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Thermodynamics of Ion Binding to Phosphatidic Acid Bilayers. Titration Calorimetry of the Heat of Dissociation of DMPA†

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Received October 2, 1991; Revised Manuscript Received February 21, 1992

ABSTRACT: The heat of dissociation of the second proton of 1,2-dimyristoylphosphatidic acid (DMPA) was studied as a function of temperature using titration calorimetry. The dissociation of the second proton of DMPA was induced by addition of NaOH. From the calorimetric titration experiment, the intrinsic pK_0 for the dissociation reaction could be determined by applying the Gouy-Chapman theory. pK_0 decreases with temperature from ca. 6.2 at 11 °C to 5.4 at 54 °C. From the total heat of reaction, the dissociation enthalpy, ΔH_{diss}, was determined by subtracting the heat of neutralization of water and the heat of dilution of NaOH. In the temperature range between 2 and 23 °C, ΔH_{diss} is endothermic with an average value of ca. 2.5 kcal·mol^{-1} and shows no clear-cut temperature dependence. In the temperature range between 23 and 52 °C, ΔH_{diss} calculated after subtraction of the heat of neutralization and dilution is not the true dissociation enthalpy but includes contributions from the phase transition enthalpy, ΔH_{trans}, as the pH jump induces a transition from the gel to the liquid-crystalline phase. The ΔC_p for the reaction enthalpy observed in this temperature range is positive. Above 53 °C, the pH jump induces again only the dissociation of the second proton, and the bilayers stay in the liquid-crystalline phase. In this temperature range, ΔH_{diss} seems to decrease with temperature. The thermodynamic data from titration calorimetry and differential scanning calorimetry as a function of pH can be combined to construct a complete enthalpy-temperature diagram of DMPA in its two ionization states.

Differential scanning calorimetry (DSC)[1] has been used extensively for the determination of thermotropic properties of lipid model membranes composed of pure or mixed lipids and for the determination of the nature of lipid-protein interactions [for reviews, see Mabrey and Sturtevant (1979), McElhaney (1982, 1986), Bach (1984), Jones (1988), and Blume (1988, 1991)]. However, reaction calorimetry (RC) and differential titration calorimetry (DTC) have rarely been

† This work was supported by the Deutsche Forschungsgemeinschaft (Bl 182/7-1) and the Fonds der Chemischen Industrie.

[1] Abbreviations: DSC, differential scanning calorimetry; DTC, differential titration calorimetry; RC, reaction calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphoric acid; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine.

0006-2960/92/0431-4636$03.00/0

used up to now in studying binding reactions to lipids or membranes. RC and DTC can be employed for studying the heat of incorporation of peptides and proteins into lipid bilayers and for the determination of the heat of ion binding to negatively charged lipids and the heat of "solubilization" of hydrophobic molecules in lipid bilayers (Jones, 1988; Blume, 1988, 1991). We report here on the use of RC and DTC to determine the dissociation enthalpy for the second proton of the phospholipid DMPA as a function of temperature. Experiments were performed in two different ways: either as batch experiments to measure the total heat of dissociation by inducing a pH jump to pH 12, where DMPA is doubly charged, or by calorimetric titration of DMPA with dilute NaOH. The reaction enthalpy was measured in a temperature range between 2 and 70 °C. We will show that the titration curves can be simulated using the Gouy–Chapman theory, yielding the intrinsic pK_0 of the dissociation reaction of DMPA as a function of temperature. In addition, we will show that the temperature dependence of the enthalpy of dissociation, ΔH_{diss}, is different from what has been known for compounds resembling the head group of DMPA, like phosphoric acid or glycerophosphoric acid. From the temperature dependence of ΔH_{diss}, the change in heat capacity, ΔC_p, can be calculated. Comparison with the apparent molar heat capacities, ${}^{\Phi}C_p$, of $DMPA^-$ and $DMPA^{2-}$ as determined by DSC gives a consistent picture about the contributions of hydrophilic and hydrophobic hydration to ${}^{\Phi}C_p$ of $DMPA^-$ and $DMPA^{2-}$ and to ΔC_p of the dissociation reaction. Thus, reaction calorimetry and titration calorimetry prove to be very valuable tools for studying binding reactions.

Materials and Methods

DMPA was prepared from DMPC (Sigma, Deisenhofen, Germany) using phospholipase D isolated from cabbage according to the method of Eibl and Kovatchev (1981). The purity of the phosphatidic acid was checked by TLC on silica gel plates (Merck, Darmstadt, Germany) using $CHCl_3$/MeOH/7 M NH_3 (230:90:15) as solvent. Phospholipids were detected by spraying with a sulfuric acid containing phosphate reagent (Hahn & Luckhaus, 1955). Other organic compounds were visualized by charring. No impurities could be detected using this procedure. DSC curves of DMPA samples showed identical transition temperatures and transition enthalpies as reported in the literature.

Sample Preparation. (*A*) *DSC.* About 5.5 mg of DMPA was dispersed in ca. 3 mL of H_2O by vigorous shaking. The desired pH was adjusted by addition of 0.1 M NaOH. After further shaking, the pH was again controlled and if necessary adjusted.

(*B*) *DTC and RC.* An appropriate amount of DMPA was dispersed in 30 mL of degassed H_2O at 70 °C in a Branson bath-type ultrasonicator for 2 min to give a 1 mM dispersion. During the sonification procedure and for all following steps, the dispersion was always kept under an argon atmosphere to prevent dissolution of carbon dioxide. The pH of the dispersion was adjusted to pH 7 with ca. 15 μL of 0.1 M NaOH.

Vesicle Characterization. Vesicles were characterized by dynamic light scattering using a Malvern Zetasizer 3 with an AZ10 cell. The sonication procedure used for the preparations for RC and DTC produced vesicles with a relatively narrow size distribution between 58 and 200 nm with a mean diameter of ca. 110 nm and a half-width of the distribution of 50 nm. These vesicles are probably unilamellar as has been shown before (Hauser & Gains, 1982; Elamrani & Blume, 1983). Vesicle suspensions for DSC were prepared by mechanical shaking at temperatures above T_m in bidistilled water. Dynamic light scattering of these suspensions at neutral pH indicated that they contained larger vesicles or liposomes with a mean diameter of ca. 2 μm and a half-width of the distribution of 0.9 μm.

pH Titration. pH titration was carried out using a Radiometer PHM 26 pH meter with a micro glass electrode (Schott and Gen., Mainz, Germany). Ten milliliters of 10 mM DMPA dispersion was titrated stepwise by addition of 10 μL of 0.5 M NaOH. The vesicle suspension was kept under a nitrogen atmosphere and was continuously stirred during the titration. pH readings were taken after the pH had stabilized after several minutes. The α vs pH curve was then calculated by subtraction of a titration curve obtained with a blank solution.

DSC. DSC was performed using a Microcal MC-2 DSC instrument. The heating rate was 60 °C/h. At least two runs were recorded with each sample. Transition enthalpies were determined using the Microcal Origin software package.

RC and DTC. Reaction calorimetry and titration calorimetry were performed using a Microcal OMEGA titration calorimeter. The experiments were carried out using two different methods.

Method A (titration experiment): The calorimetric cell was filled with 1 mM DMPA (pH 7.0) vesicle dispersion (1.3434-mL cell volume), and the reference cell was filled with water. The DMPA vesicle suspension was titrated with 0.1 M NaOH solution by stepwise addition of 5 μL of NaOH solution up to a total volume of 100 μL. The heat of dilution of NaOH was either determined in a separate experiment or calculated from the signals observed at the last additions when the dissociation reaction was essentially complete. The final pH was checked after removing the sample from the cell, and the vesicle dispersion was checked by DSC for the correct T_m.

Method B (batch experiment): The cell was filled with 0.01 M NaOH solution, and 4 or 5 times, 20 μL of 1 mM DMPA dispersion was added. In this case, the heat of dilution of NaOH was determined in a separate experiment by adding water to the NaOH solution.

Results

Phospholipids like phosphatidic acids show a pH-dependent ionization and transition behavior which has been studied in quite detail (Träuble & Eibl, 1974, 1975; van Dijck et al., 1978; Eibl & Blume, 1979; Blume & Eibl, 1979). In pure water at lipid concentrations of ca. 1 mM, the apparent pK for the dissociation reaction

$$DMPA^- + OH^- \rightleftharpoons DMPA^{2-} + H_2O \qquad (1)$$

and also for other phosphatidic acids as determined by spectroscopic and calorimetric methods is between 10 and 10.5 (Eibl & Blume, 1979; Blume & Eibl, 1979). The apparent pK is dependent on the ionic strength; thus, different values are observed when higher lipid concentrations are used in unbuffered media. Dissociation of the second proton of the phosphate ester group of DMPA leads to a decrease of the transition temperature. This is caused by an increase in surface potential due to the doubly charged head groups and a change in the tilt angle of the fatty acyl chains, leading to decreased van der Waals interactions between the chains (Jähnig et al., 1979). Figure 1 shows DSC curves of DMPA at different pH values. The data agree with previously published results and show that the apparent pK is between 10 and 10.5 (Eibl & Blume, 1979; Blume & Eibl, 1979).

The dissociation of the second proton of DMPA can be induced at constant temperature by addition of NaOH. As the permeation of H^+/OH^- through lipid bilayers is relatively

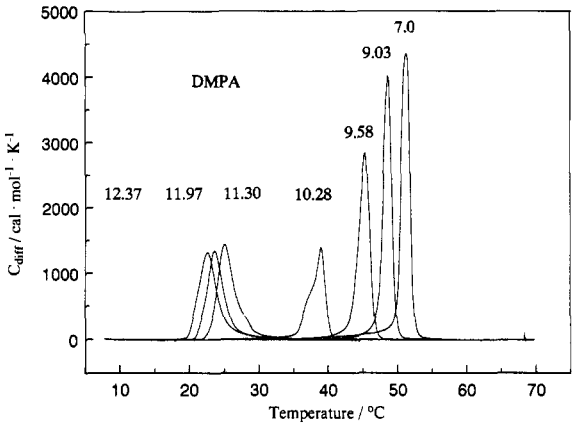

FIGURE 1: Differential scanning calorimetric curves of DMPA at different pH values as indicated.

fast with permeability coefficients between 10^{-5} and 10^{-3} cm/s (Nichols & Deamer, 1980; Elamrani & Blume, 1983), the dissociation reaction is essentially complete within the dead time of the reaction calorimeter. Permeation of H^+/OH^- is initially a fast uncompensated flux with half-times between 50 and 1000 ms below and shorter than 6 ms above T_m in unbuffered systems. This flux persists till the arising potential difference opposes a further influx of OH^- into and of H^+ out of the vesicles. In the second phase, H^+/OH^+ permeation is slow as it is controlled by counterion diffusion, i.e., the diffusion of Na^+ into the vesicles. This counterion-controlled diffusion has half-times of 0.5–3 min between 45 and 65 °C and comprises only 10% of the total amplitude of the pH indicator change. (Elamrani & Blume, 1983). Thus, even this diffusion process occurs within the observed time range of our calorimetric experiment as the waiting time between successive additions of NaOH was ca. 6 min. In the batch-type experiments, the H^+/OH^- flux thus raises the pH of the inner volume of the vesicles beyond the apparent pK of DMPA within the time of our experiments. It should be noted that Hauser et al. (1990) have reported that a pH gradient of ca. 4 pH units can exist over phosphatidic acid bilayers. These results are clearly at variance with our estimations. We have no explanations for these discrepancies, except that the experimental parameters are definitely different, as Hauser et al. used phosphatidic acid concentrations of 0.15–0.17 M. In addition, they reported that DMPA behaved differently than DLPA in that only a sharp singlet in the ^{31}P NMR spectrum was observed after the pH was raised to 12. This indicates that in DMPA indeed all head groups are deprotonated after the pH jump, in agreement with our proposition.

The observed heat of reaction, ΔH_r, will be the sum of several heat effects, namely, the dissociation enthalpy, ΔH_{diss}, of $DMPA^-$, the enthalpy of neutralization, ΔH_{neutr}, of water, the enthalpy of dilution of NaOH, and additional enthalpy changes caused by changes in head-group hydration and head-group interactions, and by rearrangements of the fatty acyl chains, i.e., induction of chain tilt in the gel phase or induction of the liquid-crystalline phase.

ΔH_{neutr} can be measured separately or can be taken from tabulated values (Landolt-Börnstein, 1961). The heat of dilution of NaOH was measured separately, so the latter two contributions could be subtracted from ΔH_r. The enthalpy ΔH_{diss} obtained after these subtractions is thus a purely operational quantity, as a clear separation of other contributions is not really possible.

The dissociation reaction according to eq 1 was studied by reaction and titration calorimetry over a temperature range

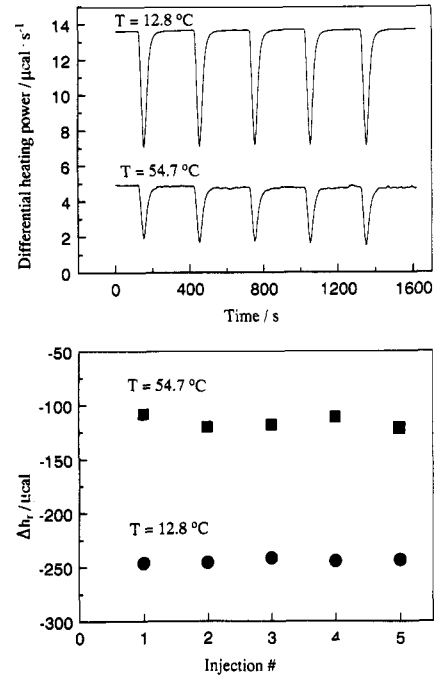

FIGURE 2: Top: Heat signals observed by adding 20 µL of a 1 mM DMPA (pH 7) vesicle suspension to 0.01 M NaOH (batch-type experiment) at two different temperatures. Bottom: Total heat of reaction (Δh_r) obtained from integration of the peaks.

between 2 and 70 °C using two different experimental approaches. In the first batch-type experiment, the total heat of reaction was measured by titrating 20 µL of a 1 mM DMPA vesicle dispersion into the reaction cell filled with 0.01 M NaOH. This was repeated several times as shown in Figure 2. The enthalpy of reaction (Δh_r) was calculated by integration of peaks. The dissociation enthalpy, ΔH_{diss}, was then determined from the average of the five additions after subtraction of the enthalpy of dilution, determined by addition of pure water to 0.01 M NaOH, and subtraction of the enthalpy of neutralization at that particular temperature (Landolt-Börnstein, 1961).

In the titration experiment, the sample cell was filled with a 1 mM DMPA vesicle suspension at pH 7 and then titrated stepwise with 5 µL of 0.1 M NaOH till the heat signal observed after 5-µL additions stayed constant, indicating that it resulted purely from the dilution of NaOH. In this experiment, the whole titration curve could be determined. From the titration curve, the pK for the dissociation reaction can be calculated as will be shown below. The total heat of reaction in the titration experiment can be compared to the heat of reaction from the batch-type experiments. Both values were the same within experimental error, indicating that possible hydrolysis reactions of DMPA during the time of the titration experiments did not play a role.

Figure 3 shows experimental titration curves of DMPA at three different temperatures. At 11 °C, the bilayers stay in the gel phase when the head group is titrated, but the chain arrangement changes from an L_β to an $L_{\beta'}$ phase (Jähnig et al., 1979). The observed heat signals are exothermic due to the large contributions of ΔH_{neutr}. At intermediate temperatures (23–52 °C), the dissociation of the second proton of $DMPA^-$ causes a change from the L_β phase of $DMPA^-$ to the L_α phase of $DMPA^{2-}$, so that the observed heat of reaction contains the transition enthalpy ΔH_{trans}. The transition takes place during the first few additions of NaOH. The observed heat signal is first endothermic due to the contribution of ΔH_{trans} overcompensating other contributions and then be-

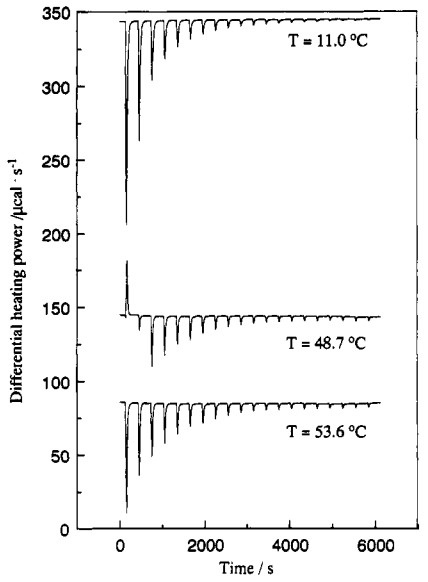

FIGURE 3: Calorimetric heat signals observed by titrating a 1 mM DMPA (pH 7) vesicle suspension with 0.1 M NaOH by stepwise addition of 5 µL of NaOH solution.

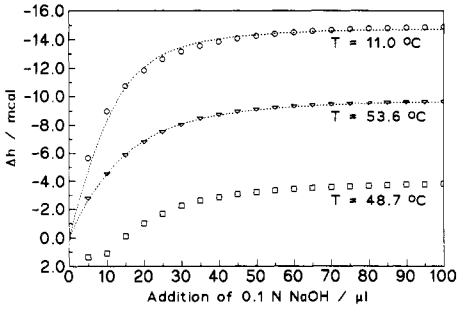

FIGURE 4: Total integrated heat of reaction (Δh) calculated from the curves in Figure 3 after subtraction of the heat of dilution of NaOH. The dashed curves for T = 11 °C and T = 53.6 °C are calculated curves using the Gouy–Chapman theory as described in the text with pK_0 values of 6.2 (11 °C) and 5.4 (53.6 °C).

comes exothermic when the transition is completed. At temperatures above 52 °C, the bilayers stay in the liquid-crystalline phase. The heat signals are again only exothermic due to the large contributions from ΔH_{neutr} but clearly smaller than at lower temperatures. Contributions from the heat of dilution of NaOH are not very large as indicated by the essentially constant signals observed for the final additions.

From the experimental curves in Figure 3, the integral heat of reaction, Δh, can be calculated by integration of the signals. Corrections have to be made for displacement of sample from the cell due to the addition of NaOH solution and for the heat of dilution. Figure 4 shows the integral heat of reaction determined from the titration curves at three different temperatures. The change in sign of Δh is evident for the curve at 48.7 °C where the gel–liquid-crystalline-phase transition is induced. From the end values, the total heat of reaction and ΔH_{diss} can be calculated. The curvature of these plots contains information on the dissociation constant K_{diss}. A method for determining ΔH_{diss} as well as K_{diss} from calorimetric titration curves was suggested by Chen and Wadsö (1982). They applied their method to the binding of small molecules to macromolecules. However, in the case of the dissociation of $DMPA^-$ in lipid bilayers, this method cannot be applied due to surface charge effects. In this case, the Gouy–Chapman theory for the electrical double layer has to be applied (Träuble et al., 1976; Jähnig, 1976). The pK_{app} is dependent on the surface potential (ψ_0) according to

$$pK_{app} = pK_0 + e\psi_0/2.303kT \quad (2)$$

with pK_0 being the intrinsic pK, e the electric charge, k the Boltzmann constant, and T the absolute temperature (Träuble et al., 1976).

The surface potential ψ_0 in turn depends on the surface charge density σ, which can be calculated from the area of the lipid molecules at the bilayer–water interface and the number of charges of the head group, in our case changing between −1 and −2 depending on the degree of dissociation α. The relation between pK_0 and α is given by

$$\alpha = K_0/\{K_0 + [H^+] \exp(e\psi_0/kT)\} \quad (3)$$

with $[H^+]$ being the bulk proton concentration.

In the regime of high surface charge density, the exponential expression $\exp(e\psi_0/kT)$ can be replaced by $(2\sigma/c)^2$ (Träuble et al., 1976) with the constant c being

$$c = (\epsilon/2\pi)(kT/e)\kappa \quad (4)$$

and the Debye length χ

$$\kappa = \{(8\pi/\epsilon)(e^2/kT)n\}^{1/2} \quad (5)$$

where ϵ is the dielectric constant of the medium and n is the number of ions per cubic centimeter.

For DMPA, the head-group charge changes from −1 to −2 between pH 7 and pH 12. The surface charge density can then be expressed as

$$\sigma = (1 + \alpha)(e/f) \quad (6)$$

with f being the molecular area for DMPA at the bilayer–water interface, which ranges between 44 and ca. 60 Å^2 (Jähnig et al., 1979; Blume, 1979). Inserting the appropriate values for e, f, and k and using ϵ = 80 and T = 300 K, all constants can be lumped together in a constant C which has a numerical value of 29.15 for f = 50 Å^2 (Träuble et al., 1976):

$$\alpha = K_0/\{K_0 + C[H^+](1 + \alpha)^2/n_m\} \quad (7)$$

with n_m being now the salt concentration in moles per liter.

The factor C changes due to temperature, changes in the dielectric constant ϵ, and changes in the molecular area f. However, increasing T decreases ϵ, leading to partly compensating effects. The effects of a change in f are somewhat larger, as f^2 goes into the calculation. Nevertheless, the calculated curves do not vary dramatically, so that for our cases C was fixed at 29.15. Using eq 7 solved for $[H^+]$, the calorimetric titration curves can be simulated by an iterative procedure under the assumption that α is proportional to the evolved integral heat of reaction. That this is essentially correct within the precision of our measurements was checked by a separate pH titration using a pH meter. Figure 5 shows a pH titration curve determined by titrating 10 mL of 0.01 M DMPA with 0.5 M NaOH. The values for α were determined from the difference in pH values between pure water serving as a blank and the DMPA vesicle suspension. In the bottom panel of Figure 5, the fractional heat of reaction ($\Delta h/\Delta h_{total}$) vs α is shown. Within experimental error, the fractional heat of reaction and α are proportional, though some deviations from linear behavior are observed at higher values of α.

Using this assumption, the titration curves can be simulated using eq 7 with pK_0 being the parameter which has to be varied to give the best fit. Figure 6 shows several calculated curves where the pK_0 was varied. These calculated curves are for a temperature of 20 °C where the ionic product for water is 10^{-14} M^2. At other temperatures, the change in the ionic product has to be taken into account (Weast, 1981). The dashed curves in Figure 4 for the curves at 11 and 53.6 °C were calculated in this way. The titration curve at 47 °C could not be sim-

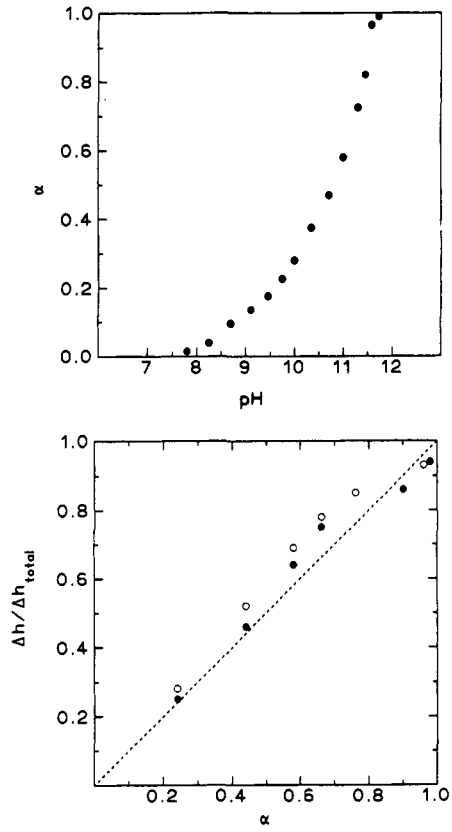

FIGURE 5: Top: Degree of dissociation (α) obtained for the titration of 10 mL of 10 mM DMPA (pH 7) with 0.5 M NaOH using a pH electrode (T = 20 °C). Bottom: Fractional heat of reaction ($\Delta h/\Delta h_{total}$) vs degree of dissociation.

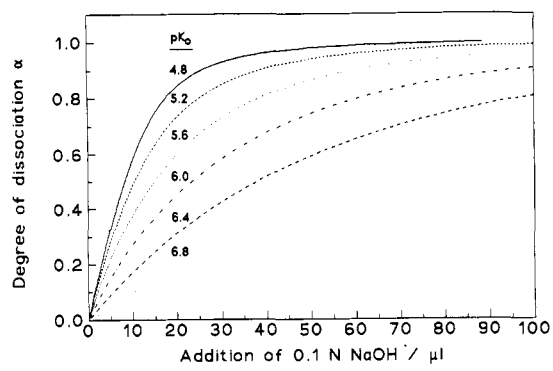

FIGURE 6: Calculated curves of degree of dissociation (α) of 1.343 mL of 1 mM DMPA vs microliters of added 0.1 M NaOH using the Gouy–Chapman theory as described in the text (T = 20 °C).

ulated due to the change in sign caused by the phase transition. The resulting pK_0 values are between 6.2 at low temperatures in the gel phase and 5.4 at temperatures above 52 °C, where the bilayers are in the liquid-crystalline phase. It is well-known that the apparent pK changes at the phase transition due to the change in surface charge density (Träuble et al., 1976; Jähnig, 1979). This leads to a decrease in the pH when DMPA goes into the liquid-crystalline phase, particularly when the system is at a pH value in the range of the apparent pK (Träuble & Eibl, 1975). The change in pK_{app} calculated from eq 2 for a change in molecular area from 44 to 60 Å^2 is, however, only ca. 0.2. Thus, the observed change in pK_0 is much larger and is caused by the heat of dissociation. In the temperature range below 23 °C and above 52 °C, the pK_0 seems to decrease with temperature. This would mean that the dissociation enthalpy determined from an Arrhenius plot of ln K_0 vs $1/T$ would be endothermic. Due to the limited temperature range of our measurements, the exact determination of ΔH_{diss} values from a van't Hoff plot is not feasible.

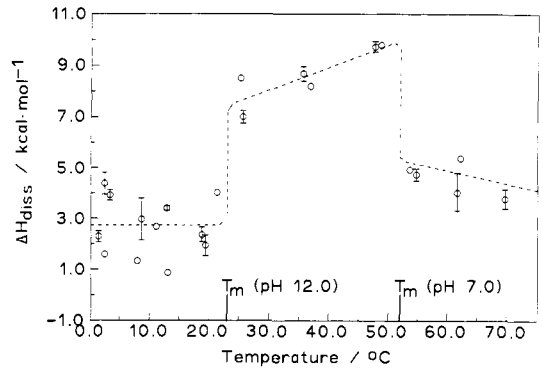

FIGURE 7: Enthalpy of dissociation (ΔH_{diss}) for DMPA as a function of temperature. Transition temperatures of $DMPA^-$ and $DMPA^{2-}$ are indicated. Experimental points with bars (maximum error) represent data obtained by batch experiments; those without bars were obtained by titration experiments. The slopes of the dashed lines in the three temperature regions (below 23 °C, between 23 and 52 °C, and above 52 °C) were calculated by linear regression. The sudden shift in ΔH_{diss} between 23 and 52 °C is caused by the pH-induced phase transition from the L_β to the L_α phase upon dissociation of the proton. Below 23 °C and above 52 °C, the bilayers remain either in the gel or in the liquid-crystalline phase.

However, in our case, we can directly determine ΔH_{diss} from the calorimetric experiments after subtracting the heat of neutralization and dilution from our experimental values for the total heat of reaction. Figure 7 shows the temperature dependence of the dissociation enthalpy as a function of temperature. Points with error bars were determined by method A, the batch-type experiments; the other points are from the end values in the titration experiments. Due to the huge contribution of the heat of neutralization of ca. 13–14 kcal·mol^{-1} to the total heat of reaction, the errors in ΔH_{diss} are relatively large. At 12.8 °C, for instance, the heat of neutralization is −14.25 kcal·mol^{-1}, and the heat of reaction after subtraction of the heat of dilution is −10.8 kcal·mol^{-1}. The precision in the determination of Δh_r is between ±1 and ±4%. Therefore, particularly at low temperatures when the heat of neutralization is large, ΔH_{diss} shows considerable scattering. Because of this effect, the temperature dependence of ΔH_{diss} below 23 °C is not clear. Linear regression analysis of the data leads to a ΔC_p of zero. The sudden jump in ΔH_{diss} between 23 and 52 °C is caused by the contribution of the transition enthalpy ΔH_{trans} to the total heat of reaction, as in this temperature range a pH jump induces the gel- to liquid-crystalline-phase transition (see Figure 1). As mentioned above, ΔH_{diss} in this temperature range includes the transition enthalpy ΔH_{trans}. Between 23 and 52 °C, the slope of the ΔH_{diss} curve is positive, giving a value of ca. +87 cal·mol^{-1}·K^{-1}. Above 52 °C, the bilayers stay in the liquid-crystalline phase during the pH jump. The temperature dependence of ΔH_{diss} gives a value of approximately −28 cal·mol^{-1}·K^{-1}.

Discussion

Reaction calorimetry can be used to obtain information on the dissociation behavior of phospholipid bilayers with dissociable groups like PA, PG, and PS. We have studied as a first example the dissociation of $DMPA^-$ as a function of temperature. From the total heat of reaction observed after a pH jump to pH 12, the dissociation enthalpy ΔH_{diss} of $DMPA^-$ can be calculated after subtraction of heat effects arising from heats of dilution and heats of neutralization. For glycero-1-phosphate, a compound resembling the head group of $DMPA^-$, the value of ΔH_{diss} was reported to be −0.145 kcal·mol^{-1} at 20 °C, decreasing to −1.82 kcal·mol^{-1} at 50 °C (Datta & Grzy-

bowski, 1958). The corresponding pK values are 6.65 (20 °C) and 6.71 (50 °C). Thus, ΔH_{diss} for glycero-1-phosphate is exothermic and shows a negative temperature dependence with a ΔC_p of −55 cal·K^{-1}·mol^{-1}. Negative ΔC_p values are characteristic for dissociation reactions in water as solvent. The dissociation enthalpy for water itself also has a negative ΔC_p of −37 cal·K^{-1}·mol^{-1} (Landolt-Börnstein, 1961). The negative ΔC_p values arise from changes in the structure of the water molecules in the hydration sphere of the ions, where they are oriented by ion–dipole interactions. For DMPA$^-$, we measured dissociation enthalpies ΔH_{diss} of approximately +2.5 kcal·mol^{-1} at 20 °C. A comparison of the absolute vlaues of ΔH_{diss} between these two systems is difficult because they are in completely different states. In a solution of glycero-1-phosphate, the distance between the charged molecules is much larger. In a 1 mM solution, the available volume per singly charged molecule is ca. 1.66 × 10^6 Å^3, corresponding to an average distance of ca. 150 Å. In lipid membranes, the head groups are all in one plane having an average available area of ca. 50 Å^2 per charge. This corresponds to a distance of 7 Å between charges. Thus, the hydration spheres of the head groups overlap, and intermolecular interactions via hydrogen bonds are possible between the phosphatidic acid head groups. Dissocation changes these interactions and also the hydration of the head groups. In addition, the pH jump to 12 causes a slight change in chain packing, as the tilt angle for DMPA^{2-} is larger than for DMPA$^-$ (Jähnig et al., 1979). An endothermic contribution would be expected from this effect because the van der Waals interactions between the chains are reduced and the chains become slightly more disordered as detected by FT-IR (Tuchtenhagen & Blume, 1991). That the transition enthalpy for doubly charged phosphatidic acids is lower than that of the singly charged form is obviously a consequence of the increase in disorder in the gel phase upon dissociation of the second proton (Blume & Eibl, 1979). Thus, this increase in chain tilt and slight disordering of the chains certainly contributes to the endothermic ΔH_{diss} value. However, various other enthalpic contributions arising from changes in hydrogen bonding between head groups and hydration of the head groups cannot be neglected, so that the observed endothermic ΔH_{diss} value cannot be interpreted in an unambiguous fashion.

The temperature dependence of ΔH_{diss} in the temperature range between 2 and 23 °C cannot be determined with any confidence because of the relatively large scattering of data. ΔC_p for the dissociation enthalpy of glycero-1-phosphate is negative with −55 cal·mol^{-1}·K^{-1}. In DSC experiments, we determined the apparent molar heat capacities, $^{\Phi}C_p$, of DMPA$^-$ and DMPA^{2-} and found a somewhat higher value for DMPA^{2-} below 23 °C (Blume, 1983). Thus, a positive ΔC_p should be expected for the dissociation reaction. The previous finding was interpreted as being caused by an increase in the exposure of hydrophobic groups to water and an increase in counterion condensation. Exposure of hydrophobic groups to water is connected with a positive ΔC_p, a characteristic feature of "hydrophobic hydration" (Tanford, 1980). The increased separation of the doubly charged head groups, as evaluated from low-angle X-ray scattering (Jähnig et al., 1979), is also evident in the FT-IR spectra as the C=O vibrational bands indicate increased contacts with water (Blume et al., 1988; Tuchtenhagen & Blume, 1991). Increased counterion condensation to the highly charged surface can also give a positive ΔC_p contribution. Due to a sharing of hydration spheres, "free water" is created, which has a higher heat capacity than the water molecules in the hydration sphere of the ions. Both effects can compensate or even overcompensate the normally negative ΔC_p for dissociation reactions.

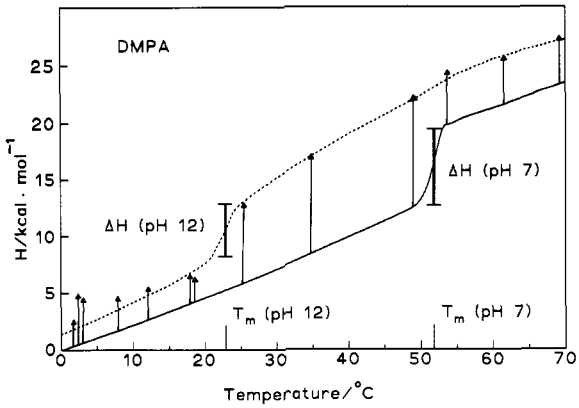

FIGURE 8: Enthalpy vs temperature diagram for the system DMPA$^-$ → DMPA^{2-} as obtained from differential scanning and reaction calorimetry. The bars represent the transition enthalpies for singly and doubly charged DMPA as obtained by DSC. The arrows indicate the enthalpy change observed by reaction calorimetry using the batch-type experiment with a pH jump from 7 to 12. The solid and the dashed lines represent the enthalpy of DMPA$^-$ and DMPA^{2-}, respectively, the slopes being determined by the apparent molar heat capacities ($^{\Phi}C_p$) evaluated from the DSC curves.

While this compensating effect is not apparent below 23 °C, it seems to be present in the temperature range between 23 and 53 °C. ΔH_{diss} is much more endothermic due to the contribution from the transition enthalpy ΔH_{trans}. The sudden increase of ΔH_{diss} at 23 °C corresponds to ΔH_{trans} for the doubly charged DMPA, which is ca. 4.1 kcal·mol^{-1} as measured by DSC. Likewise, the drop in ΔH_{diss} at 53 °C is caused by an ΔH_{trans} of 5.7 kcal·mol^{-1} of singly charged DMPA. The changes as determined from titration calorimetry are both ca. 4.5 kcal·mol^{-1} and are thus in rough agreement with the DSC values. Between 23 and 53 °C, ΔH_{diss} increases with temperature with a ΔC_p of ca. 87 cal·mol^{-1}·K^{-1}. The effects responsible for the positive ΔC_p have been discussed above. Contributions of "hydrophobic hydration" to ΔC_p are probably larger in this temeprature range, because of the transition from the tightly packed L_β phase of DMPA$^-$ to the liquid-crystalline L_α phase of DMPA^{2-}. Above 52 °C, no phase transition is induced by the pH jump, but only the dissociation of the second proton. ΔH_{diss} seems to decrease in this temperature range, as is normally found for dissociation reactions. Apparently, contributions from "hydrophobic hydration" and counterion condensation are negligible in this temperature range where the bilayers are in the liquid-crystalline phase and the head groups have a larger available area.

The results obtained for the temperature dependence of ΔH_{diss} can be plotted in an enthalpy vs temperature diagram as shown in Figure 8. The solid line represents the enthalpy of DMPA$^-$ as a function of temperature, arbitrarily set to zero at 0 °C, the slope being determined by the apparent molar heat capacity $^{\Phi}C_p$ of DMPA$^-$ as determined by a DSC experiment (Blume, 1983). The arrows schematically represent the changes in enthalpy induced by a pH jump as performed in the RC experiment. The dashed line is the enthalpy of DMPA^{2-}, the slope determined again by $^{\Phi}C_p$ evaluated from DSC experiments. The thermodynamic data for ΔH_{trans} for singly and doubly charged DMPA are shown as vertical bars. The thermodynamic data obtained by DSC (ΔH_{trans} and $^{\Phi}C_p$) and by RC (ΔH_{diss}) are consistent with each other and can thus be put together in a common diagram.

The titration experiments shown in Figure 4 allow the determination of the intrinsic pK for the dissociation reaction.

The data could be evaluated by assuming that the Gouy–Chapman theory in its simplest form is valid (Träuble et al., 1976; Cevc, 1990). Within the precision of our measurements and with the assumption that the observed integral heat of reaction is directly proportional to the degree of dissociation (α), a relatively good fit between measured and calculated curves could be obtained. The intrinsic pK_0 determined at 11 °C is 6.2 and thus comparable to values found for glycero-1-phosphate (6.65) and other monoesters of phosphoric acid like adenosine 5′-phosphate ($pK = 6.1$) or cytidine 5′-phosphate ($pK = 6.3$). For glycero-1-phosphate, the pK increases with temperature, but only slightly. Our measurements on $DMPA^-$ indicate a decrease in pK_0 with increasing temperature. At 53 °C, we determined a pK_0 of 5.4. This decrease is not due to simple surface charge effects. The apparent pK is of course dependent on the surface charge density, σ, leading to a change in the apparent pK at the transition which results in a proton pulse (Träuble & Eibl, 1975; Träuble et al., 1976). The decrease in pK_0 must have other reasons. The bilayer expansion changes the intermolecular interactions between head groups. In phosphatidic acids, the high transition temperature is thought to be partly caused by intermolecular hydrogen bonding between the phosphate head groups (Eibl & Blume, 1979; Eibl & Woolley, 1979; Boggs, 1987). This intermolecular hydrogen bonding network will probably influence the dissociation constant. When the bilayers are in the liquid-crystalline phase, the molecules become more mobile, because the average distance between head groups and chains increases. Presumably, hydrogen bonding can still occur but over increased distances and with changed dynamics. The reduced hydrogen bond strength because of the larger distance between phosphate groups may lead to a decrease in the intrinsic pK_0. Additionally or alternatively, hydration changes upon bilayer expansion can influence the dissociation constant (Cevc, 1990). At present, a consistent interpretation of the observed effects is not possible. It is clear, however, that the simple Gouy–Chapman theory can describe our titration curves but not the temperature dependence of pK_0. The decrease in pK_0 at higher temperatures is at least consistent with the direct calorimetric measurements of ΔH_{diss}. From the decrease in pK_0, a positive ΔH_{diss} value of ca. 5–7 kcal·mol^{-1} can be estimated by applying the van't Hoff equation. This agrees in sign and magnitude with the calorimetric ΔH_{diss} values shown in Figure 7. Thus, the thermodynamic data determined directly by RC and from the temperature dependence of the dissociation constant are consistent.

Summary

Reaction calorimetry in combination with differential scanning calorimetry is a valuable tool to determine the thermodynamic properties of binding reactions in lipid bilayers. Combination of data obtained by these two methods allows the construction of enthalpy/temperature diagrams which contain complete information on the thermodynamic properties of the system. We have shown here the application to the dissociation reaction of $DMPA^-$. Reaction calorimetry can also be used to study binding reactions of monovalent and divalent cations to negatively charged phospholipids. The thermodynamics of Ca^{2+} binding to $DMPA^-$ and $DMPG^-$ is one example which will be reported in a separate paper.

Registry No. DMPA, 28874-52-4.

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