in Table 1. Corresponding values of the free energy and entropy of micelle formation obtained from Eqs. 13 and 14, respectively, are also included in this table. It is notable that the CMCs of the negatively charged lipids are much lower in 0.15 M NaCl than they are in the absence of salt (see also King and Marsh (4)), whereas the enthalpies are much more comparable. Also, the CMC of phosphatidic acid in the singly charged state at pH 5.0 is much smaller than that in the doubly charged state at pH 8.2. Note that the effective $pK_{a,2}$ for micellar transfer of phosphatidic acid is 7.4 (4). Changes in the thermodynamics of self-assembly with increasing ionic strength arise from screening of electrostatic interactions, because for zwitterionic phosphatidylcholine they are unchanged by addition of NaCl (7). On the other hand, pH titration can give rise to changes in phospholipid hydration, not simply changes in electrostatic charge (10,11).

Lipid self-assembly

Fig. 2 gives the dependence of the monomer concentration (*squares*) on total $(8:0)_2$PG concentration at 50°C. These data are obtained from the ITC titration points given in the lower panel of Fig. 1 by numerical integration according to Eq. 3. The solid line gives a nonlinear least-squares fit of Eq. 9 to this titration. Beyond the CMC as defined in Fig. 1, the monomer concentration levels off to a constant value that is only slightly larger than the CMC. Correspondingly, the concentration of micelles, obtained from Eq. 4 (*circles*), remains close to zero up to the CMC and then increases linearly with added lipid shortly thereafter. Electrode measurements with anionic surfactants have revealed that the monomer activity begins to decrease with increasing surfactant concentration above the CMC, because of the increasing micelle concentration (12–14). If this also happens with $(8:0)_2$PG, it does not appear to be associated with an appreciable heat evolution or absorption (see Fig. 1).

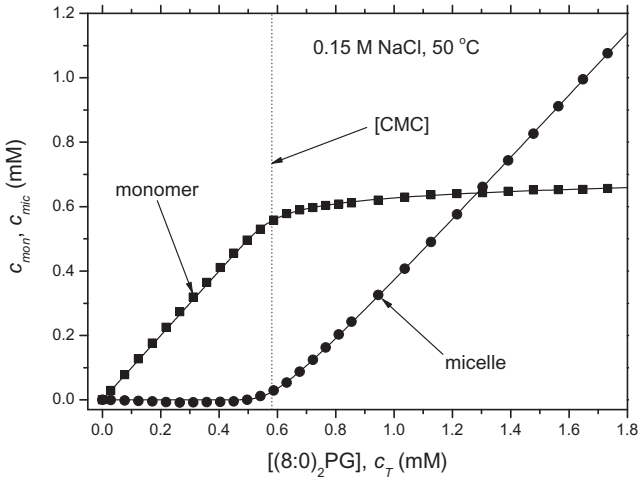

FIGURE 2 Integrated titration curve according to Eq. 3 for the monomer concentration (*squares*) deduced from the demicellization data of $(8:0)_2$PG, in 0.15 M NaCl at 50°C (given in the *lower panel* of Fig. 1). (*Circles*) Corresponding micelle concentration (in moles of lipid) according to Eq. 4. (*Dotted vertical line*) Critical micelle concentration (defined as the point of maximum slope in the *lower panel* of Fig. 1). (*Solid lines*) Nonlinear least-squares fits of Eq. 9 ($K = 1.32 \pm 0.02$ mM^{-1}, $m = 22.5 \pm 0.8$, and $\Delta H_o = 8.82 \pm 0.15$ kJ.mol^{-1}).

Numerical differentiation of the fitted monomer progress curve in Fig. 2 gives the solid line shown for the 50°C data in the lower panel of Fig. 1. As for the fit in Fig. 2, this gives a reasonable description of the experimental titration data,

TABLE 1 Thermodynamic parameters of micelle formation for charged phospholipids (see Eqs. 5, 13, and 14)

Lipid	pH	[NaCl] (M)	T (°C)	[CMC] (mM)	ΔG^o_{tr} (kJ.mol^{-1})	ΔH^o_{tr} (kJ.mol^{-1})	ΔS^o_{tr} (J.mol^{-1} K^{-1})
$(8:0)_2$PG	7.4	0	5	2.49 ± 0.01	−23.16 ± 0.01	7.16 ± 0.08	109.0 ± 0.3
			10	1.25 ± 0.01	−25.19 ± 0.02	7.08 ± 0.14	114.0 ± 0.5
			40	1.18 ± 0.02	−28.02 ± 0.05	−7.44 ± 0.25	65.7 ± 1.0
			50	2.68 ± 0.02	−26.70 ± 0.02	−6.78 ± 0.17	61.7 ± 0.6
		0.15	5	0.552 ± 0.004	−26.64 ± 0.02	6.80 ± 0.12	120.2 ± 0.5
			10	0.272 ± 0.005	−28.79 ± 0.04	5.28 ± 0.18	120.3 ± 0.8
			40	0.417 ± 0.006	−30.72 ± 0.04	−6.80 ± 0.21	76.4 ± 0.8
			50	0.581 ± 0.005	−30.81 ± 0.02	−9.10 ± 0.20	67.2 ± 0.7
$(8:0)_2$PS	7.4	0	5	2.29 ± 0.01	−23.35 ± 0.01	9.84 ± 0.14	119.3 ± 0.5
			10	1.22 ± 0.02	−25.25 ± 0.03	8.14 ± 0.28	117.9 ± 1.1
			40	1.34	−27.68	−5.18 ± 0.59	71.8
			50	2.38 ± 0.03	−27.02 ± 0.03	−9.87 ± 0.44	53.1 ± 1.5
		0.15	5	0.457 ± 0.010	−27.07 ± 0.05	7.23 ± 0.30	123.3 ± 1.3
			10	0.333 ± 0.002	−28.31 ± 0.02	5.76 ± 0.07	120.3 ± 0.3
			40	0.389 ± 0.002	−30.90 ± 0.01	−9.45 ± 0.10	68.5 ± 0.3
			50	0.631 ± 0.019	−30.59 ± 0.08	−9.44 ± 0.77	65.5 ± 2.6
$(8:0)_2$PA	5.0	0	5	0.509 ± 0.026	−26.83 ± 0.12	2.64 ± 0.15	105.9 ± 1.0
			50	0.483 ± 0.011	−31.31 ± 0.06	−27.2 ± 0.9	12.6 ± 2.8
	8.2	0	5	4.21 ± 0.02	−21.94 ± 0.01	28.71 ± 0.33	182.1 ± 1.2
			40	4.00 ± 0.05	−24.83 ± 0.03	6.64 ± 0.20	100.5 ± 0.7
			50	3.012 ± 0.073	−26.39 ± 0.06	5.80 ± 0.24	99.6 ± 1.0

and gives a consistency check on the former. A similar procedure gives the solid line shown for the 5°C data in the lower panel of Fig. 1. In the latter case, the effective micelle size required for the fit is larger ($m = 78 \pm 4$) than that for the 50°C data ($m = 23 \pm 1$), both with 0.15 M NaCl. Effective values obtained by the same procedure for $(8:0)_2$PG micelles in the absence of salt ($m = 36 \pm 2$ and 14 ± 0.1 at 5 and 50°C, respectively) are lower than those for the micelles in 0.15 M NaCl. For comparison, aggregation numbers for the single-chain anionic surfactant, sodium dodecyl sulfate (SDS), at 25°C obtained from light scattering are $m_{agg} = 77$ and 101 in 0.01 and 0.1 M NaCl, respectively (15). In addition, a decrease in aggregation number with increasing temperature is found for SDS micelles, from $m_{agg} = 75$ at 21.1°C to $m_{agg} = 49$ at 51.4°C (16). Micelles of the zwitterionic glycerophospholipid $(8:0)_2$PC are much larger than these anionic micelles: $m_{agg} \geq 470$ (17).

Temperature dependence

Fig. 3 shows the temperature dependence of the enthalpy and free energy of micelle formation by $(8:0)_2$PG in 0 and 0.15 M NaCl. It has been shown previously that the large negative heat capacity, ΔC_P^o, of phospholipid micelle formation is practically independent of temperature between 5 and 50°C (7), as for the hydrophobic effect (3). Thus, two temperatures sufficiently far removed on either side of T^* are enough to define the temperature dependence of the enthalpy (see Eq. 10). One further parameter, ΔS^*, is required to define the temperature dependence of the free energy (see Eq. 12). This is done by simultaneous fitting

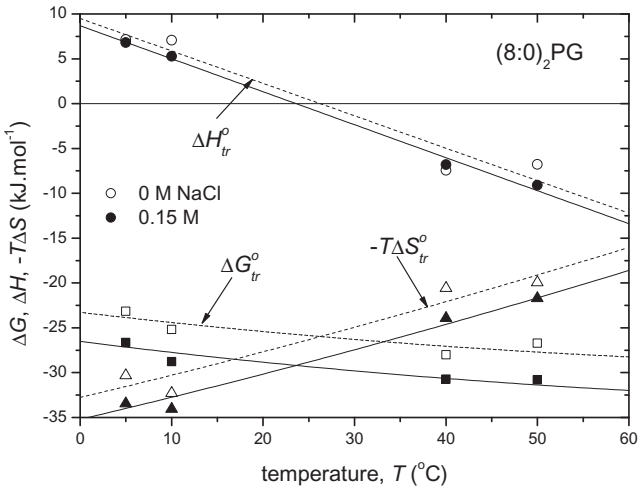

FIGURE 3 Temperature dependence of the enthalpy, ΔH_{tr}^o (*circles*), and free energy, ΔG_{tr}^o (*squares*), of micelle formation by $(8:0)_2$PG in the presence (*solid symbols*) and absence (*open symbols*) of 0.15 M NaCl. Solid (dashed) lines are simultaneous least-squares fits of Eqs. 10 and 12, respectively, for micelles in the presence (absence) of 0.15 M NaCl. For comparison, the entropic contributions, $-T\Delta S_{tr}^o$ (*triangles*), are also included.

to the temperature dependences of both enthalpy and free energy with Eqs. 10 and 12 (see Fig. 3). The parameters determined in this way for the anionic lipids are listed in Table 2. Evaluations of previous data for zwitterionic 1,2-diacyl phosphatidylcholines, $(n:0)_2$PC, and 1-acyl-2-lyso phosphatidylcholines, (*n*:0/0:0)PC (7), are also included in the table for comparison. Note that, extrapolated linearly to $(8:0)_2$PC, the value of $\Delta S^* = 100.6$ J.mol^{-1} K^{-1} for the zwitterionic lipid is close to that for the ionic lipids $(8:0)_2$PG and $(8:0)_2$PS in 0.15 M NaCl, where the electrostatic contribution is screened.

Application of the same method to published ITC data for demicellization of SDS surfactant (5), over the same temperature range, gives $\Delta C_P^o = -459 \pm 9$ J.mol^{-1} K^{-1}, $\Delta S^* = 73.6 \pm 0.1$ J.mol^{-1} K^{-1}, and $T^* = 25.1 \pm 0.3$°C in the absence of salt; and at higher ionic strength, $\Delta C_P^o = -379 \pm 9$ J.mol^{-1} K^{-1}, $\Delta S^* = 87.1 \pm 0.1$ J.mol^{-1} K^{-1}, and $T^* = 19.1 \pm 0.4$°C in 0.1 M NaCl. These values are comparable to those for $(8:0)_2$PG and $(8:0)_2$PS in Table 2, as is the direction of the changes on screening by salt, except that for SDS the heat capacity also changes. The latter arises from a change in hydrophobic exposure at the micelle surface of the single-chain surfactant (5), which evidently is absent for the two-chain glycerophospholipids.

DISCUSSION

The integrated demicellization titrations obtained from ITC (Fig. 1, *lower panel*) can be described adequately by Eq. 9, with a single effective micelle size, *m*. This then yields values for both the enthalpy of transfer (ΔH_{tr}^o) and the free energy of transfer (ΔG_{tr}^o, or equivalently, CMC). The temperature dependences of the thermodynamic parameters of transfer are given by Eqs. 10–12 (see Fig. 3). These are characterized by a large constant heat capacity (ΔC_P^o), which is indicative of the hydrophobic effect, the temperature (T^*) at which the enthalpy of transfer vanishes (also typical of the hydrophobic effect), and the corresponding entropy (ΔS^*).

Critical micelle concentrations

Previous experimental determinations of the CMC for anionic dioctanoyl phospholipids are 1.2 mM for $(8:0)_2$PG in the absence of salt at 21.5°C (18), and 2.18 mM and 1.7 mM for $(8:0)_2$PS in the absence of salt at 21.5 and 25°C, respectively (18,19). Corresponding values interpolated from the present measurements by using Eqs. 12 and 13 with data from Table 2 are 1.6 mM for $(8:0)_2$PG in the absence of salt at 21.5°C, and 1.5 mM for $(8:0)_2$PS in the absence of salt at both 21.5 and 25°C. The agreement with the previous measurements is, therefore, quite good. A similar interpolation for $(8:0)_2$PG in 0.15 M NaCl at 21.5°C gives a CMC of 0.4 mM, which is similar to the value

TABLE 2 Parameters determining the temperature dependence of the free energy and enthalpy of micelle formation for phospholipids with different polar headgroups (see Eqs. 10–12)

Lipid	pH	[NaCl] (M)	T^* (°C)	ΔC_P^o (J.mol^{-1} K^{-1})	ΔS^* (J.mol^{-1} K^{-1})	Ref.
$(8:0)_2$PG	7.4	0	26.7 ± 7.1	−373 ± 40	86.8 ± 9.2	This work
		0.15	23.7 ± 3.2	−379 ± 18	98.5 ± 4.3	
$(8:0)_2$PS	7.4	0	27.9 ± 2.9	−448 ± 19	87.2 ± 4.5	This work
		0.15	22.8 ± 4.6	−423 ± 29	98.5 ± 6.8	
$(8:0)_2$PA	5.0	0	7.8 ± 1.5	−626 ± 11	99.8 ± 3.3	This work
	8.2	0	59.1 ± 1.7	−547 ± 11	84.7 ± 2.8	
$(5:0)_2$PC	7.4	0.15	57.0 ± 1.4	−280 ± 12	54.9 ± 0.5	(7)
$(6:0)_2$PC	7.4	0.15	42.4 ± 0.5	−384 ± 9	68.8 ± 0.4	(7)
$(7:0)_2$PC	7.4	0.15	30.4 ± 0.1	−455 ± 2	85.7 ± 0.1	(7)
(10:0/0:0)PC	7.4	0.15	31.7 ± 0.4	−376 ± 9	74.8 ± 0.5	(7)
(12:0/0:0)PC	7.4	0.15	23.6 ± 0.4	−481 ± 10	93.7 ± 0.6	(7)
(14:0/0:0)PC	7.4	0.15	17.3 ± 0.3	−604 ± 10	116.2 ± 0.5	(7)
(16:0/0:0)PC*	7.4	0.15	13.4 ± 0.3	−669 ± 24	134.4 ± 0.8	(7)

*Measurements of ΔH_{tr}^o restricted to $T \leq 26$°C.

of 0.27 mM measured for the corresponding zwitterionic phospholipid, $(8:0)_2$PC (20). This confirms that electrostatic contributions to self-assembly of phosphatidylglycerol are mostly screened in 0.15 M NaCl (see also King and Marsh (4)).

Critical micelle concentrations of phosphatidylglycerol and phosphatidylserine can be predicted from measurements on spin-labeled phospholipids relative to phosphatidylcholine (4,21). Predictions for $(8:0)_2$PG in 0 and 0.15 M NaCl at 20°C are 2.2 and 0.37 mM, respectively; corresponding predictions for $(8:0)_2$PS are 3.7 and 0.50 mM (see Marsh and King (21)). Comparison with Table 1, shows these predictions to be reasonably reliable, particularly as regards the effect of ionic strength on the anionic lipids.

For spin-labeled phosphatidic acid in 0.1 M NaCl at 20°C, the CMC increases by a factor ~7 on titrating from pH 5 to pH 8 (4). The corresponding increase for $(8:0)_2$PA on going from the singly negatively charged state at pH 5.0 to the doubly negatively charged state at pH 8.2, in the absence of salt, is by a factor of 8.3 and 6.2 at 5 and 50°C, respectively (see Table 1). This reflects a similar influence of electrostatics on micelle formation of charged lipids to that exhibited by the ionic strength dependence for PG and PS.

Effect of electrostatics on the thermodynamics of self-assembly

The results in Table 1 and Fig. 3 for charged lipids in the presence and absence of salt show that the increase in CMC (and corresponding decrease in micelle stability) by surface electrostatics is principally entropic in origin. In terms of Eqs. 10 and 11, the changes in enthalpy ($\Delta\Delta H_{tr}^o$) and entropy ($\Delta\Delta S_{tr}^o$) of transfer that arise from a change in ionic strength, or from pH titration, are characterized by changes, $\Delta\Delta C_P^o$, ΔT^*, and $\Delta\Delta S^*$, in the parameters, ΔC_P^o, T^*, and ΔS^*, respectively. Relative to the screened or less charged state, the appropriate expressions at temperature $T^*+\Delta T^*$ are

$$\Delta\Delta H_{tr}^o(T^* + \Delta T^*) = -\Delta C_P^{o,o}\Delta T^*, \quad (15)$$

$$\Delta\Delta S_{tr}^o(T^* + \Delta T^*) = \Delta\Delta S^* - \Delta C_P^{o,o} \ln\left(1 + \frac{\Delta T^*}{T^*}\right), \quad (16)$$

where $\Delta C_P^{o,o}$ is the heat capacity of transfer in the screened or less-charged state. Relative to the screened state in 0.15 M NaCl (see Table 2), the change in enthalpy of transfer for the charged state is relatively small: $\Delta\Delta H_{tr}^o = 1.1 \pm 4.0$ and 2.2 ± 3.3 kJ.mol^{-1} for $(8:0)_2$PG and $(8:0)_2$PS, respectively. The contribution to the entropy of transfer, on the other hand, is much larger: $-(T^*+\Delta T^*)\Delta\Delta S_{tr}^o = -10.1 \pm 5.8$ and -22.3 ± 7.0 kJ.mol^{-1} for $(8:0)_2$PG and $(8:0)_2$PS, respectively. As will be seen in the following section, the electrostatic entropy is stored in the diffuse double layer of the counterion cloud, which contracts with increasing ionic strength. At high surface potentials, this dominates the free energy of the electrostatic double layer (22). Note that the heat capacity of transfer, ΔC_P^o, is determined by the change in apparent water-accessible apolar surface area (3,7) and does not change on increasing the ionic strength (see Table 2).

The effects of electrostatics are also seen upon titrating the second phosphate pK_a in the polar headgroup of phosphatidic acid. In this case, however, larger effects are seen in the thermodynamics of lipid transfer than are induced by simply ionic screening with phosphatidylglycerol or phosphatidylserine. This is because proton binding to lipid polar groups has effects additional to simply altering the headgroup charge. pH titration can also result in considerable changes of the degree of hydration of the lipid polar groups (10,11), and hydrogen bonding is thought also to play a role in the titration behavior of phosphatidic acid (23). These changes clearly will have a considerable further influence on the thermodynamics of lipid self-assembly. Note that the heat capacity of transfer, ΔC_P^o, is larger for phosphatidic acid than for phosphatidylglycerol

and phosphatidylserine (see Table 2). This indicates a greater change in water-accessible apolar surface area, which correlates with the smaller polar headgroup of phosphatidic acid.

Thermodynamics of the electrostatic double layer

According to classical electrostatic double-layer theory, the surface potential, ψ_o, of a planar surface with charge density σ is given by (see, e.g., Cevc and Marsh (2))

$$\sinh\left(\frac{ze\psi_o}{2k_BT}\right) = \frac{\sigma}{\sqrt{8\varepsilon_r\varepsilon_o nk_BT}}, \tag{17}$$

where e is the electronic charge, ε_r is the effective dielectric constant in the double layer, ε_o is the dielectric permittivity of free space, z is the valence, and n the concentration (in ions/m^3) of the assumed symmetrical electrolyte, and k_B is Boltzmann's constant.

The contribution, ΔH_{el}, to the enthalpy per mole of lipid that arises from the internal energy of the double-layer electric field ($E_x = -d\psi/dx$) is (2,22)

$$\begin{aligned}\Delta H_{el} &= \frac{1}{2}\varepsilon_r\varepsilon_o N_A A_l \int_0^\infty \left(\frac{d\psi}{dx}\right)^2 dx \\ &= 2N_A k_B T A_l \frac{\sqrt{2\varepsilon_r\varepsilon_o nk_BT}}{ze}\left[\cosh\left(\frac{ze\psi_o}{2k_BT}\right) - 1\right],\end{aligned} \tag{18}$$

where A_l is the surface area per lipid, and N_A is Avogadro's number. The contribution, ΔG_{el}, to the free energy per mole of lipid from the electrostatic double layer, is given by (2,24)

$$\begin{aligned}\Delta G_{el} &= N_A A_l \int_o^{Z_l e/A_l} \psi_o(\sigma).d\sigma \\ &= 2N_A k_B T\left\{\frac{Z_l}{z}\left(\frac{ze\psi_o}{2k_BT}\right)\right. \\ &\quad \left. - 2A_l\frac{\sqrt{2\varepsilon_r\varepsilon_o nk_BT}}{ze}\left[\cosh\left(\frac{ze\psi_o}{2k_BT}\right) - 1\right]\right\},\end{aligned} \tag{19}$$

where $Z_l e$ is the charge on each lipid. The entropy, ΔS_{el}, contributed by the double layer is then obtained from $\Delta S_{el} = (\Delta H_{el} - \Delta G_{el})/T$.

The dependences of the thermodynamic parameters of the electrostatic double layer on ionic strength are shown in Fig. 4, with the lower x axis as a scaled functional quantity that is linear in the square-root of the ionic strength, and the upper x axis as a nonlinear scale of the molar electrolyte concentration, $c_M = n/10^3N_A$, for specific values of ε_r, A_l, Z_l, and T. Qualitatively, the dependence of the electrostatic free energy on salt concentration given in Fig. 4 is very similar to that observed experimentally for micelle formation by spin-labeled phosphatidylglycerol (4), and for anionic n-alkyl sulfate surfactants (14,25).

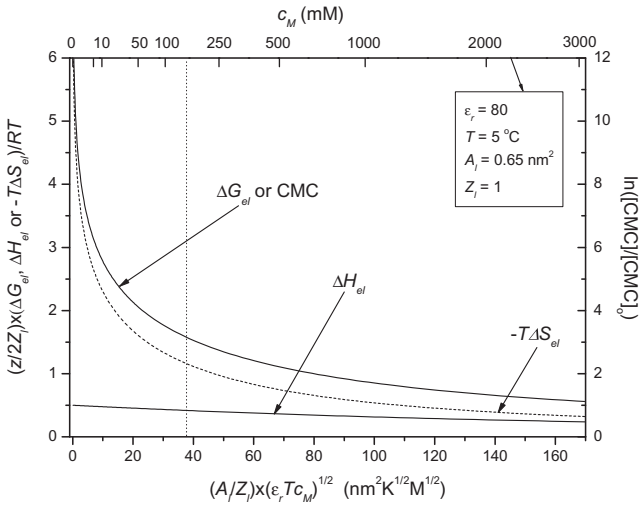

FIGURE 4 Contributions, per mole of lipid, to the electrostatic free energy (ΔG_{el}) of a charged lipid assembly from the enthalpy (ΔH_{el}) and entropy ($-T\Delta S_{el}$) of the electrostatic double layer. The functional dependence on $\sqrt{\varepsilon_r Tc_M}$ is calculated from Eqs. 17–19 for a planar surface. The thermodynamic quantities are scaled by the factor ($z/2Z_l$) and expressed in units of RT. The lower x axis is scaled by the factor (A_l/Z_l), and the upper x axis is the molar concentration, c_M, of symmetrical electrolyte for a double-layer dielectric constant $\varepsilon_r = 80$, and area per singly charged lipid ($Z_l = 1$) of $A_l = 0.65$ nm^2, at 5°C. The right-hand ordinate represents the CMC relative to that in the absence of electrostatics (i.e., $[CMC]_o$) for $Z_l = 1$ and $z = 1$. (*Vertical dotted line*) Monovalent salt concentration of 0.15 M.

For high potentials ($ze\psi_o \gg k_BT$), the first term on the right in Eq. 19 dominates and the free energy is almost entirely entropic:

$$\Delta G_{el} \approx -T\Delta S_{el} \approx 2N_A k_B T\frac{Z_l}{z}\ln\left(\frac{Z_l e}{A_l\sqrt{2\varepsilon_r\varepsilon_o nk_BT}}\right). \tag{20}$$

See Fig. 4 at low ionic strength. Correspondingly, the contribution to the heat capacity is negligible. For low potentials ($ze\psi_o \ll k_BT$), entropic and enthalpic contributions to the free energy are comparable:

$$\Delta G_{el} \approx 2\Delta H_{el} \approx N_A k_B T\frac{Z_l^2 e}{A_l\sqrt{8\varepsilon_r\varepsilon_o nk_BT}}. \tag{21}$$

See Fig. 4 at high ionic strength. Under these conditions, however, the electrostatic contribution is, of course, small.

The results in Fig. 4 are given for planar lipid surfaces because analytical solutions are possible. Nonetheless, they reproduce rather well the qualitative thermodynamic features found by calorimetry. At an NaCl concentration in the region of 150 mM, the major part of the electrostatic contribution to the free energy of micellization has been removed, corresponding to a large reduction in the measured

CMC (see Table 1). On the other hand, the electrostatic contribution to the enthalpy of micellization and to the heat capacity is hardly changed, as is found for the experimental results in Tables 1 and 2 and in Fig. 3. Note that the planar model becomes more appropriate the larger is the micelle, and certainly is found to be a better approximation than Debye-Hückel theory (14). A similar reduction in electrostatic enhancement of the CMC is found for the single-chain anionic surfactant, sodium dodecyl sulfate, in 0.1 M NaCl (5). As already noted, however, the heat capacity and correspondingly enthalpy are reduced in this case because of the decreased exposure of the single chains to water.

CONCLUSION

As for phosphatidylcholine (7), the thermodynamics of self-assembly of phosphatidylglycerol, phosphatidylserine, and phosphatidic acid are characterized by a large heat capacity, with the enthalpy going through zero (corresponding to a purely entropy-driven process) at a temperature within the accessible range. These features correspond to the determining role of the hydrophobic effect in the self-assembly of membranes and micelles.

On the other hand, self-assembly of negatively charged lipids is destabilized (the CMC is increased manyfold), relative to zwitterionic phosphatidylcholine or to the charged lipid at high ionic strength. The source of the destabilization is primarily entropic and corresponds to building up the counterion cloud in the electrostatic double layer of the anionic lipid assembly. This destabilizing of membranes by charged lipids will have various functional consequences, including those for lipid exchange, membrane fission and fusion, and membrane curvature and vesicle formation.

Finally, it should be mentioned that the thermodynamic methods used in this study are more widely applicable to other fields of interest in biophysics. For instance, it holds quite generally that entropy of the double layer makes a major contribution to the electrostatic free energy of polyelectrolytes. It also is known that electrostatic free energy is an important determinant of the chain-melting temperature of charged lipid-bilayer membranes, as well as that for other significant phase transitions (e.g., those involved in lamellar-nonlamellar polymorphism) (2,22,24). In addition, interactions with peptides and proteins will affect membrane stability according to the above thermodynamic principles. If such an interaction affects only membrane electrostatics it is expected to have little effect on the enthalpy of formation and consequently not to change T^* (see Eqs. 10 and 15). Changes in hydrophobic exposure, on the other hand, will be reflected in the heat capacity of transfer (3,5,7), with consequent shifts in the characteristic temperature, T^*. In such cases, the membrane/micelle and peptide/protein system must, of course, be considered as a whole, and changes in electrostatics and hydrophobic interactions of the peptide/protein itself will be subject to the same thermodynamic considerations.

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REFERENCES

1. Tanford, C. 1980. The Hydrophobic Effect. Wiley, New York.
2. Cevc, G., and D. Marsh. 1987. Phospholipid Bilayers. Physical Principles and Models. Wiley-Interscience, New York.
3. Gill, S. J., and I. Wadsö. 1976. An equation of state describing hydrophobic interactions. *Proc. Natl. Acad. Sci. USA.* 73:2955–2958.
4. King, M. D., and D. Marsh. 1987. Head group and chain length dependence of phospholipid self-assembly studied by spin-label electron spin resonance. *Biochemistry.* 26:1224–1231.
5. Paula, S., W. Süs, …, A. Blume. 1995. Thermodynamics of micelle formation as a function of temperature: a high sensitivity titration calorimetry study. *J. Phys. Chem.* 99:11742–11751.
6. Heerklotz, H., G. Lantzsch, …, A. Blume. 1996. Thermodynamic characterization of dilute aqueous lipid/detergent mixtures of POPC and $C_{12}EO_8$ by means of isothermal titration calorimetry. *J. Phys. Chem.* 100:6764–6774.
7. Heerklotz, H. H., and R. M. Epand. 2001. The enthalpy of acyl chain packing and the apparent water-accessible apolar surface area of phospholipids. *Biophys. J.* 80:271–279.
8. Gill, S. J., S. F. Dec, …, I. Wadsö. 1985. Anomalous heat capacity of hydrophobic solvation. *J. Phys. Chem.* 89:3758–3761.
9. Kresheck, G. C. 1998. A calorimetric determination of the standard enthalpy and heat capacity changes that accompany micelle formation for four long chain alkyldimethylphosphine oxides in H_2O and D_2O solution from 15 to 79°C. *J. Am. Chem. Soc.* 120:10964–10969.
10. Cevc, G., A. Watts, and D. Marsh. 1980. Non-electrostatic contribution to the titration of the ordered-fluid phase transition of phosphatidylglycerol bilayers. *FEBS Lett.* 120:267–270.
11. Cevc, G., A. Watts, and D. Marsh. 1981. Titration of the phase transition of phosphatidylserine bilayer membranes. Effects of pH, surface electrostatics, ion binding, and head-group hydration. *Biochemistry.* 20:4955–4965.
12. Vikingstad, E. 1979. The mean activity and the activities of the separate ions of sodium decanoate above and below the CMC determined by a surfactant selective silver/silver decanoate electrode. *J. Colloid Interface Sci.* 72:68–74.
13. Cutler, S. G., P. Meares, and D. G. Hall. 1978. Ionic activities in sodium dodecyl sulphate solutions from electromotive force measurements. *J. Chem. Soc., Faraday Trans. I.* 74:1758–1767.
14. Gunnarsson, G., B. Jönsson, and H. Wennerström. 1980. Surfactant association into micelles. An electrostatic approach. *J. Phys. Chem.* 84:3114–3121.
15. Hayashi, S., and S. Ikeda. 1980. Micelle size and shape of sodium dodecyl sulfate in concentrated NaCl solutions. *J. Phys. Chem.* 84:744–751.
16. Malliaris, A., J. Le Moigne, …, R. Zana. 1985. Temperature dependence of the micelle aggregation number and rate of intramicellar excimer formation in aqueous surfactant solutions. *J. Phys. Chem.* 89:2709–2713.
17. Tausk, R. J. M., J. van Esch, …, J. T. Overbeek. 1974. Physical chemical studies of short-chain lecithin homologues. II. Micellar weights of dihexanoyl- and diheptanoyllecithin. *Biophys. Chem.* 1:184–203.

18. Kleinschmidt, J. H., and L. K. Tamm. 2002. Structural transitions in short-chain lipid assemblies studied by ^{31}P-NMR spectroscopy. *Biophys. J.* 83:994–1003.

19. Walker, J. M., E. C. Homan, and J. J. Sando. 1990. Differential activation of protein kinase C isozymes by short chain phosphatidylserines and phosphatidylcholines. *J. Biol. Chem.* 265:8016–8021.

20. Tausk, R. J. M., J. Karmiggelt, …, J. T. Overbeek. 1974. Physical chemical studies of short-chain lecithin homologues. I. Influence of the chain length of the fatty acid ester and of electrolytes on the critical micelle concentration. *Biophys. Chem.* 1:175–183.

21. Marsh, D., and M. D. King. 1986. Prediction of the critical micelle concentrations of mono- and di-acyl phospholipids. *Chem. Phys. Lipids.* 42:271–277.

22. Jähnig, F. 1976. Electrostatic free energy and shift of the phase transition for charged lipid membranes. *Biophys. Chem.* 4:309–318.

23. Eibl, H., and P. Woolley. 1979. Electrostatic interactions at charged lipid membranes. Hydrogen bonds in lipid membrane surfaces. *Biophys. Chem.* 10:261–271.

24. Träuble, H., M. Teubner, …, H. Eibl. 1976. Electrostatic interactions at charged lipid membranes. I. Effects of pH and univalent cations on membrane structure. *Biophys. Chem.* 4:319–342.

25. Huisman, H. F. 1964. Light scattering of solutions of ionic detergents. *Koninkl. Ned. Akad. Wetenschap. Proc. Ser. B.* 64:367–424.