Chapter 4

Differential Scanning Calorimetry of Protein–Lipid Interactions

Olga Cañadas and Cristina Casals

Abstract

Differential scanning calorimetry (DSC) is a highly sensitive non-perturbing technique for measuring the thermodynamic properties of thermally induced transitions. This technique is particularly useful for the characterization of lipid/protein interactions. This chapter presents an introduction to DSC instrumentation, basic theory, and methods and describes DSC applications for characterizing protein effects on model lipid membranes. Examples of the use of DSC for the evaluation of protein effects on modulation of membrane domains and membrane stability are given.

Key words: Differential scanning calorimetry, Lipids, Proteins, Lipid–protein interaction, Antimicrobial peptides, Data analysis

1. Introduction

Differential scanning calorimetry (DSC) is an effective analytical tool to characterize the thermotropic properties of protein–lipid interactions. This chapter attempts to provide the reader with an understanding of the capabilities of DSC instrumentation and the type of information that can be achieved from DSC studies of lipid–protein interaction. In particular, we describe in detail the analysis of DSC data to assess the effects of proteins on model lipid bilayers.

In DSC experiments, the sample under study and an appropriate reference material (a material which does not undergo a phase transition within the temperature range of interest) are simultaneously heated or cooled at identical, predetermined rates. If the sample undergoes a phase transition induced by temperature, absorption (or release) of heat from the sample will occur. As a result, a temperature differential (ΔT) develops between the sample and reference cells. This temperature differential is minimized by the instrumental control system, which supplies more (or less) heat

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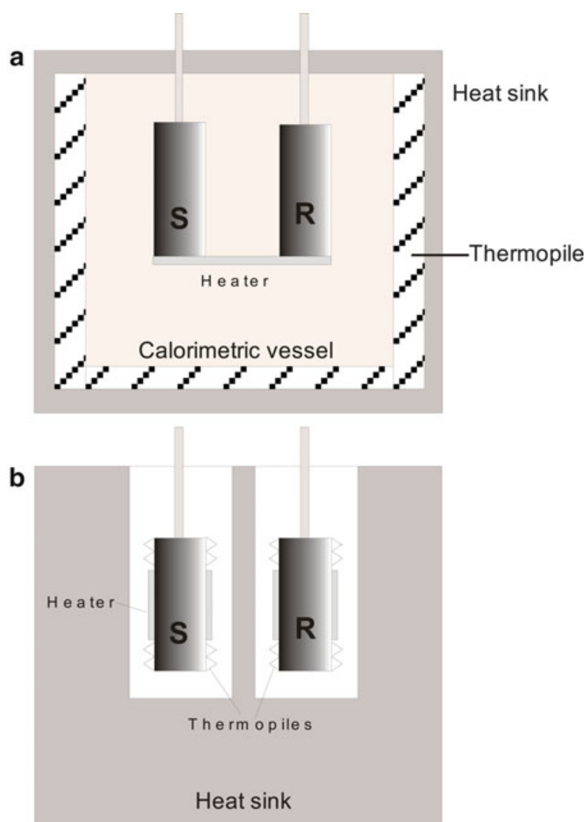

Fig. 1. Different types of differential scanning calorimeters: (**a**) heat conduction calorimeter; (**b**) power compensation calorimeter.

to the sample cell so as to maintain its temperature equal to that of the reference cell. Based on the mechanism of operation, DSC instruments can be classified into two types.

Heat Conduction Calorimeters: The calorimetric vessel is in thermal contact with a heat flow sensor which is placed on a heat sink, usually a constant temperature bath, and both sample and reference cells are heated or cooled indirectly by contact with the heat sink (Fig. 1a). The heat released (or absorbed) during the calorimetric event is allowed to flow to (or from) the surrounding heat sink. Usually, the sensor of the heat flow is a thermopile located between the sample cell and the heat sink. The temperature difference between the sample and reference cells and the heat sink results in an electrical power difference over the thermopile. The output differential power generated by the heat flow sensor is related to the flow of heat from the calorimetric vessel to the heat sink. These calorimeters have a large time constant of the measurement system. Therefore, it is necessary to operate at very slow scan rates to maintain thermal equilibrium.

Power Compensation Calorimeters: Sample and reference cells are surrounded by a cooling jacket that is maintained some 10–20°C below the temperature of the calorimetric vessel. Sample and reference cells are heated (or cooled) by independent heat sources (Fig. 1b). Both cells are maintained at the desired temperature by continuously manipulating the power supplied to an electrical heating element placed within the vessel. At the beginning of the experiment, cooling power and electrical heat are in balance. As the calorimetric event occurs, the electrical power is varied in order to maintain the desired process temperature. The difference between the electrical power at the beginning and at any time during the measurement is proportional to the heat absorbed or liberated by the calorimetric process. These calorimeters have a very short response time and operate at considerably faster scanning rates than those of heat conduction instruments.

DSC instruments are characterized by high sensitivity, a stable instrumental baseline, and a wide operational range, being able to scan aqueous solutions from 0°C up to 100°C. To supercool the sample below 0°C, the instrument must be equipped with an automatic system that heats the cells to avoid the freezing of water. On the other hand, to heat the samples above 100°C, an excess pressure of 2 atm should be applied to instruments equipped with pressure-resistant sample cells. This overpressure allows raising the temperature to 120°C and dissolves bubbles forming in the solution upon heating. Since DSC instruments compare the heat capacities of identical volumes (1), it is important to avoid the formation of bubbles that would affect the accuracy of the volume.

A calorimetric experiment usually consists of three or more scans. The first one corresponds to a buffer scan; successive scans are of the sample (see Note 1). These scans are performed to assess the reversibility of the sample since DSC data analysis is performed on equilibrium data, and samples that undergo irreversible transitions cannot be analyzed by equilibrium thermodynamics-based approaches. The equilibrium criterion usually applied is the reproducibility of the sample DSC trace in a second heating.

1.1. Lipid Thermotropic Phase Behavior

The output of any DSC experiment is the heat flow rate as a function of time. The time integral of the measured differential heat flow provides the energy of the sample (2). Figure 2 shows the typical DSC thermogram for the two-state gel (L_β)-to-liquid crystalline (L_α) phase transition exhibited by an aqueous dispersion of a single phospholipid. This phase transition is characterized by a number of thermodynamic parameters, that is, the phase transition temperature (T_m), the relative cooperativity of the phase transition, and the enthalpy of the calorimetric event (ΔH) that can be directly determined from the thermogram. The phase transition temperature, T_m, for lipid hydrocarbon chain-melting transitions corresponds to the maximum excess specific heat absorbed by the

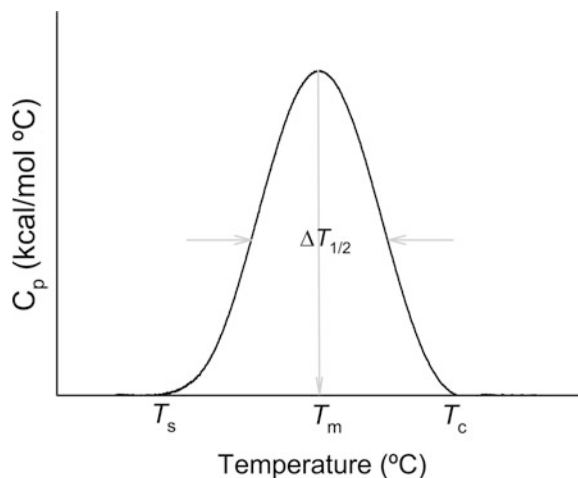

Fig. 2. DSC thermogram for a gel-to-liquid crystalline endothermic phase transition of a pure phospholipid that has reached a thermodynamic equilibrium. T_m, the transition midpoint temperature; $\Delta T_{1/2}$, the transition with at half-height; *T*s, the onset or lower boundary of the phase transition; *T*c, the completion or upper boundary temperature.

system (3–5). For lipid samples that have reached a thermodynamic equilibrium and hence show a symmetric DSC trace, T_m represents the temperature at which the gel-to-liquid crystalline phase transition is half complete (6) (see Note 2). At any point under the transition endotherm, the sample comprises a mixture of lipids in the gel and fluid phases. Below the onset temperature (T_s), the membrane is in the gel state, and above the completion temperature (T_L), the sample is in the liquid crystalline phase.

The relative cooperativity of a phase transition can be determined directly from the DSC curve since it is related to the sharpness of the transition peak, which is given by the temperature width at half-height, $\Delta T_{1/2}$, of the DSC trace. Pure single lipids usually have $\Delta T_{1/2}$ values less than 0.1°C. The presence of impurities within the bilayers broadens the transition peak. Thus, for complex lipid mixtures $\Delta T_{1/2}$ reaches values of 10–15°C (3–5). The cooperativity of a pure lipid transition is described by the cooperative unit (CU) that measures the degree of cooperation between lipid molecules. CU reaches a zero value for noncooperative processes, whereas large values of CU indicate a high cooperativity (3).

The enthalpy of the endothermic or exothermic event is evaluated by the integration of the area of the scan rate-normalized DSC peak relative to the experimental baseline (3, 4, 7).

In addition to the gel-to-liquid crystalline lamellar phases, other metastable lipid phases can occur. Examples are the pre-transition from a gel to a rippled gel ($P_{\beta'}$) phase of disaturated phosphatidylcholines and the lamellar to inverted hexagonal (H_{II}) phase transition of unsaturated phospholipids that contain small, strongly interacting polar headgroups such as phosphatidylethanolamines.

These thermotropic interconversions do not proceed under equilibrium conditions but are kinetically controlled, that is, these processes approach thermodynamic equilibrium at a slower rate than the temperature scanning rate. As a consequence, both the shape and the transition temperature of the phase transition vary with the scanning rate, and the only accessible thermodynamic parameter is the enthalpy of the process. Thus, to obtain reliable data from these types of lipid phase transitions, it is necessary to perform the experiments at slow heating rates.

1.2. Theory

Differential scanning calorimetry measures the excess heat capacity, C_p, of the sample solution with respect to the reference material, which usually is the same buffer used in the preparation of the sample solution. The calorimetric enthalpy of the transition, ΔH_{cal}, can be determined by integrating the DSC peak:

$$\Delta H_{cal} = \int C_p \cdot dT \tag{1}$$

and the entropy of the transition is given by

$$\Delta S = \frac{\Delta H_{cal}}{T_m} \tag{2}$$

where T_m is the transition temperature.

The dependence of the equilibrium constant, K, for a simple two-state transition (A $\leftrightarrows$ B) on enthalpy is given by the van't Hoff equation:

$$\left(\frac{\partial \ln K}{\partial T}\right)_p = \frac{\Delta H_{vH}}{RT^2} \tag{3}$$

where ΔH_{vH} is the van't Hoff enthalpy and R is the gas constant.

Since the standard free energy change that occurs during the transition, ΔG^0, is related to the standard enthalpy and entropy changes:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{4}$$

and

$$\Delta G^0 = -RT \ln K \tag{5}$$

it follows that

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{6}$$

Therefore, a plot of the natural logarithm of K versus $1/T$ gives a straight line, the slope of which is equal to minus the van't Hoff enthalpy divided by the gas constant, $-\Delta H^0/R$, and the intercept corresponds to the standard entropy change divided by the gas constant, $\Delta S^0/R$ (see Note 3).

The van't Hoff enthalpy is the amount of heat required for each cooperative unit to undergo the phase transition. Comparison of ΔH_{vH} with the calorimetric enthalpy provides information about the nature of the transition. For a first-order two-state transition, ΔH_{vH} is equal to the calorimetric enthalpy, whereas for systems that involve intermediate stages, the van't Hoff enthalpy is smaller than the calorimetric enthalpy. Finally, when oligomers are involved in the thermotropic transition, the van't Hoff enthalpy is larger than the calorimetric enthalpy.

The number of molecules (or moles) per cooperative unit, CU, is given by the ratio:

$$CU = \frac{\Delta H_{vH}}{\Delta H_{cal}} \tag{7}$$

Comparison of the CU values for phospholipid bilayers, in the absence and presence of different amounts of reconstituted protein, allows evaluation of the effects of the protein on the cooperativity of the phase transition.

2. Materials

2.1. Equipment

1. DSC instrument (MicroCal VP-DSC, TA NanoDSC, Setaram μDSC7 evo, or equivalent).
2. Degassing equipment (vacuum desiccator, magnetic stirrer).
3. Dialysis tubing or cassettes (e.g., Pierce Slide-A-Lyzer Dialysis Cassettes).
4. Polystyrene beads (e.g., Sigma-Aldrich Amberlite XAD-25).
5. Gel filtration columns (e.g., Sephadex G-50).
6. Centrifuge (Beckman XL-90, or equivalent).

2.2. Reagents

2.2.1. Buffer

A buffer with a small or zero enthalpy of ionization is preferable since this eliminates any ionization contributions in the measurement. Buffers such as phosphate, formate, and acetate are ideal since they have negligible ionization heats and their pH remains constant with changes in temperature. However, phosphate buffer should not be used when the phospholipid concentration is determined by phosphorus assay, since the presence of inorganic phosphorus in the buffer would interfere in the measurement. The pH and ionic strength of the buffer solution should be chosen to avoid sample aggregation (see Note 4). Although the choice of buffer depends on the type of study, the buffer most widely used to study lipid–protein interactions is 10 mM HEPES, 0.1 mM NaCl, pH 7.4.

2.2.2. Organic Solvents

Usually, a chloroform/methanol mixture is used to dissolve the lipids. When the reconstitution of hydrophobic membrane proteins into proteoliposomes is performed by co-solving lipids and protein in organic solvents, the chosen solvents should affect neither the structure nor the function of the protein.

2.2.3. Detergent

The most common detergents used for protein reconstitution into proteoliposomes are octylglucoside, CHAPS, or Triton X-100, since these mild detergents preserve the native biological structure and activity of the proteins.

2.2.4. Sample

The lipid component should exhibit phase transition temperatures below the thermal denaturation temperature of the protein of interest (see Note 5).

3. Methods

3.1. Sample Preparation

Given the variety of lipids and proteins that can be studied, each one with its own thermodynamic characteristics, there is no single protocol optimal for the preparation and characterization of all the possible lipoprotein complexes that can be formed. Therefore, we present some general guidelines for sample preparation.

3.1.1. Preparation of Proteoliposomes

Only the purest lipid and protein samples should be used in lipid–protein interaction studies. Phospholipid vesicles that contain integral membrane proteins are known as proteoliposomes and are an excellent tool for studying the structure and function of membrane proteins as well as protein effects on the physical properties of membranes. Some extremely hydrophobic membrane proteins are reconstituted into proteoliposomes by co-solving both components in an organic solvent that is subsequently evaporated to dryness under a stream of nitrogen or argon, leaving a lipid–protein film. This film is rehydrated for 1 h in an aqueous buffer at a temperature above the gel-to-liquid phase transition temperature of the lipid or lipid mixture to form multilamellar vesicles (MLVs) (see Note 6). However, most integral membrane proteins are isolated and reconstituted using detergents. There are two common approaches for reconstituting proteoliposomes via a detergent-mediated pathway (8) which involve the transfer of an integral membrane protein from a detergent solution to phospholipid vesicles. The first is a simple dilution approach: If a protein–detergent mixture is diluted in a liposome solution, the protein would transfer into the liposome when the concentration of detergent falls below its critical micelle concentration (CMC). The second approach involves introducing detergent to preformed liposomes such that the liposome bilayer becomes saturated with detergent. The detergent disrupts

lipid–lipid interactions, which results in a more permeable bilayer. These structures are more receptive to protein uptake, and after protein is introduced, the excess detergent can be removed by several methods (dialysis, column chromatography, or incubation with detergent-adsorbing beads). Dialysis is done against the hydration buffer devoid of detergents, the dialysate is centrifuged, and the pellet is resuspended in the buffer of interest. For detergent adsorption, polystyrene beads such as Amberlite XAD-25 can be used. Finally, for gel filtration the lipoprotein complex is passed through a gel filtration column that has been previously equilibrated with the buffer used for film hydration devoid of detergent. Turbid fractions containing the lipoprotein complex are collected, the sample centrifuged, and the pellet suspended in the buffer of interest.

3.1.2. Proteoliposome Characterization

The mode and extent of interaction of membrane proteins with phospholipid vesicles might depend on the conditions of preparation of lipid–protein samples (9), and care should be taken in the interpretation of findings from reconstituted systems. Prior to the DSC measurement, it is necessary to determine the chemical composition of the reconstituted proteoliposomes and characterize their morphology (9). It is also necessary to check the presence of residual solvents or detergents in the sample (see Note 7).

3.1.3. Sample Degassing

To avoid bubble formation, which would affect the accuracy of the volume and add experimental noise to the thermogram, it is necessary to degas under vacuum with gentle stirring both the sample and reference solutions (see Note 8).

3.2. DSC Procedure

3.2.1. Loading the Calorimeter

Load the degassed buffer solution into the sample and reference cells and scan the calorimeter up and down several times until the baseline stabilizes. Without stopping scanning, replace the solvent in one of the calorimetric cells with the sample solution. This must be done when the instrument is cooling down the sample and the temperature of the cells is close to room temperature.

3.2.2. Application of Excess Pressure

Apply an excess pressure not lower than 2 atm to the sample and reference solutions.

3.3. Data Analysis

3.3.1. Reference Substraction

In most experiments both sample and reference data are obtained, hence the first step in data analysis is to subtract the reference from the sample data.

3.3.2. Baseline Substraction

After normalizing the result by concentration, the following step is to subtract a pre-transition to post-transition baseline from the overall curve. Due to ΔC_p effects, the pre- and post-transition baselines usually differ. This makes it necessary to estimate the behavior of the baseline in the region between the onset and completion of the transition, where a mixture of lipid molecules

in different phase states occurs. To that end, the temperature range scanned during a DSC assay should be broad enough for the instrument to establish stable baselines before and after the transition peak. Different approaches can be used to establish a proper baseline, as the progress, step, linear, or quadratic interpolations of pre- and post-transition baselines. Given that each possible baseline somehow affects the apparent shape and area under the transition, which in turn results in errors in ΔH_{cal} and ΔH_{vH}, it is desirable to check several baseline procedures to get an idea of the uncertainties derived from each baseline type.

3.3.3. Data Deconvolution

The final step is to deconvolute the data to obtain the thermotropic parameters T_m, $\Delta T_{1/2}$, and ΔH_{cal}, from which it is possible to calculate the van't Hoff enthalpy, entropy, and Gibbs free energy of the transition.

To properly characterize the effect of a protein on the thermotropic behavior of a lipid bilayer, it is necessary to determine, in the first place, the thermodynamic parameters of the lipids alone, that is, T_m, $\Delta T_{1/2}$, and ΔH_{cal}.

3.4. Data Interpretation

The sensibility of the phase transitions to the presence of exogenously added compounds has converted DSC in a valuable tool to study lipid–protein interactions. Monitoring changes in T_m, $\Delta T_{1/2}$, and ΔH can yield information concerning the ability of proteins to interact with lipid membranes and affect the lipid acyl chain packing, which provides insight into their interaction mechanism.

According to Papahadjopoulos (10), the binding mode of the lipid–protein interaction can be determined from the effect of the protein on the gel-to-liquid crystalline lipid phase transition. Thus, hydrophilic proteins that interact with the bilayer surface through electrostatic forces produce no change or a slight increase in the T_m and the $\Delta T_{1/2}$ of the transition, and a substantial increase in ΔH in a dose-dependent manner. These proteins normally do not expand phospholipid monolayers, nor alter the permeability of phospholipid vesicles into which they are incorporated. These peripheral membrane proteins are thought to interact with the surface of the phospholipid bilayer exclusively by electrostatic forces. Hence, protein interaction induces stronger effects on the phase transitions of negatively charged phospholipids rather than on zwitterionic phospholipids bearing no net charge. An example of this behavior would be the interaction of pulmonary surfactant protein A (SP-A) with surfactant-like membranes (11). The interaction of SP-A with liposomes consisting of 1,2-dipalmitoyl-phosphatidylcholine (DPPC)/1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG)/palmitic acid (PA) (28:9:5.6, w/w/w) leads to a protein concentration-dependent increase in the T_m (Fig. 3). Moreover, SP-A leads to a narrowing of the phase transition, indicating that this peripheral protein mediates stabilization of DPPC-rich assemblies (11).

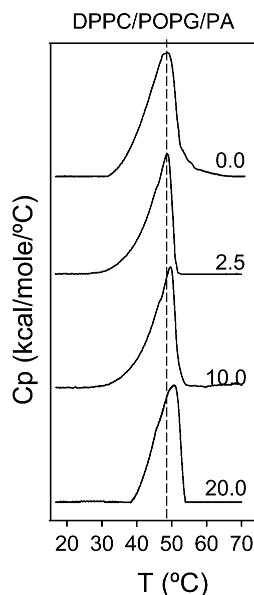

Fig. 3. Effect of SP-A on DSC heating scans of DPPC/POPG/PA vesicles (1 mM). The % SP-A/lipid weight ratio is indicated on each thermogram. Calorimetric scans were performed at a rate of 0.5°C/min. For clarity of presentation, the tick labels of heat capacity (*C*p) are not shown. For each thermogram, the scale of *C*p was from 0 to 1.4. One representative experiment of three experiments is shown (Adapted from (11), © Cell Press, 2007).

Another example is the interaction of the basic polypeptide, poly (L-lysine), with phosphatidylglycerol membranes that leads to a substantial increase in ΔH in a dose-dependent manner (4). However, with other anionic phospholipids, the ΔH of the phospholipid gel-to-liquid crystalline phase transition is reduced rather than increased. McElhaney (4) suggests that in the poly(L-lysine)-phospholipid interaction two competing effects occur: an electrostatic, charge-neutralization interaction at the bilayer surface, which condenses and stabilizes the gel-state phospholipid bilayer, and a hydrophobic interaction due to a partial penetration of the amino acid side chains into the lipid bilayer core, which decreases the ΔH as the amount of polypeptide added is increased.

In fact, if the binding of the peripheral membrane protein to the bilayer surface is followed by the partial penetration of the protein into the bilayer, where it interacts with the lipid hydrocarbon chains, a decrease in T_m and/or ΔH is observed, which might be explained by a decrease of van der Waals interactions between lipid acyl chains after protein insertion. These proteins are believed to interact with phospholipid bilayers by a combination of electrostatic and hydrophobic forces. These peripheral proteins initially adsorb to the charged polar head groups of the phospholipids and subsequently partially penetrate the hydrophilic–hydrophobic

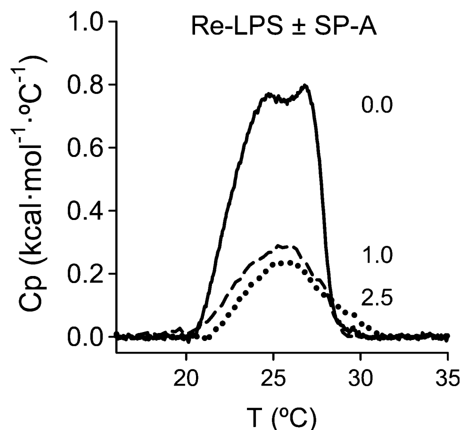

Fig. 4. Effect of SP-A on the DSC heating scans of Re-LPS membranes (0.4 mM) in 5 mM Tris–HCl, 150 mM NaCl, pH 7.4, containing 150 μM $CaCl_2$. Experiments were performed in the presence of different SP-A/Re-LPS weight ratios: 0 % (*solid line*), 1 % (*dotted line*), and 2.5 % (*filled circles*). Calorimetric scans were performed at a rate of 0.5 °C/min. Data shown are the means of four thermograms (Adapted from (13), © Cell Press, 2008).

interface of the bilayer to interact with a portion of the lipid hydrocarbon chains. These proteins normally expand phospholipid monolayers and alter the permeability of phospholipid vesicles into which they are incorporated. A good example of this type of proteins is the C-reactive protein (CRP) (12), which is a plasma protein considered the prototypical acute-phase protein in humans and other animal species. The presence of packing defects produced by coexisting ordered/disordered domains within membranes facilitates the partial insertion of CRP in the membrane (12). Another example is the effect of pulmonary surfactant protein A (SP-A) on model bacterial membranes composed of deep rough lipopolysaccharide (Re-LPS) (13). SP-A favors the penetration of water molecules in Re-LPS membranes and causes a sharp decrease of the overall transition enthalpy of these membranes (Fig. 4). Such SP-A effects are due to protein-induced perturbation of the lipid packing. SP-A induces Re-LPS molecular loss by promoting the formation of calcium-mediated protein aggregates that contain LPS. This would result in decreased van der Waals interactions between LPS hydrocarbon chains, consistent with SP-A-mediated decrease in the overall transition enthalpy of Re-LPS membranes observed by DSC. Other examples of peripheral proteins that reduce the T_m and significantly reduce the ΔH of the chain-melting transition of anionic phospholipids include the myelin basic protein and cytochrome c, indicating that hydrophobic as well as electrostatic lipid–protein interactions are important in these systems (4).

Finally, hydrophobic integral membrane proteins, which deeply penetrate into the hydrophobic core of the lipid bilayers, prevent

some lipid molecules from participating in the melting transition. As a consequence, these proteins have little effect on T_m, but reduce the cooperativity of gel-to-liquid crystalline phase transitions and decrease ΔH usually linearly, with increasing protein concentration. In this case, the dependence of the transition enthalpy on the protein/lipid molar ratio is given by (14):

$$\Delta H = \Delta H_0 \cdot \left[1 - m \cdot \frac{P}{L}\right] \quad (8)$$

where ΔH_0 and ΔH are the transition enthalpies in the absence and presence of protein, respectively; P/L is the protein/lipid molar ratio; and m is the number of phospholipid molecules prevented from participating in the gel-to-liquid crystalline phase transition by one molecule of protein.

The number of phospholipid molecules that do not participate in the transition can be determined by extrapolating ΔH to zero in the ΔH versus protein/lipid molar ratio plot (see Note 9).

The decrease of the T_m induced by the protein, known as the van't Hoff freezing point depression, can be estimated by the following equation:

$$\Delta T_m = -\left(\frac{RT_m^2}{\Delta H}\right)X \quad (9)$$

where ΔH is the lipid-melting enthalpy of the lipid bilayers, R the universal gas constant, T_m the lipid-melting temperature, and X the molar fraction of the protein or peptide incorporated into the membrane. In the case of nonideal mixing:

$$\Delta T_m = -\left(\frac{RT_m^2}{\Delta H}\right)X(1 - wX) \quad (10)$$

where w is the nonideal mixing parameter.

There are a number of integral transmembrane proteins that exhibit this behavior (increase in $\Delta T_{1/2}$ and substantial decrease in ΔH). These include the VSV G protein (15), myelin proteolipid protein (3, 16), glycophorin (14), bacteriorhodopsin (17), cytochrome oxidase (18), cytochrome b_5 (19), $(Ca^{2++}\ Mg^{2+})$–ATPase (20), the transmembrane pulmonary surfactant peptide SP-C (21), and the monotopic integral membrane surfactant protein SP-B (9).

Information on miscibility of lipids and proteins can also be obtained by studying the effect of proteins on the transition temperature and shape of the DSC peaks. If one protein exhibits ideal mixing with both lipid phases, a symmetric broadening of the DSC curve is observed (22). Conversely, if the protein does not bind well with both lipid phases, it aggregates into clusters at all temperatures, reducing its interface with the lipids and slightly affecting the transition. For proteins that mix well with the gel phase, the thermogram shifts upward, broadening the high-temperature side of

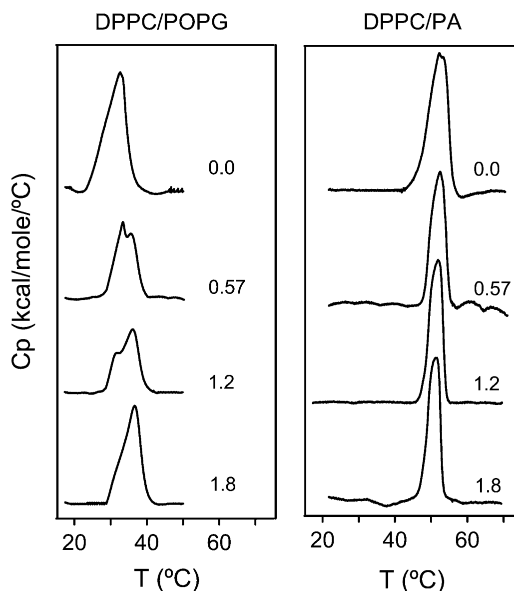

Fig. 5. DSC heating scans of DPPC/POPG and DPPC/PA multilamellar vesicles (1 mM) in the absence and presence of different concentrations of KL4. The mole percentage of KL4 is indicated on each thermogram. Calorimetric scans were performed at a rate of 0.5 °C/min (Adapted from (23), © Wiley, 2006).

the transition. In the case of proteins that show good mixing with the fluid phase lipids, the protein accumulates in the fluid phase, shifting the DSC curve to lower temperatures and broadening the low-temperature side of the transition.

The specific interaction of a protein or peptide with a phospholipid class could induce the formation of domains within lipid membranes. The appearance of shoulders or double peaks in the DSC trace of lipid mixtures indicates that the protein has a higher affinity for one lipid species (23, 24). Figure 5 shows an example of the application of DSC to investigate peptide-induced redistribution of lipids in DPPC/POPG bilayers (23). DSC thermograms indicated that, in the presence of a cationic and hydrophobic 21-residue peptide KLLLLKLLLLKLLLLKLLLLK, KL4, the thermal transition of DPPC/POPG vesicles is characterized by a double peak, which is not observed in DPPC/PA vesicles. It must be generated by electrostatic interactions between the positively charged lysine residues of KL4 and the anionic headgroup of POPG. Another example is that of penetratin, an antimicrobial peptide that induces the appearance of a second peak at around 41°C in DPPC/cardiolipin bilayers (24). Without the peptide, these vesicles show a single, broad phase transition due to lipid intercalation, with a Tm of 28°C. The penetratin-induced second peak corresponds to the temperature at which the phase transition of pure DPPC occurs. This indicates that penetratin preferentially binds and recruits cardiolipin, leaving a domain highly enriched in DPPC.

The stabilizing or destabilizing effect of a peptide or protein is related to the induction of a curvature strain in the membrane. DSC can be used to assess this curvature stress exerted on the membrane. In particular, the lamellar (L_α) to type II hexagonal (H_{II}) transition of suitable model lipids, such as 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), is usually studied as the lamellar phase has no curvature, whereas the type II hexagonal phase is negatively curved. Compounds that stabilize the membrane by favoring the lamellar phase increase the L_α–H_{II} phase transition temperature, shifting the formation of the H_{II} phase to a higher temperature, whereas a downward shift of the L_α–H_{II} phase transition indicates the promotion of the H_{II} phase (25), that is, the destabilization of the membrane.

4. Conclusions

The diversity of lipid thermotropic phases and the different ways in which proteins and peptides can affect them constitute the main problem in the application of DSC to the study of lipid/protein interactions. In order to optimize the protocols for data acquisition, analysis, and interpretation, the user should have general knowledge of the thermodynamically and kinetically controlled lipid phase transitions as well as the capabilities and limitations of the available instruments.

5. Notes

1. The first scan is less reproducible than the following scans because the DSC instrument has a different thermal history prior to this scan. Therefore, the first scan usually corresponds to a buffer scan, and the next scans correspond to the sample under study. To obtain comparable results during a series of related experiments, each sample solution should be treated exactly the same. In order to improve the repeatability of the thermal scanning, it is necessary to allow the DSC instrument to remain in cycle, programming identical scan parameters: that is, equilibration prior to the run, scan rate, starting and final temperatures, filtering period, gain, cooldown, and re-equilibration. This ensures that the DSC thermal history is the same from the 2nd to the *n*th scan, which increases the repeatability of scans.

   For pure lipids that exhibit a sharp transition, scan rates of around 0.1 K/min or less, filter periods of 1–5 s, and high gain feedback are usually appropriate.

2. When the phase transition does not proceed under equilibrium conditions, as in many lipid mixtures, the DSC curve is asymmetric, and the T_m and the phase transition midpoint temperature do not coincide (3).

3. This plot is linear only in certain simple cases and over relatively narrow ranges of temperature. In general, a curvature of the van't Hoff plot is observed due to the temperature dependence of ΔH. It is necessary to subject the data to nonlinear least squares analysis employing the integrated form of the van't Hoff equation (26):

$$\ln \frac{K}{K_0} = \frac{\Delta H_0 - T_0 \Delta C_p}{R} \left(\frac{1}{T_0} - \frac{1}{T} \right) + \frac{\Delta C_p}{R} \ln \frac{T}{T_0} \qquad (11)$$

where ΔC_p is the temperature-dependent heat capacity change, T_0 is an arbitrarily selected reference temperature, and K_0 and ΔH_0 are the equilibrium constant and the van't Hoff enthalpy at that temperature.

4. Sample aggregation causes a rapid downward shift and/or an increase in baseline noise, hampering data analysis. This can be reduced by decreasing the concentration, changing the pH, and/or using a different buffer.

5. Usually, DSC studies have been performed with model bilayers composed of a single phospholipid class, that is, phosphatidylcholine to model eukaryotic cell membranes and anionic phospholipids to model bacterial cell membranes. However, lipid mixtures should be used to examine the role of different parameters, such as electrostatic interactions, lipid bilayer fluidity, or the preferential interaction of a protein with a certain lipid component, on the lipid–protein interaction. When designing a lipid mixture, it is necessary to consider the miscibility of the lipids since not all the lipids are miscible.

6. Multilamellar vesicles are usually used for DSC studies since they provide the clearest resolution of the phase transitions (7, 27).

7. For proteins that partition between water and the membrane, the concentration of protein that interacts with the membrane decreases depending on the lipid/water ratio. Since the accuracy of the estimated ΔH_{cal} depends critically on the purity of the sample, it is necessary to remove any uncomplexed lipid or protein and determine the lipid/protein ratio of the reconstituted lipoprotein complex. Protein concentration is usually determined by measuring the absorbance at 280 nm, provided a reliable molar extinction coefficient is available, whereas lipid concentration is evaluated through a phosphorus assay or by inclusion of trace quantities of ^{14}C lipids in the reconstitution mixture followed by liquid scintillation counting of the reconstituted proteoliposomes. On the other hand, because of

the preparation procedures of the proteoliposomes, the thermotropic properties of the reconstituted lipoprotein complex can be affected by detergent or solvent traces (3). Therefore, it is necessary to check the proteoliposomes for the presence of residual organic solvent or detergent molecules. Finally, alterations in lipid vesicle size and homogeneity induced by the reconstitution procedure or the presence of the protein can modify the thermodynamic properties of lipid chain-melting transitions (3). Hence, to prevent the failure of the DSC study, it is also necessary to characterize the morphology of the lipoprotein complex.

8. The amount of air that can be dissolved in water decreases with the temperature. Thus, the sample should be equilibrated at room temperature before the degassing is started. If the sample is being stirred, it will take only 5 min of vacuum to completely degas the sample and reference solutions.
9. Sometimes, positive deviations of linearity are observed. This indicates that either the protein aggregates within the bilayers, and hence the number of lipid molecules that are affected by the protein decreases, or the conformation of the hydrophilic regions of the protein has changed, modifying their interaction with the bilayer.

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