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High sensitivity differential scanning calorimetry investigation of the interaction between liposomes, lactate dehydrogenase and tyrosinase

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Abstract

High sensitivity differential scanning calorimetry (HSDSC) has been used to study the interaction of the model proteins lactate dehydrogenase (LDH) and tyrosinase with dimyristoylphosphatidylcholine (DMPC) liposomes, and relate this to the thermal and physical stability of the proteins.

On heating, both LDH and tyrosinase denatured irreversibly in a time-dependent manner and modified the phase transition behaviour of DMPC liposomes at all concentrations investigated. The most marked effects occurred for the pretransition rather than the main phospholipid phase transition. The effects on the bilayer are likely to result from electrostatic interactions of the hydrophilic proteins with the head-groups of DMPC molecules, whilst due to their hydrophilic nature they do not penetrate into the bilayer. Tyrosinase is more highly ionised than LDH at the pH of the investigation, which may explain why tyrosinase has a greater effect than LDH on the HSDSC scans at mg/ml protein concentrations.

Keywords: High sensitivity differential scanning calorimetry; Lactate dehydrogenase; Liposome; Phospholipid; Protein; Tyrosinase

1. Introduction

The nature of the distribution of protein drugs within a liposomal carrier is important because any interactions between the lipid membranes and protein may alter the physiochemical properties of the protein and affect its release rate and bioavailability following administration (Hamilton et al., 1991; Gasset and Onaderra, 1991).

Differential scanning calorimetry (DSC) provides information on the temperature dependence of the excess specific heat over a wide temperature range. This allows the determination of thermodynamic functions for well-defined molecular systems. DSC interpretation is normally based on the equilibrium thermodynamic expression shown in Eq. (1):

$$(\mathrm{d}\ln K/\mathrm{d}T)_P = \Delta H_{vH}/RT^2 \quad (1)$$

where K is the equilibrium constant, T the absolute temperature, ΔH_{vH} the Van't Hoff enthalpy (a standard state quantity, which reflects the shape of the transition), R the gas constant and P is the constant pressure (Sturtevant, 1987). The equation is applicable to a two-state process, i.e. reactions in which intermediate states between the initial and final states are not significantly populated at equilibrium. For two-state processes the Van't Hoff expression (Eq. (2)) also applies:

$$\Delta H_{vH} = ART^2 C_{ex1/2}/\Delta H_{cal} \quad (2)$$

where ΔH_{cal} is the calorimetric specific enthalpy, T is the absolute temperature at which the process is half completed, $C_{ex1/2}$ is the excess specific heat at T, and A is a constant which for two-state processes has the value of 4.0.

The ratio of ΔH_{vH} to ΔH_{cal} can be used to give an estimate of the cooperativity of the transition (Ford and Timmins, 1989). Attempts can then be made to relate these macroscopic thermodynamic parameters to data on microscopic structural/conformational changes occurring in the sample.

DSC has been used to characterise proteins. As the protein is heated the transition from the native to the unfolded state

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results in an exothermic peak on the DSC scan. Small globular proteins, which are monomeric, denature in a simple two-stage process due to the equality of ΔH_{cal} and ΔH_{vH} and hence have a cooperativity value of 1 (Sturtevant, 1987). Cooperativity values above 1 indicate the presence of one or more thermally distinct intermediate states in protein unfolding, whilst cooperativity below 1 is indicative of complex systems, which lack thermally distinct intermediate states in their unfolding. This is typical of proteins, which are oligomers (Privalov, 1980).

DSC has been used to study how proteins interact with liposomes and attempts have been made to classify proteins according to their effects on liposomal phase transition behaviour (Papahadjopoulos et al., 1975). The location of proteins within liposomes will ultimately determine the stability, loading efficacy, and release rate of proteins from their carriers following administration.

Lo and Rahman (1995) used both DSC and high sensitivity DSC (HSDSC) to investigate the location of proteins in liposomes. HSDSC has several advantages over traditional DSC. The resolution of the instrument is greater, the thermopile is more sensitive than traditional DSC instruments and it is suitable for use with liquids and a manageable sample volume of around 1 ml can be used. Hydrophobic proteins had a profound effect on the phase transition behaviour of liposomes by partially integrating into the bilayer structure, whereas hydrophilic proteins did not interact with the bilayer to an extent, which altered the phase transition behaviour of the liposomes. HSDSC has shown that the thermal stability of ribonuclease A and cytochrome c was improved by binding to the liposomal phospholipid bilayers (Lo and Rahman, 1998).

In this study HSDSC was employed to study the interaction between the model proteins, lactate dehydrogenase and tyrosinase, and liposomal formulations.

2. Materials and methods

2.1. HSDSC analysis of proteins

Lactate dehydrogenase (LDH), Type II from rabbit muscle, was obtained from Sigma, UK in a stabilising solution of ammonium sulphate, pH 6. This was dialysed into Sørensen's modified phosphate buffer, pH 7.4 (SMPB) in accordance with the method of Harris and Angel (1989) and made up to concentrations of 25 μg/ml and 1 mg/ml using SMPB.

Tyrosinase, with an activity of 5350 units/mg was obtained from Sigma, UK as a lyophilized powder isolated from the mushroom species *Agaricus bisporus*. This was dissolved in SMPB, pH 7.4 to give solutions of 50 μg/ml and 2 mg/ml.

Solutions (0.8 ml) were placed in the HSDSC sample vessels of the Microcal DSC III (Setaram, France), which has an internal calibration system. SMPB (0.8 ml) was placed in the reference vessel and a scan rate of 1 °C/min from 5 to 90 °C was employed. In addition, an isothermal and ramping experiment was conducted in which the sample was heated to 40 °C at 1 °C/min, held isothermally for 30 min and then the temperature increased by 5 °C at a scan rate of 1 °C/min and held at 45 °C for 30 min. This process was repeated up to an isothermal hold of 90 °C.

2.2. Protocol for HSDSC analysis of liposomes

Multilamellar vesicles were prepared from DMPC by hydrating thin films as described by Leung et al. (1996) with either SMPB to produce 'empty' liposomes or with LDH solution (25 μg/ml and 1 mg/ml) or tyrosinase solution (50 μg/ml and 2 mg/ml) to give a final lipid concentration of 10 mg/ml. HSDSC studies were performed on liposomes using the Microcal DSC III (Setaram, France). A fill volume of 0.8 ml and a scan rate of 1 °C/min over a temperature range 5–35 °C were employed. The peak temperatures, peak width at half height and enthalpies of the pretransition and main phospholipid phase transition were recorded. A DSC trace of enthalpy versus temperature, allows the excess specific heat of the transition to be calculated. The derivative of the curve is equal to the temperature rate of heat absorption and equivalent to the excess heat capacity. The heat capacity curve is used as follows: the integral under the curve is equal to the enthalpy of transition, the melting temperature is approximately the temperature at which C_p is maximum, the half height width (HHW) of the transition is related to both the purity of the system and the cooperative nature of the transition. The ΔH_{vH} and the cooperativity of the transitions were calculated from the calorimetric data obtained (Eq. (2)). Where appropriate, the scans were extended to include temperatures up to 90 °C to determine whether the presence of the liposomes affected the protein denaturation profile.

3. Results and discussion

3.1. Thermal analysis of LDH

In this study, the calorimeter was not sufficiently sensitive to detect dissociation into sub-units, or protein unfolding at a concentration of 25 μg/ml. Fig. 1 shows a typical HSDSC trace obtained when the protein was examined at a concentration of 1 mg/ml. The magnitude of the peak observed is in the order of 0.3 mW, which is very small in comparison to the peaks obtained for the phospholipid phase transitions (see later). The scan began to deviate from the baseline at between 50 and 60 °C, which corresponds to the previously reported denaturation temperature of 56 °C for LDH (Stellwagen and Wilgus, 1974) with a small endothermic peak. This may represent a small conformational change in protein structure, which is responsible for loss of enzymatic activity. The exothermic peak seen at around 70 °C has been suggested to represent break up of the tetramer into the monomer sub-units, breakage of disulphide bonds and unfolding of the tertiary structure of proteins (Privalov, 1980).

In order to determine whether the denaturation process was reversible the area in which protein unfolding was observed in the heating cycle was also studied during the cooling cycle. No peaks were observed in the cooling cycle indicating that the thermal denaturation of the protein was irreversible.

In the isothermal and ramping experiment, no changes in heat flow were seen when the temperature was held at 40 °C

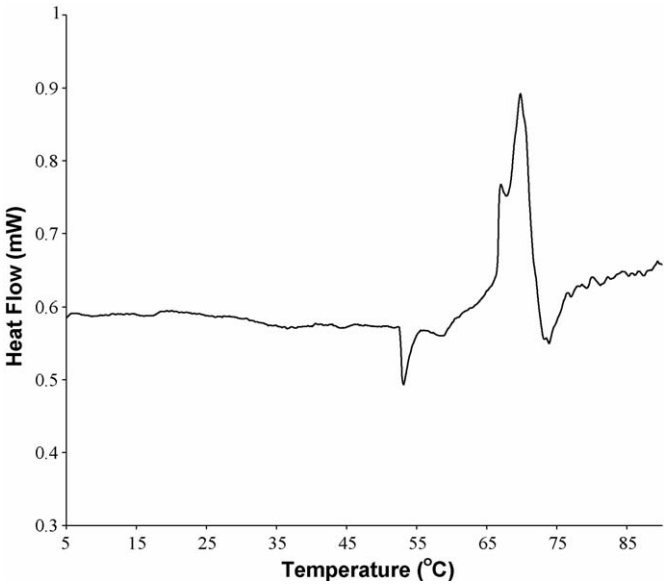

Fig. 1. A typical HSDSC trace obtained when LDH 1 mg/ml is heated from 5 to 90 °C at a scan rate of 1 °C/min.

(Fig. 2). At the 45 and 50 °C isothermal hold there was a small but detectable heat flow change in the sample. At the 55 °C isothermal hold (close to the denaturation temperature of the enzyme) a larger change in heat flow in the order of 0.1 mW was observed. No changes in heat flow were seen at holding temperatures of 60 °C and above. No peaks representative of protein unfolding were observed at 70 °C. It is possible that the protein may have uncoiled at lower temperatures as a consequence of the isothermal holding periods. These experiments indicate the progressive denaturation of LDH.

3.2. Thermal analysis of tyrosinase

At 50 μg/ml tyrosinase, no changes in heat flow with scanning temperatures from 5 to 90 °C were observed. At 2 mg/ml, exothermic peaks were observed but the shape and position of the peaks were not reproducible. This may be because the protein is a natural product, which may contain impurities, which result in poor reproducibility. The supplier was unable to provide any details concerning the purity of this enzyme. The HSDSC trace obtained on cooling samples from 90 to 5 °C was reproducible, showing the same profile as buffer alone, indicating that any changes occurring within the sample due to heating were irreversible.

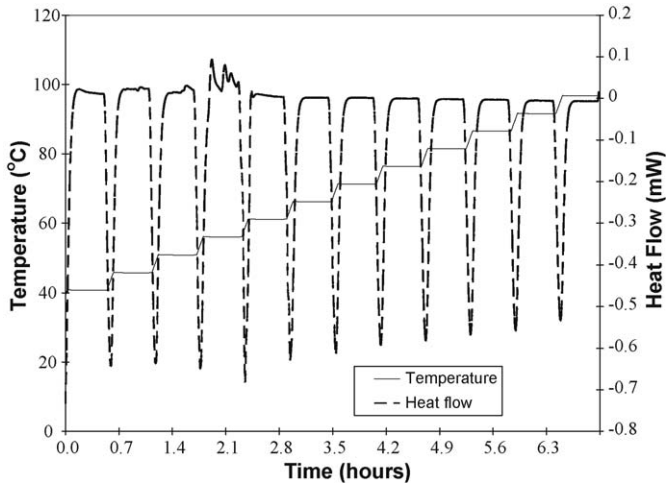

Fig. 2. A typical HSDSC trace showing the effects of repeatedly ramping at 1 °C/min for 5 min and isothermal holds of 30 min on LDH 1 mg/ml.

3.3. Thermal analysis of liposomal/protein systems

In all liposomal/protein systems, clear and reproducible pretransitions and main phospholipid phase transitions were observed. The pretransition has been interpreted as transition of the planar gel phase, to a gel phase in which ripples are formed in the bilayer. This may be due to rotation of the phospholipid polar head-groups or cooperative movement of the hydrocarbon chains prior to "melting" (Hinz and Sturtevant, 1972). When the liposomal bilayer is heated further, it undergoes the main gel to liquid–crystalline transition, which is a cooperative "melting" of the phospholipid hydrocarbon chains.

Fractional areas of the HSDSC traces were used to calculate mole fractions over the temperature range of the transitions and hence to calculate K. A graph of $\ln K$ against $1/T$ will have a gradient of $-\Delta H_{vH}/R$ and therefore ΔH_{vH} could be calculated using the integrated form of the equilibrium thermodynamic (Van't Hoff) expression (Eq. (2)).

When all data sets were compared there were significant differences in peak temperatures, half height width (HHW), ΔH_{cal} and ΔH_{vH} of both the pretransition and the main phospholipid phase transition and the cooperativity of the pretransition ($p < 0.05$). The only parameter that was the same for all data sets was the cooperativity of the main phospholipid phase transition ($p < 0.05$) (Tables 1 and 2).

The effect of LDH at 25 μg/ml and 1 mg/ml on the liposome phase transitions is shown in Fig. 3. Addition of LDH resulted in significant shifts to higher peak temperatures in the pre and main phospholipid phase transitions ($p < 0.05$). Increasing the concentration of LDH did not affect the peak temperatures ($p < 0.05$).

In the presence of LDH, the pretransition peaks were significantly sharpened, with a significant decrease in HHW at both concentrations (<0.05). However, the HHW of the main phospholipid phase transition did not change in the presence of LDH. When the two concentrations were compared there was no difference in the HHW measurements of the pretransition or the main phospholipid phase transition ($p < 0.05$).

LDH at 1 mg/ml produced no change in the ΔH_{cal} of the pretransition or the main phospholipid phase transition ($p < 0.05$). LDH at 25 μg/ml gave a significantly larger ΔH_{cal} for both the pre and main transitions compared to 1 mg/ml. When LDH 25 μg/ml was compared to DMPC there was a significant increase in the ΔH_{cal} of the pretransition but no significant difference in the ΔH_{cal} of the main phospholipid phase transition ($p < 0.05$) (Tables 1 and 2). Higher ΔH_{cal} values in the presence of LDH are likely to be indicative of LDH stabilising the bilayer. It is unclear why this effect was not observed at the higher LDH concentration but it can be hypothesized that there is an opti-

Table 1
Calorimetric data for the pretransition of DMPC liposomes with and without entrapped LDH or tyrosinase (mean ± S.D., $n = 6$)

	DMPC	LDH		Tyrosinase	
		25 (μg/ml)	1 (mg/ml)	50 (μg/ml)	2 (mg/ml)
Peak temperature (°C)	13.2 ± 1.2	15.3 ± 0.2	15.2 ± 0.1	15.7 ± 0.6	17.9 ± 0.4
Half height width (°C)	2.9 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.2	2.9 ± 0.3
ΔH_{cal} (J/mol)	3009.9 ± 613.4	4231.2 ± 366.6	2768.1 ± 224.8	3629.8 ± 629.7	2651.6 ± 262.8
ΔH_{vH} (J/mol)	2400.0 ± 200.4	3500.1 ± 300.0	3175.1 ± 431.7	3761.7 ± 512.7	4100.6 ± 414.4
Cooperativity	0.8 ± 0.3	1.3 ± 0.2	1.1 ± 0.3	1.1 ± 0.3	1.5 ± 0.4

Table 2
Calorimetric data for the main phospholipid phase transition of DMPC liposomes with and without entrapped LDH or tyrosinase (mean ± S.D., $n = 6$)

	DMPC	LDH		Tyrosinase	
		25 (μg/ml)	1 (mg/ml)	50 (μg/ml)	2 (mg/ml)
Peak temperature (°C)	23.8 ± 0.4	24.8 ± 0.4	24.5 ± 0.1	24.8 ± 0.2	25.7 ± 0.1
Half height width (°C)	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.9 ± <0.1
ΔH_{cal} (J/mol)	22927.7 ± 1295.3	25396.4 ± 1323.7	21707.5 ± 759.5	23880.3 ± 2788.3	22063.7 ± 902.6
ΔH_{vH} (J/mol)	9100.0 ± 900.1	10299.9 ± 900.5	10697.2 ± 1364.3	11406.3 ± 1455.6	10536.1 ± 789.6
Cooperativity	0.4 ± 0.1	0.5 ± <0.1	0.5 ± 0.1	0.4 ± 0.2	0.5 ± <0.1

mal concentration at which LDH can stabilize the phospholipid bilayer and the lower concentration of LDH may be closer to this optimal value.

LDH increased the ΔH_{vH} of the pretransition at both concentrations, but when the concentrations were compared there was no significant difference in the observed ΔH_{vH} of the pretransition. However, when the main phospholipid phase transition was examined no differences were observed in the ΔH_{vH} ($p < 0.05$). Changes in the ΔH_{vH} are indicative of changes in the shape of the transition. This indicates that the presence of LDH influences the shape of the pretransition and the extent of this change is independent of LDH concentration. The results demonstrate that the shape of the main transition is less sensitive to the presence of LDH and support the hypothesis that LDH, although a hydrophilic protein, is interacting with the lipid bilayer.

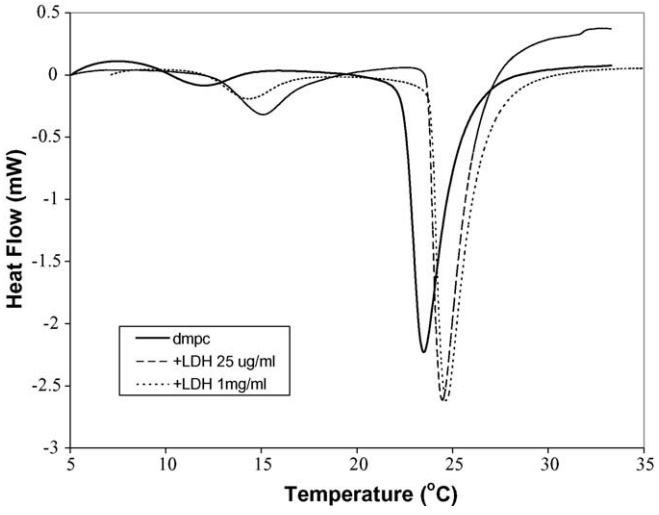

Fig. 3. Typical HSDSC traces of heating DMPC liposomes with and without LDH 25 μg/ml and 1 mg/ml.

The cooperativity of the phase transitions is given in Tables 1 and 2. LDH within the liposomes increased the cooperativity of the pretransition but had no effect on the cooperativity of the main phospholipid phase transition ($p < 0.05$). When the protein systems were compared at the high and low LDH concentrations no differences in cooperativity were seen for either the pretransition or the main phospholipid phase transition ($p < 0.05$). Generally, the presence of impurities within liposome bilayers reduces the cooperativity of lipid phase transitions with a resultant broadening of the main transition endotherm (Jain et al., 1975). The increase in cooperativity of the pretransition may be indicative of the interactions of LDH with the bilayer giving rise to the presence of more than one thermally distinct state (Privalov, 1980).

The peak previously seen for LDH at approximately 70 °C was not observed in the presence of the liposomes. Without liposomes, this peak was reproducibly seen at this temperature, with a magnitude in the order of 0.3 mW (Fig. 1), which may represent the conformational change in protein structure, which is responsible for loss of enzymatic activity. This unfolding may not be detected in the liposomal systems because the location of the protein in close proximity to the bilayer may hinder the complete unfolding of the protein's tertiary structure. This would have the effect of minimising the calorimetric changes associated with the phenomenon of denaturation to a level, which was too small for detection by HSDSC.

Fig. 4 shows the effect of 50 μg/ml and 2 mg/ml tyrosinase on the thermal behaviour of the liposomes. The shape and position of the peaks were reproducible, unlike for tyrosinase alone, indicating that the thermal response was dominated by the properties of the DMPC. A shift to higher peak temperatures was observed for both the pretransition and main phospholipid phase transition. In contrast to the LDH data, the peak temperatures increased with increasing protein concentration ($p < 0.05$). The HHW measurements were the same for the pretransitions of

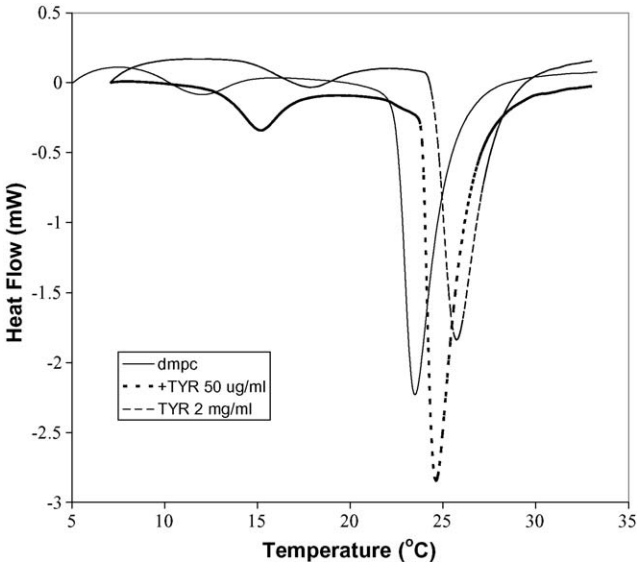

Fig. 4. Typical HSDSC traces of heating DMPC liposomes with and without tyrosinase 50 μg/ml and 2 mg/ml.

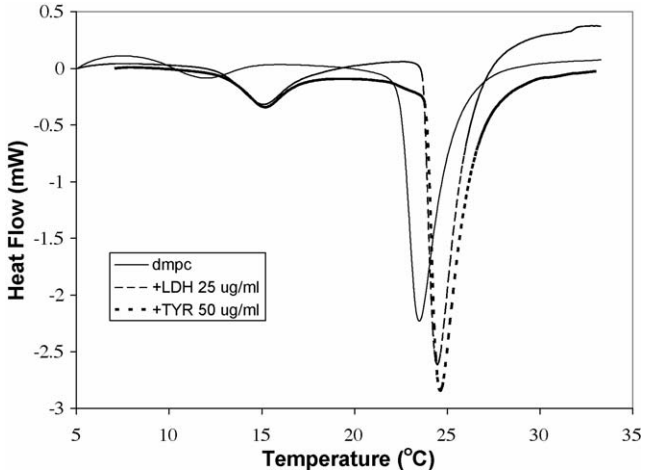

Fig. 5. Typical HSDSC traces of heating DMPC liposomes with and without tyrosinase (50 μg/ml) and LDH (25 μg/ml).

DMPC and tyrosinase at the higher concentration but in all other cases differed ($p < 0.05$, Tables 1 and 2). The pretransition and main phase transitions were sharper in the presence of tyrosinase at the lower concentration, whilst a higher concentration resulted in a broadening of the main phospholipid phase transition.

Entrapment of tyrosinase produced no significant difference in the ΔH_{cal} of either the pretransition or the main phospholipid phase transition in all cases, except for a small but significant difference ($p < 0.05$) when the pretransitions for the liposomal tyrosinase formulations were compared.

The ΔH_{vH} of the transitions were the same when the protein at the two concentrations were compared and when the main transition of DMPC and tyrosinase 50 μg/ml were compared ($p < 0.05$). In all other cases the results differed (Tables 1 and 2). The presence of tyrosinase significantly increased the ΔH_{vH} of both phase transitions and increased the cooperativity of the pretransition in a concentration dependent manner without affecting the cooperativity of the main phospholipid phase transition.

Fig. 5 compares the HSDSC scans obtained when LDH and tyrosinase were entrapped in liposomes at 25 and 50 μg/ml concentrations, respectively. The presence of protein shifts the scan to higher peak temperatures. However, interestingly, there was no difference in the peak temperatures, HHW measurements, ΔH_{cal}, ΔH_{vH} and cooperativity observed when the pretransition and the main transitions of these two systems were compared at the lower protein concentration ($p < 0.05$) (Tables 1 and 2).

In contrast, Fig. 6 shows the thermal behaviour of DMPC liposomes when 1 and 2 mg/ml concentrations of LDH and tyrosinase respectively were entrapped. At these higher concentrations, tyrosinase shifts the phase transitions to higher temperatures, and produces broader peaks than LDH. The ΔH_{cal} of the protein liposomal transitions are the same. The ΔH_{vH} of the main phospholipid phase transitions are the same but the pretransition of LDH is smaller than that of tyrosinase ($p < 0.05$). This leads to an increase in the cooperativity of the pretransition of tyrosinase 2 mg/ml in comparison to the pretransition of LDH 1 mg/ml.

The results demonstrate that both proteins interact with the liposomal bilayers and affect the phase transition behaviour of the systems. LDH and tyrosinase are globular enzymes and are hydrophilic in nature. It is highly unlikely that they have penetrated into the bilayer as greater changes in phase behaviour, particularly for the pre-transition which is very sensitive to the inclusion of materials in the hydrophobic region of the bilayer (Fildes and Oliver, 1978; Taylor and Craig, 2003) would have been observed.

The isoelectric point of LDH is approximately 6.0 (Sigma Technical Services) and that of tyrosinase 4.7–5.0 (Robb and Gutteridge, 1981) and hence both will be negatively charged in the buffer system. Tyrosinase will have the greater extent of ionisation at pH 7.4 as employed in this study. It is proposed that the proteins may be interacting electrostatically with zwitterionic polar head-groups of the phospholipid molecules within the bilayer, thereby restricting movement of molecules

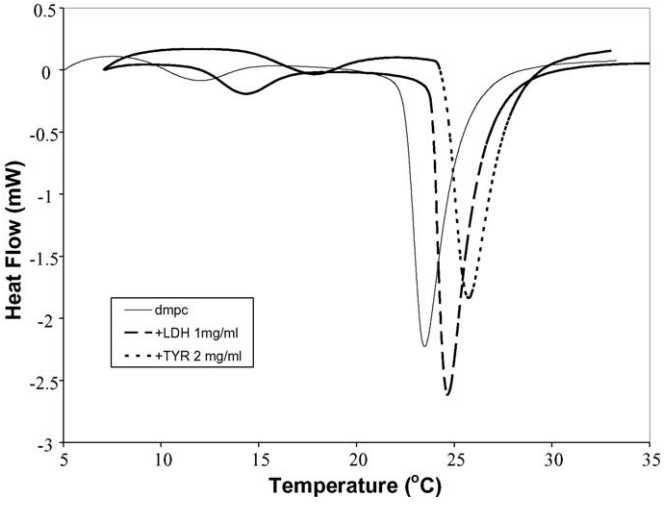

Fig. 6. Typical HSDSC traces of heating DMPC liposomes with and without tyrosinase (2 mg/ml) and LDH (1 mg/ml).

within the bilayer. This may explain why phase transitions are seen at higher temperatures in the presence of the proteins, as higher energy input is required to induce conformational changes within the bilayer due to the presence of the protein. This greater ionisation of tyrosinase may explain why tyrosinase has a greater effect than LDH on the HSDSC scans at mg/ml protein concentrations. Increasing the concentration of tyrosinase increased this interaction but increasing the concentration of LDH did not. The proteins are of similar size and shape so it is unclear why this should be the case, but it could be due differences in the acidic/basic nature of the surface of the proteins leading to different interactions with the phospholipid bilayer headgroups.

Papahadjopoulos et al. (1975) proposed that proteins could be classified into three categories based upon their effects on the thermotropic behaviour of the phospholipid. Category 1 proteins are hydrophilic, e.g. polylysine and ribonuclease A. These are believed to be adsorbed onto the bilayer surface, usually by electrostatic interactions. Category 2 proteins partially embed into the bilayers using a combination of electrostatic and hydrophobic forces, e.g. cytochrome *c*. Category 3 proteins penetrate into the core of anionic or zwitterionic lipid bilayers as a consequence of hydrophobic interactions, e.g. Gramicidin a. Being hydrophilic, LDH and tyrosinase would be predicted to fall into Category 1, and the results presented here suggest that LDH and tyrosinase were interacting electrostatically with the phospholipid bilayers, and modifying the phase transition behaviour of DMPC bilayers.

However, according to Papahadjopoulos et al. (1975) there would have been no observed change in the thermotropic behaviour of the phospholipid if these were true Category 1 proteins. However, it should be noted that Papahadjopoulos et al. (1975) used conventional DSC rather than more sensitive HSDSC employed here and they only studied positively charged proteins.

4. Concusions

HSDSC studies have revealed that LDH and tyrosinase are thermolabile and this thermal denaturation is irreversible. Tyrosinase degradation could not be reproducibly studied using HSDSC, whilst LDH denatured in a time-dependent manner. Both proteins were found to significantly affect the phase transition behaviour of DMPC liposomes at all concentrations investigated. The most marked changes were seen in the pretransition, which is a more sensitive indicator of interactions with the bilayer than the main phospholipid phase transition (Veiro et al., 1987; Castile et al., 1999). Unlike LDH, tyrosinase interacted with the bilayers in a concentration dependent manner and at mg/ml concentrations had the greatest influence on DMPC phase transition behaviour. It is believed that the proteins, which are negatively charged at the pH of this investigation, interact electrostatically with the polar head-groups of DMPC molecules in the bilayer and due to their hydrophilic nature do not penetrate into the bilayer.

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