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Thermodynamics of α-lactalbumin–DL-α-dipalmitoylphosphatidylcholine interactions and effect of the antioxidant nicotinamide on these interactions

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Abstract

Differential scanning calorimetry has been used to understand the thermodynamics of the interactions of DL-α-dipalmitoylphosphatidylcholine (DPPC) with α-lactalbumin and the effect of the antioxidant nicotinamide on these interactions. Nicotinamide decreases the thermal transition temperature of both the lipid and the protein at high concentrations. The thermal unfolding transitions of the protein were two state and calorimetrically reversible. There was no significant change in the shape and thermodynamic parameters accompanying the lipid endotherms, suggesting that nicotinamide did not penetrate the lipid bilayer. The thermal unfoldings of α-lactalbumin in the presence of DPPC as cosolute also adhered to two-state reversible mechanism. The changes in the thermodynamic parameters accompanying the thermal transitions were small, indicating no significant interaction of α-lactalbumin with DPPC. The changes in the thermodynamic parameters indicate that the lipid bilayer organization, as well as the partitioning of the extrinsic protein α-lactalbumin into the bilayer, is not affected in the entire studied concentration range of the lipid. It is observed that the presence of increasing concentration of nicotinamide (as high as 1.0 mol dm^{-3}) in the lipid–protein mixture does not affect its partitioning into the lipid bilayer, although nicotinamide preferentially interacts with α-lactalbumin. The change in the effect of nicotinamide on lipid transition temperature in the mixture and literature report suggests that nicotinamide may be forming a hydrogen-bonded complex with the protein through its amide functionality. The surface tension data of aqueous nicotinamide in combination with the thermal denaturation results of protein in presence of nicotinamide confirmed that surface tension effect does not have any significant contribution to the effect of nicotinamide on protein.

Keywords: Differential scanning calorimetry; α-Lactalbumin; DL-α-dipalmitoylphosphatidylcholine; Nicotinamide

1. Introduction

Proteins and lipids are integral parts of the biomembranes. Membrane proteins are classified as extrinsic or intrinsic depending on their association with the membrane structure. Phosphatidylcholine or 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine (or "lecithin") is a complex phospholipid which is usually the most abundant lipid in the membranes of animal tissues and is often a major lipid component of plant membranes. The saturated fatty acids are mainly concentrated in the *sn*-1 position [1]. Phosphatidylcholine in some organs contains relatively high proportions of disaturated molecular species. For example, it is well known that lung phosphatidylcholine in all animal species studied to date contains a high proportion (50% or more) of dipalmitoylphosphatidylcholine [2]. It appears that this is the main surface-active component, providing alveolar stability by decreasing the surface tension at the alveolar surface to a very low level. In addition to its role as a membrane constituent, phosphatidylcholine may have a role in signaling via the generation of diacylglycerols.

In the biological membranes, the interaction between lipid and protein is a normal phenomenon. There has

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been an increasing interest in biological processes where soluble proteins transiently bind to membranes. Such systems are essential for understanding enzyme function, cellular communications [3–5], the mechanism of action of certain toxins, pathogens, protein insertion into membranes, and protein translocation across biological membranes [6]. Many reports are available in literature showing the interaction of lipid and proteins in a qualitative manner [3–10]. The conformational changes in proteins in the presence of lipids have also been extensively studied [7,9,10]. However, quantitative studies on the lipid–protein interaction are generally rare. Understanding of the energetics associated with the lipid–protein interactions is important due to their biological significance and the role of proteins in membrane functions.

We have studied the thermodynamics of lipid–protein interactions and effect of nicotinamide on lipid and protein transitions separately and in the lipid–protein mixture. The lipid chosen is DL-α-dipalmitoylphosphatidylcholine (DPPC) and the protein chosen is α-lactalbumin for their well-established structures [11]. In vitro α-lactalbumin reversibly interacts with phospholipid membranes and has been extensively studied by NMR and CD [10,12]. α-Lactalbumin has lipophilic properties, interacting with hydrophobic peptides [13] and model lipid membranes [14,15]. However, significant association between α-lactalbumin and fatty acid has not been observed [16]. α-Lactalbumin has been found to be associated with milk fat globule membranes, suggesting a possible role for this protein in fat digestion or transport [17]. α-Lactalbumin is one of the two components of lactose synthase, which catalyzes the final step of lactose biosynthesis in lactating mammary gland. The other component is galactosyltransferase (GT), which catalyzes the transfer from UDP-galactose to either endogeneous glycoproteins, in the carbohydrate portion of glycoproteins, or to glucose when α-lactalbumin is present [18].

$$\text{UDP} - \text{galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}$$

Nicotinamide has been found to act as an antioxidant, inhibit lipid peroxidation, and stabilize membranes [19]. Aerobic cells are exposed to the damaging effect of oxidants. Lipid peroxidation is the oxidative degradation of phospholipids, cholesterol, and other unsaturated lipids by reactive oxygen species [20]. The resulting lipid hydroperoxides are detrimental to cell membranes, both structurally and functionally. Nicotinamide or vitamin B_3 preferentially inhibited the production of the species $^1O_2$, thus inhibiting lipid peroxidation [19].

The main objective of this study is to understand the lipid–protein interactions quantitatively using differential scanning calorimetry and effect of antioxidant on these interactions. Quantitative thermodynamic parameters accompanying the thermal unfolding of α-lactalbumin and gel to crystalline phase transitions of lipid have been studied in absence and presence of nicotinamide and correlated with lipid bilayer organization and partitioning of the protein in the bilayer. The interactions in the ternary system of protein–lipid–nicotinamide have also been examined quantitatively.

2. Experimental methodology

2.1. Materials

Bovine α-lactalbumin, DL-α-dipalmitoylphosphatidylcholine (DPPC), nicotinamide, NaCl, and KH_2PO_4 were purchased from Sigma (USA). KCl, Na_2HPO_4, and methanol were procured from E. Merck (India), Fluka (Switzerland), and SRL (India), respectively. $CaCl_2$ was procured from E. Merck (Germany). The compounds were of the best available purity grade. HCl and NaOH used for adjusting the pH of buffers were of reagent grade.

2.2. Method for preparation of multilamellar vesicles (MLV)

Known weight of lipid was dissolved in methanol, and the solvent was dried in a stream of dry nitrogen. It was further evaporated by keeping it in vacuum oven at 303.15 K for 1 h. One milliliter of buffer (or solution of additive or protein in the same buffer) was added to it and vortexed twice for 1 min each at a temperature above the phase transition temperature of the lipid. The buffer used was phosphate buffer saline (PBS) for studying lipid transitions. Phosphate buffer saline contained 1.5×10^{-3} mol dm^{-3} KH_2PO_4, 8.1×10^{-3} mol dm^{-3} Na_2HPO_4, 2.7×10^{-3} mol dm^{-3} KCl, 137.0×10^{-3} mol dm^{-3} NaCl, and the pH of the solution was adjusted to 7.4. Tris buffer containing 0.1 mol dm^{-3} NaCl at pH 7.0 was used to study the protein transitions.

2.3. Differential scanning calorimetry

The studies on thermal transitions of α-lactalbumin and DPPC were performed on micro-DSC from SETARAM, France. The samples for experiments were prepared and loaded in the instrument, as described earlier [21]. The scan rate was 6 K h^{-1} for studying lipid transitions and 30 K h^{-1}for protein transitions. The scan rate for protein transitions was 24 K h^{-1} when the experiments were done in presence of lipid and protein together. The reversibility was checked for all the transitions by heating the sample to just past the transition temperature, cooling, and then heating again. The thermal transition profiles of protein were deconvoluted using EXAM program of Kirchoff [22]. The protein concentration maintained in all the experiments was 0.28×10^{-3} mol dm^{-3}. For lipid transitions, the area under the curve was determined by the EXAM program

to calculate enthalpy of transition. The concentration of the lipid was 1.36×10^{-3} mol dm^{-3}, except for experiments where the lipid concentration was varied. The maximum standard deviation of the fit to the heat capacity profiles of α-lactalbumin was 0.105 kJ K^{-1} mol^{-1} and to those of DPPC was 0.013 kJ K^{-1} mol^{-1}.

2.4. Surface tension measurements

The surface tension of the aqueous solutions was measured by the drop weight method as described earlier [23].

3. Results and discussion

3.1. Differential scanning calorimetric studies on thermal transitions of α-lactalbumin and DPPC in presence of nicotinamide

The thermal denaturation of α-lactalbumin in presence of varying concentrations of nicotinamide was studied. The transition temperature and calorimetric enthalpy of α-lactalbumin in Tris buffer at pH 7.0, containing 0.1 mol dm^{-3} NaCl, were 337.8 ±0.1 K and 270±10 kJ K^{-1} mol^{-1}, respectively. The DSC endotherms of α-lactalbumin unfolding in presence of nicotinamide are shown in Fig. 1A. The thermodynamic parameters accompanying these thermal transitions are presented in Table 1A.

At high concentrations, nicotinamide acted as a destabilizer of α-lactalbumin as reflected by the decrease in transition temperature. All the thermal transitions of α-lactalbumin in the presence of nicotinamide were observed to be calorimetrically reversible. The average ratio of van't Hoff to calorimetric enthalpy is 0.95±0.04. Thus, the thermal unfolding of α-lactalbumin in the presence of nicotinamide adheres to two-state, N⇌D mechanism. The transition temperature and calorimetric enthalpy decrease with increasing concentration of nicotinamide. The change in the heat capacity of the native protein upon denaturation was 4.3±0.3 kJ K^{-1} mol^{-1}. The denaturational change in heat capacity in presence of nicotinamide, obtained from slope of the plot of ΔH_{cal} against $T_{1/2}$ was 3.5±0.5 kJ K^{-1} mol^{-1}. Thus, the alteration in the structure of the solvent upon denaturation of the protein does not differ appreciably with nicotinamide as the cosolute.

The denaturational change in preferential solvation parameter ($\Delta\Gamma_{23}$) of the protein (component 2) by nicotinamide (component 3) was calculated using the data in Table 1A and the following equation [24],

$$\Delta\Gamma_{23} = \Gamma_{D3} - \Gamma_{N3} = -\frac{\Delta H\left(\frac{\partial T_{1/2}}{\partial x_3}\right)_{pH}}{RT_{1/2}^2\left(\frac{\partial \ln a_3}{\partial x_3}\right)_{T_{1/2}}} \quad (1)$$

where, Γ_{D3} and Γ_{N3} are preferential solvation of the protein by alcohol in the denatured and native state, respectively. Here, x is the mole fraction and a is the activity of nicotinamide. The highest mole fraction of nicotinamide used in the experiments is 0.035. We have assumed unit activity coefficient in this concentration range of nicotinamide. The values of $\Delta\Gamma_{23}$ against concentration of

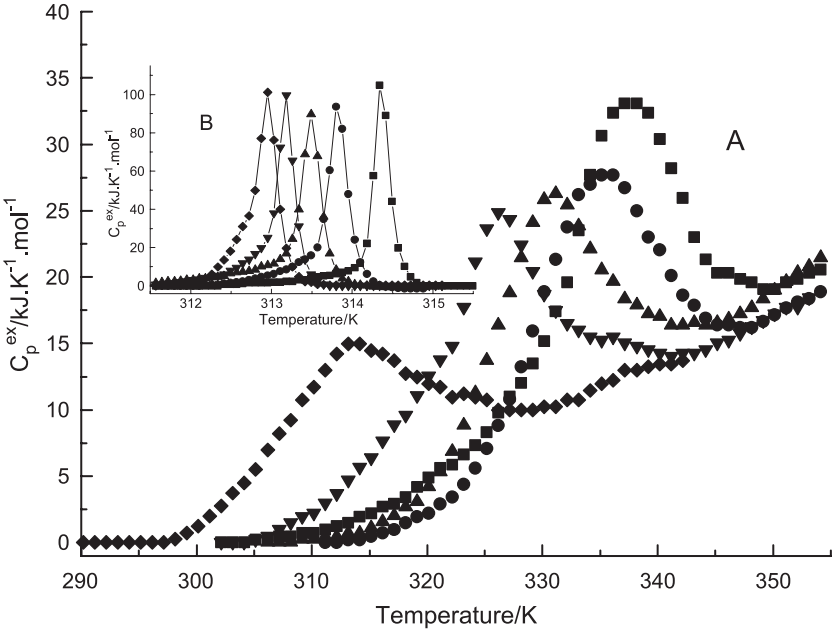

Fig. 1. (A) DSC profiles of thermal denaturation of 0.28×10^{-3} mol dm^{-3} α-lactalbumin at pH 7.0 in buffer (■), and in presence of 0.25 (●), 0.50 (▲), 1.00 (▼), 2.00 (♦) mol dm^{-3} nicotinamide; (B) calorimetric transitions of 1.36×10^{-3} mol dm^{-3} DPPC at pH 7.4 in PBS buffer (■), and in presence of 0.25 (●), 0.50 (▲), 1.00 (▼), 2.00 (♦) mol dm^{-3} nicotinamide.

Table 1
Thermodynamic parameters associated with the thermal unfolding of 0.28×10^{-3} mol dm^{-3} α-lactalbumin in 20×10^{-3} mol dm^{-3} Tris–HCl buffer containing 0.1 mol dm^{-3} NaCl at pH 7.0 at different conditions[a]

Nicotinamide (mol dm^{-3})	$T_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔC_P (kJ K^{-1} mol^{-1})	ΔS (kJ K^{-1} mol^{-1})	β ($\Delta H_{vH}/\Delta H_{cal}$)
(A) Nicotinamide concentration dependence					
0.00	337.8±0.1	270	4.3±0.3	0.800	1.04
0.25	332.6±0.3	246	3.9±0.5	0.740	0.97
0.50	330.5±0.4	242	3.4±0.3	0.732	0.92
1.00	325.7±0.2	208	3.4±0.5	0.639	0.94
2.00	313.8±0.2	182	3.1±0.1	0.581	0.97
Lipid/protein (mole ratio)	$T_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔC_P (kJ K^{-1} mol^{-1})	ΔS (kJ K^{-1} mol^{-1})	β ($\Delta H_{vH}/\Delta H_{cal}$)
(B) Lipid/protein molar ration dependence					
0.0	337.8±0.0	270	4.3±0.3	0.800	1.04
5.0	337.5±0.4	276	3.2±0.0	0.818	1.00
20.0	336.4±0.2	257	3.2±0.5	0.762	0.98
40.0	336.5±0.1	264	2.1±0.1	0.783	1.04
80.0	336.4±0.2	259	3.4±0.3	0.770	0.88
Nicotinamide (mol dm^{-3})	$T_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔC_P (kJ K^{-1} mol^{-1})	ΔS (kJ K^{-1} mol^{-1})	β ($\Delta H_{vH}/\Delta H_{cal}$)
(C) Nicotinamide concentration dependence at a lipid to protein molar ratio 5:1					
0.00	337.5±0.4	276	3.2±0.0	0.818	1.00
0.25	332.3±0.2	262	2.7±0.4	0.790	0.95
0.50	329.8±0.6	238	1.4±0.1	0.723	0.98
1.00	325.9±0.3	221	−0.5±0.3	0.678	0.93

[a] Including the errors in sample preparation, calibration constant, and reproducibility, the error assigned to the values of ΔH_{cal} and ΔS is 2% and 3%, respectively.

nicotinamide at the transition temperature of the protein are given in Table 2.

Nicotinamide is an amphiphilic molecule having a hydrophobic aromatic part, as well as a polar amide part. Hence, it is capable of interacting with both hydrophobic and hydrophilic groups in the protein. The values of $\Delta\Gamma_{23}$ are positive, indicating that there are more nicotinamide molecules in the immediate vicinity of the protein in the denatured state than in the native state, shifting the native (N) and denatured (D) equilibrium to the denatured side. It agrees with the calorimetric results that nicotinamide destabilizes the native state of protein.

The endotherms of lipid (DPPC) in PBS buffer in the absence and presence of different concentrations of nicotinamide are given in Fig. 1B. The thermodynamic parameters accompanying the transition: calorimetric enthalpy, van't Hoff enthalpy, and transition temperature are given in Table 3A. The calorimetric enthalpy of the transition was measured by integration of the thermograms by the EXAM program of Kirchoff [22]. From the fraction of the area under the curves of the lipid thermograms, the extent of reaction of the lipids were calculated [25] and plotted against temperature as shown in Fig. 2. Since the heating was carried out at constant scan rate, the slope of this plot could be used to calculate the van't Hoff enthalpy [26,27], ΔH_{vH} of the transition, using the equation,

$$\Delta H_{vH} = 4RT^{L}_{1/2}(d\theta/dT)^{L}_{T_{1/2}} \quad (2)$$

where $T^{L}_{1/2}$ is the transition temperature, and θ is the fraction of lipid in the liquid crystalline state that was calculated using

$$\theta = (A_T - A_N)/(A_D - A_N) \quad (3)$$

where A_N, A_T, and A_D pertain to the measured property at temperatures corresponding to pretransition, in between pre- and posttransition, and posttransition, respectively.

For DPPC, the transition temperature of 314.3±0.1 K and calorimetric enthalpy of 30.3±0.1 kJ K^{-1} mol^{-1} in PBS buffer, in absence of any additive, agreed well with that reported in literature [28]. It is seen that the shape of the lipid transition does not change upon addition of nicotinamide (Fig. 1B). The only observable change is a gradual decrease in the gel to liquid crystalline transition temperature with a maximum destabilization of 1.35 K in the presence of 2.0 mol dm^{-3} nicotinamide (Table 3A). The van't Hoff enthalpy and the size of the cooperative unit, given by the ratio of van't Hoff to calorimetric enthalpy,

Table 2
Denaturational change in the preferential solvation parameter ($\Delta\Gamma_{23}$) accompanying the thermal unfolding of 0.28×10^{-3} mol dm^{-3} α-lactalbumin in presence of nicotinamide at pH 7.0

Nicotinamide (mole fraction)	($\Delta\Gamma_{23}$)
0.0045	0.8
0.0089	1.5
0.0177	2.6
0.0347	4.9

Table 3
Thermodynamic parameters associated with the thermal unfolding of DPPC in PBS buffer at pH 7.4 in different conditions[a]

Nicotinamide (mol dm^{-3})	$T^{L}_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})	$(\Delta H_{vH}/\Delta H_{cal})$
(A) Nicotinamide concentration dependence (lipid: 1.36×10^{-3} mol dm^{-3})				
0.00	314.3±0.1	30.3±0.6	7407±20	244
0.10	314.1±0.0	31.9±0.6	7170±27	228
0.25	313.8±0.1	32.2±0.6	6521±39	200
0.50	313.5±0.0	31.1±0.6	6417±30	206
1.00	313.2±0.0	31.5±0.6	5747±38	180
2.00	313.0±0.0	37.5±0.7	7669±20	200
Lipid/protein (molar ratio)	$T^{L}_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})	$(\Delta H_{vH}/\Delta H_{cal})$
(B) Lipid/protein molar ratio dependence				
0.0	314.3±0.1	30.3±0.67	7407±20	243
5.0	314.4±0.0	31.4±0.6	5959±36	194
20.0	314.6±0.0	31.9±0.6	6609±40	204
40.0	314.4±0.0	28.5±0.6	4608±40	167
80.0	313.9±0.2	31.6±0.6	6037±12	197
Nicotinamide (mol dm^{-3})	$T^{L}_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})	$(\Delta H_{vH}/\Delta H_{cal})$
(C) Nicotinamide concentration dependence at a lipid to protein molar ratio 5:1				
0.00	314.4±0.0	31.4±0.6	5959±36	194
0.25	314.4±0.0	32.5±0.6	5365±35	166
0.50	314.0±0.1	32.7±0.7	3581±37	112
1.00	313.9±0.0	31.1±0.6	3282±39	106

[a] The calorimetric enthalpies for thermal unfolding of lipids are given up to one significant place in view of their smaller values compared to the calorimetric enthalpies of the thermal unfolding of proteins. Including the errors in sample preparation, calibration constant, and reproducibility, the error assigned to the value of ΔH_{cal} is 2%.

remain nearly the same over the concentration range of nicotinamide studied. This indicates that the lipid bilayer organization is not affected even in presence of 2.0 mol dm^{-3} nicotinamide. The result is in agreement with the literature reports that small solutes did not change the enthalpy of the phase transition of lipids significantly [29,30]. Nicotinamide acts as an antioxidant of lipid peroxidation by preventing the formation of the reactive oxygen species. The highest efficiency of nicotinamide as an antioxidant has been reported by Kamat and Devasagayam [19] at 40×10^{-3} mol dm^{-3} concentration. At this concentration, nicotinamide has no effect on thermal transition of the lipid. However, at higher concentrations, it lowers the transition temperature of the lipid slightly. Incidentally, this vitamin also occurs in the mammalian tissues in millimolar concentration range.

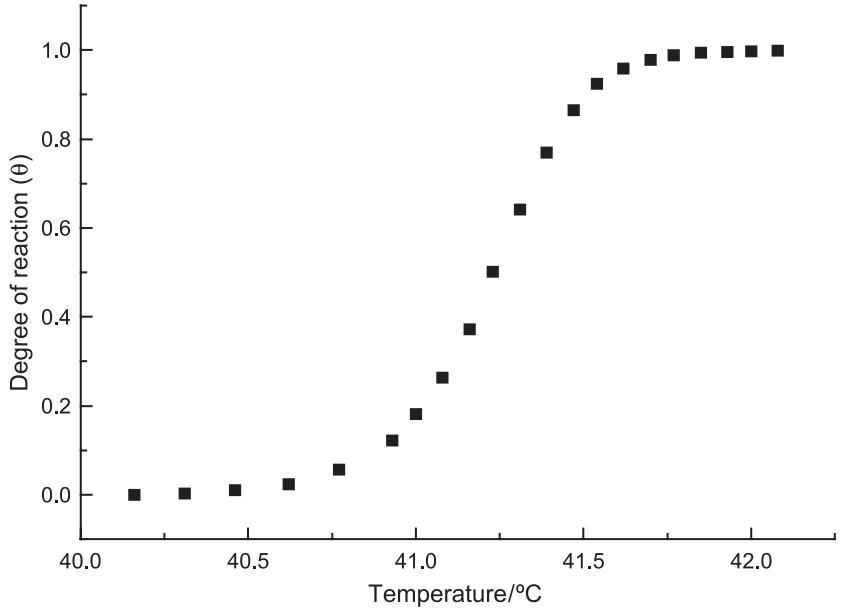

Fig. 2. Degree of gel to liquid crystalline transition of DPPC plotted against temperature.

3.2. Differential scanning calorimetric studies on thermal transitions of α-lactalbumin and DPPC in increasing molar ratio of DPPC

The effect of increasing concentration of lipid on α-lactalbumin transitions was studied. Fig. 3A shows the DSC endotherms of the thermal denaturation of protein in presence of lipid. Table 1B gives the corresponding thermodynamic parameters accompanying the protein thermal transitions. The variation in the values of transition temperature and calorimetric enthalpy is from 337.8 to 336.4 K and from 270.3 to 257.6 kJ mol^{-1}, respectively. For a lipid to protein mole ratio as high as 80:1, this variation is small. The results show that there is no significant change in the thermodynamic parameters accompanying thermal unfolding of protein in presence of increasing concentration of lipid. The very small variation in the transition temperature or the calorimetric enthalpy of the protein unfolding in presence of high concentrations of lipid supports that at the physiological pH (7.4), α-lactalbumin adsorbs only to the outer surface of the phospholipid vesicles by electrostatic attraction [31]. It also supports that calcium-enriched α-lactalbumin has a low affinity towards association to model membranes [17]. The average value of the ratio of van't Hoff to calorimetric enthalpy is 0.99±0.07, indicating that the thermal unfolding of protein in presence of DPPC is reversible and two state.

From the same set of experiments, the transition temperature and enthalpy accompanying the lipid transitions were also determined. The results are shown in Fig. 3B and Table 3B. The presence of protein also did not affect the thermal transitions of the lipid significantly. It is not surprising in view of the fact that we have changed the lipid concentration and kept the concentration of protein fixed to get the desired mole ratio, the lipid has experienced only the presence of 0.28×10^{-3} mol dm^{-3} α-lactalbumin.

3.3. Differential scanning calorimetric studies on thermal transitions of mixture of DPPC and α-lactalbumin in presence of nicotinamide

The effect of nicotinamide on lipid and protein transitions in lipid–protein mixture was also studied. These experiments were done at a lipid to protein molar ratio of 5:1. The DSC scans for the protein transitions are shown in Fig. 4A, corresponding thermodynamic parameters are given in Table 1C. The effect of nicotinamide on protein transitions was compared to those in the lipid–protein mixture. This comparison is shown in Fig. 4B. In presence of 0.25 mol dm^{-3} nicotinamide, the transition temperature and calorimetric enthalpy for the protein unfolding in absence of lipid were 332.6 K and 246 kJ mol^{-1}, respectively (Table 1A). The transition temperature and calorimetric enthalpy for the protein unfolding in presence of 0.25 mol dm^{-3} nicotinamide in the lipid–protein mixture were 332.3 K and 262 kJ mol^{-1}, respectively (Table 1C). Hence, the thermodynamic parameters accompanying the thermal denaturation of protein, in presence of nicotinamide, remain unaffected by the presence of lipid. It has been observed that α-lactalbumin and dimyristoyl phosphatidylcholine (DMPC) vesicles do not form a complex at pH 7.0. The protein behaves as uncomplexed protein, and the vesicles keep their original dimensions [32]. Our results suggest that similar to DMPC, DPPC also does not undergo

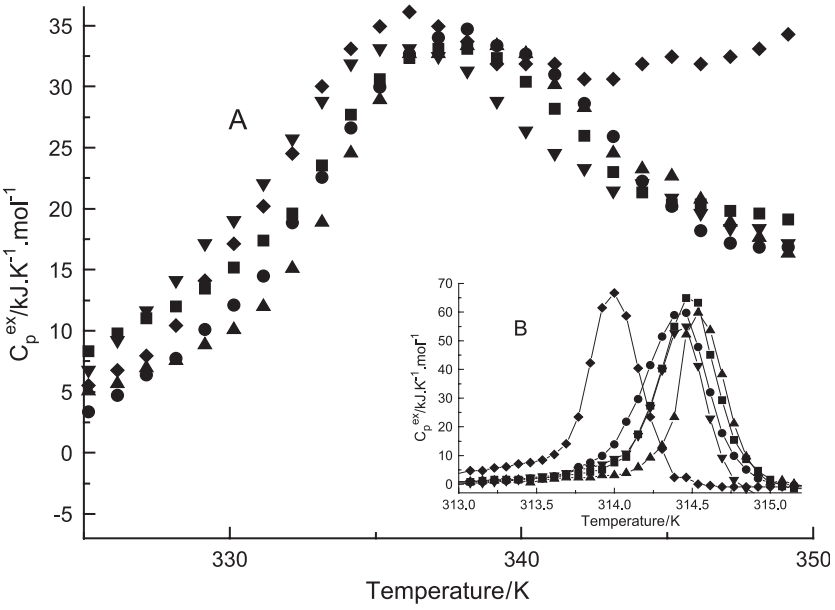

Fig. 3. (A) DSC profiles of thermal denaturation of 0.28×10^{-3} mol dm^{-3} α-lactalbumin at pH 7.0 in varying lipid to protein molar ratio (L/P), L/P=0 (■), L/P=4 (●), L/P=20 (▲), L/P=40 (▼), L/P=80 (♦). (B) Calorimetric transitions of 1.36×10^{-3} mol dm^{-3} DPPC at pH 7.4 in presence of varying lipid to protein molar ratio (L/P), L/P=0 (■), L/P=4 (●), L/P=20 (▲), L/P=40 (▼), L/P=80 (♦).

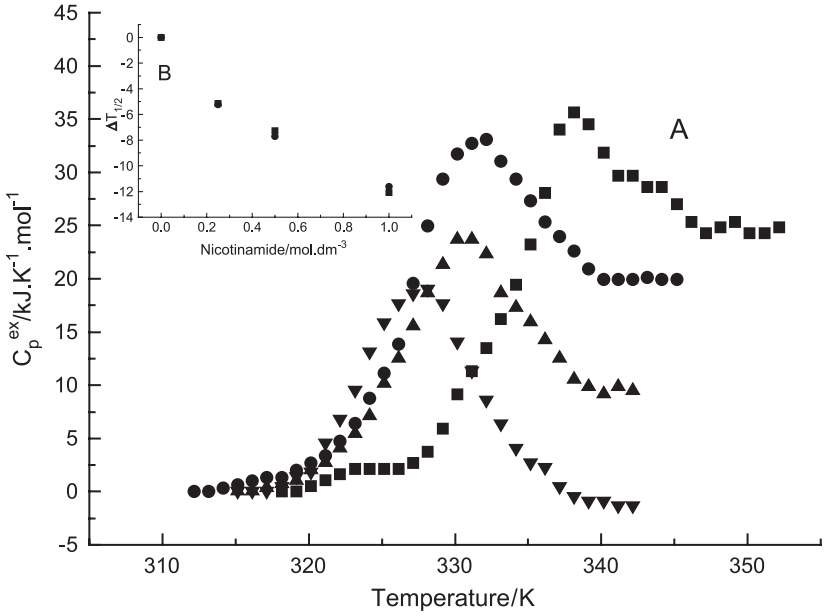

Fig. 4. (A) DSC profiles of thermal denaturation of 0.28×10^{-3} mol dm^{-3} α-lactalbumin at pH 7.0 in presence of 1.36×10^{-3} mol dm^{-3} DPPC in buffer (■) and in presence of 0.25 (●), 0.50 (▲), 1.0 (▼) mol dm^{-3} nicotinamide. (B) Plot for $\Delta T_{1/2}$ [$T_{1/2}$ (in buffer)−$T_{1/2}$ (in nicotinamide)] for 0.28×10^{-3} mol dm^{-3} α-lactalbumin at pH 7.0 against the concentration of nicotinamide in absence (■) and presence of (●) 1.36×10^{-3} mol dm^{-3} DPPC.

any complexation in presence of α-lactalbumin, as there is no significant change in the thermodynamic parameters.

The DSC thermograms and the thermodynamic parameters accompanying the lipid transitions in presence of nicotinamide in the mixture of lipid and protein at molar ratio 5:1 are given in Fig. 5A and Table 3C.

In presence of 0.25 mol dm^{-3} nicotinamide, the transition temperature and calorimetric enthalpy of DPPC unfolding in absence of protein were 313.8 K and 32.2 kJ mol^{-1}, respectively (Table 3A). The transition temperature and calorimetric enthalpy for the lipid transitions in presence of 0.25 mol dm^{-3} nicotinamide in the lipid–protein mixture were 314.4 K and 32.5 kJ mol^{-1}, respectively (Table 3C). Hence, the thermodynamic parameters accompanying the thermal transitions of lipid, in presence of nicotinamide are slightly affected by the presence of protein. A comparative plot of the transition temperature of lipid in presence of nicotinamide with and

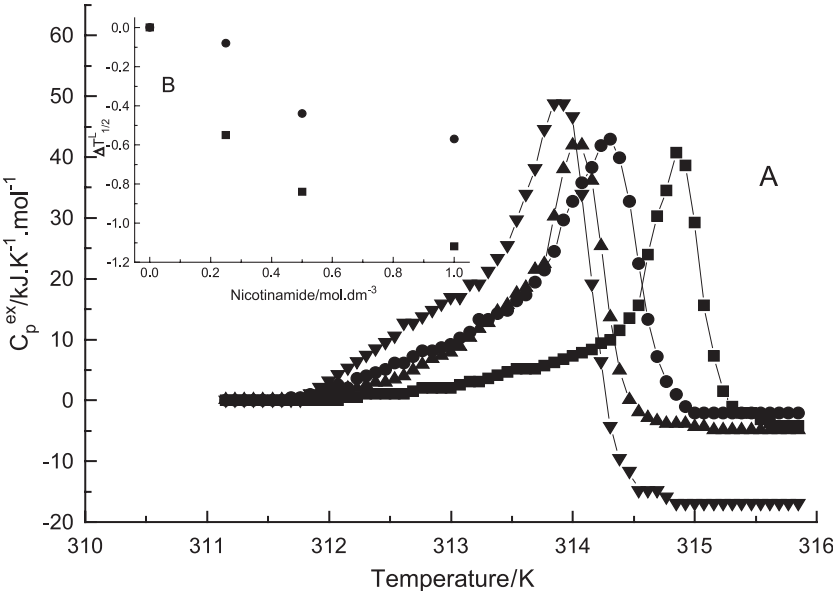

Fig. 5. (A) DSC profiles of thermal denaturation of 1.36×10^{-3} mol dm^{-3} DPPC at pH 7.0 in presence of 0.28×10^{-3} mol dm^{-3} α-lactalbumin in buffer (■) and in presence of 0.25 (●), 0.50 (▲), 1.00 (▼) mol dm^{-3} nicotinamide. (B) Plot for $\Delta T^L_{1/2}$ [$T^L_{1/2}$ (in buffer)−$T^L_{1/2}$ (in nicotinamide)] of 1.36×10^{-3} mol dm^{-3} DPPC at pH 7.0 against the concentration of nicotinamide in absence (■) and in presence (●) of 0.28×10^{-3} mol dm^{-3} α-lactalbumin.

without protein is given in Fig. 5B. The coenzymes, NAD, NADP, and NADPH have been reported to form complexes with various proteins [33–37]. It has been shown that complexation of the protein and the coenzymes are through hydrogen bonding interaction between carboxymide group of the pyridine ring and different amino acid residues in protein. Residues, Ala-6, Gln-7, Ile-13, Gly-14 of dihydrofolate reductase (DHFR) are directly involved in hydrogen bonding with the nicotinamide carboxamide group [37]. Therefore, in such a complex, the hydrogen-bonded nicotinamide is less active towards lipid interaction in the lipid-protein mixture, and consequently, the interaction of nicotinamide with DPPC is hindered in the presence of α-lactalbumin.

The stability of proteins are effected by the perturbation of the concentration of the cosolute at the protein–water interface. The surface tension of aqueous nicotinamide was determined at 298.15 K, and it was found to decrease with increase in concentration of nicotinamide. The data on surface tension are shown in Fig. 6B. The relative excess number of nicotinamide molecules with respect to the bulk solvent water at the protein–water interface has been estimated by surface tension measurements. The exclusion of water by perturbation of the surface free energy was calculated [38] by equation

$$n_i^* = \left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu 3}^{\text{surface tension}} = -\frac{sa_3}{RT}\left(\frac{\partial \sigma}{\partial a_3}\right)_{T,p} \quad (4)$$

In this expression, m_2, m_3, μ_3, a_3, σ, s, T, P, and R are, respectively, the molality of protein, molality of cosolvent, chemical potential of cosolvent, activity of cosolvent, surface tension of the solution, molar surface area of the protein, temperature, pressure, and gas constant. n_i^* is the binding parameter and represents only a relative picture since they have been calculated from the alteration of surface free energy of water by the additives, i.e., from surface tension measurements only.

Table 4
The binding parameter for α-lactalbumin in presence of varying concentrations of HFIP at 298.15 K

Nicotinamide (mol dm^{-3})	$(\partial m_3/\partial m_2)_{T,P,\mu 3}^{\text{surface tension}}$
0.25	13.5
0.50	25.9
1.00	46.8
2.00	73.9

According to Eq. (4), the compounds that lead to a positive increase in the surface tension of water with increasing concentration of the cosolvent should give a negative binding parameter, n_i*, and hence lead to preferential exclusion of the cosolvent molecules from the protein surface. Assuming the structural similarity between baboon [3] and bovine α-lactalbumin and using the surface area [39] as 0.7241×10^{-16} m^2, the values of binding parameter from surface tension measurements are reported in Table 4.

The values show that as the concentration of nicotinamide increases in solution, more and more nicotinamide molecules accumulate at the protein–water interface.

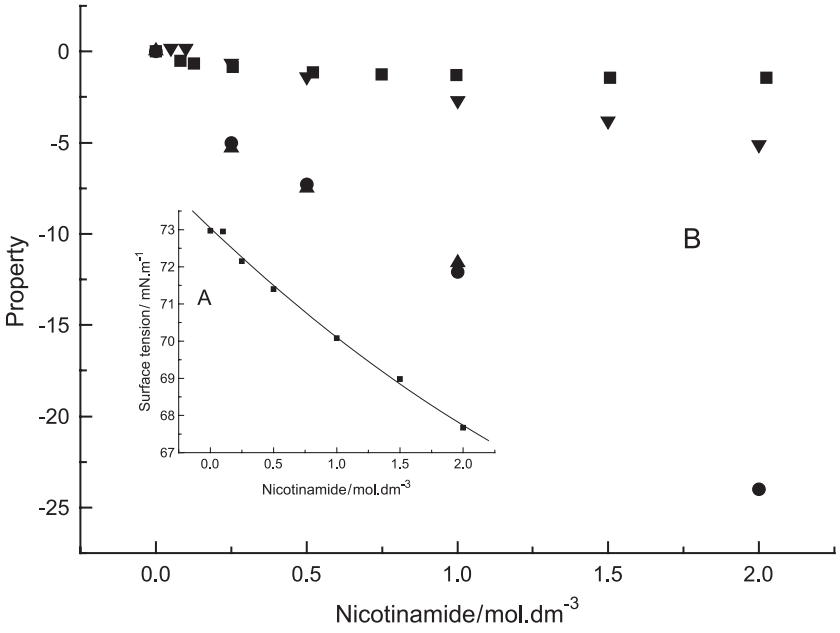

Fig. 6. (A) Variation of surface tension of nicotinamide as a function of concentration; (B) plot of change in volume, ΔV_ϕ ($V_{2,m}^0 - V_{\phi,2}$), at 298.15 K (■), change in surface tension, $\Delta\sigma$ ($\sigma_{\text{water}} - \sigma_{\text{alcohol}}$) at 298.15 K (▼), change in transition temperature, $\Delta T_{1/2}$ [$T_{1/2}$ (in nicotinamide)$-T_{1/2}$ (in buffer)] of α-lactalbumin in absence of DPPC (●), in presence of DPPC (▲) as a function of nicotinamide concentration.

3.4. Correlation of the observed thermodynamic properties of aqueous nicotinamide and thermal transitions of α-lactalbumin and DPPC in presence of nicotinamide

Fig. 6B compares change in transition temperature of α-lactalbumin against concentration of aqueous nicotinamide, with that of change in the apparent molar volume [40] and surface tension of water at 298.15 K in presence of nicotinamide. The figure demonstrates the relative effect of increasing concentration of nicotinamide on the thermal stability of α-lactalbumin in the absence and presence of the lipid DPPC. Since the curves representing $\Delta T_{1/2}$ of α-lactalbumin in both the cases overlap, it further suggests that nicotinamide does not affect the lipid bilayer organization when α-lactalbumin is present in the mixture, rather it interacts with the protein. The ΔV_ϕ plot and the $\Delta\sigma$ plot also run almost parallel to the concentration axis. It indicates that the surface tension effect and association of nicotinamide molecules in aqueous solution do not play an appreciable role in its interaction with α-lactalbumin. The binding parameter, $(\partial m_3/\partial m_2)_{T,P,\mu 3}^{\text{surface tension}}$ is significantly positive, representing the excess concentration of cosolute at the interface of native protein, the positive values of $\Delta\Gamma_{23}$ explains the shift of the N⇌D equilibrium towards right, hence reduction in the transition temperature of protein.

Fig. 7 compares change in transition temperature of lipid against concentration of nicotinamide in combination with that of change in the apparent molar volume.

It is seen that ΔV_ϕ of aqueous nicotinamide and $\Delta T_{1/2}^{L}$ of lipid transitions in the presence of nicotinamide (in absence of protein) correlate very well. This indicates that association of nicotinamide molecules in water leads to their increased interaction with the lipid, although the effect is very small. However, when α-lactalbumin is present in the mixture, there is no correlation between ΔV_ϕ and $\Delta T_{1/2}^{L}$ of lipid. This clearly demonstrates that nicotinamide interacts favorably with α-lactalbumin leading to its reduced interaction with the lipid.

4. Conclusions

Nicotinamide decreases the thermal transition temperature of both lipid and protein at sufficiently high concentrations. The maximum change in the transition temperature of α-lactalbumin is 24 K in 2.00 mol dm^{-3} nicotinamide at pH 7.4. All the protein transitions were two state and reversible under the conditions studied with an average β value of 0.95±0.06. The shape and thermodynamic parameters of the lipid endotherms in the presence of nicotinamide did not change significantly, suggesting that nicotinamide did not penetrate the lipid bilayer. The thermal denaturation of protein in presence of DPPC as cosolute also showed two-state reversible transitions with an average β value of 0.99±0.07. The changes in the thermodynamic parameters accompanying the thermal transitions were small, indicating no significant interaction of α-lactalbumin with DPPC. The changes in the thermodynamic parameters indicate that the lipid bilayer organization, as well as the partitioning of the extrinsic protein α-lactalbumin into the bilayer, is not affected in the entire studied concentration range of lipid. The results demonstrate quantitatively that at physiological pH, α-lactalbumin does not penetrate the lipid bilayer. It is observed that the presence of increasing concentration of nicotinamide (as high as 1.0 mol dm^{-3}) in the lipid–protein mixture does not affect its partitioning

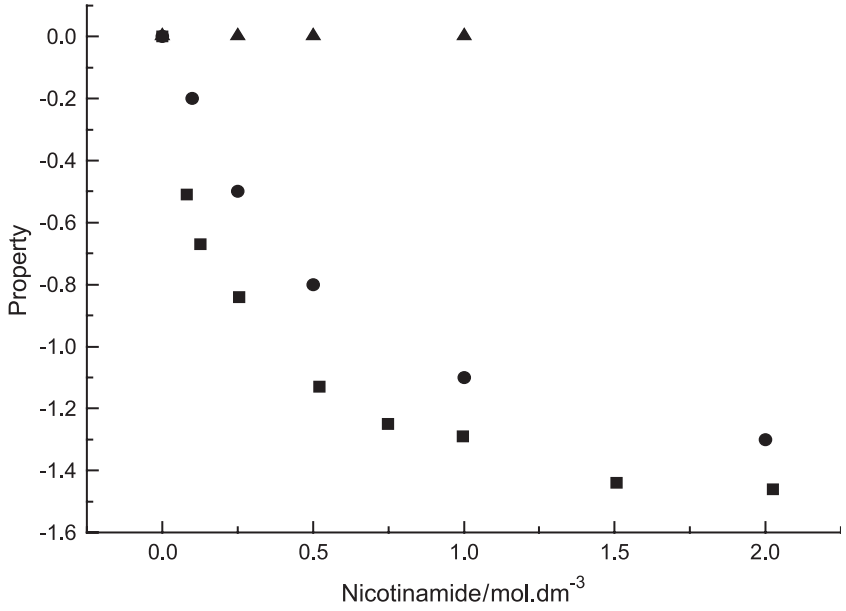

Fig. 7. Plot of change in volume, ΔV_ϕ ($V_{2,m}^{0}-V_{2,m}$), at 298.15 K (■), change in transition temperature, $\Delta T_{1/2}^{L}$ [$T_{1/2}^{L}$ (in nicotinamide)−$T_{1/2}^{L}$ (in buffer)] of DPPC in absence of α-lactalbumin (●), in presence of α-lactalbumin (▲) as a function of nicotinamide concentration.

into the lipid bilayer, although nicotinamide preferentially interacts with α-lactalbumin. The change in the effect of nicotinamide on lipid transition temperature in the mixture and literature report suggests that nicotinamide may be forming a hydrogen-bonded complex with the protein through its amide functionality. The surface tension data of aqueous nicotinamide in combination with the thermal denaturation results of protein in presence of nicotinamide confirmed that surface tension effect does not have any significant contribution to the effect of nicotinamide on protein.

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References

[1] R. Wood, R.D. Harlow, Structural studies of neutral glycerides and phosphoglycerides of rat liver, Arch. Biochem. Biophys. 131 (1969) 495–501.
[2] R. Dhand, J. Young, S. Krishnasamy, F. Possmayer, N.J. Gross, Influence of phospholipid composition on the properties of reconstituted surfactants, Lung 177 (1999) 127–138.
[3] R. Jahn, T.C. Sudhof, Membrane fusion and exocytosis, Annu. Rev. Biochem. 68 (1999) 863–911.
[4] M.H. Gelb, J. Min, M.K. Jain, Do membrane-bound enzymes access their substrates from the membrane or aqueous phase: interfacial versus non-interfacial enzymes, Biochim. Biophys. Acta 1488 (2000) 20–27.
[5] J.E. Johnson, R.B. Cornell, Amphitropic proteins: regulation by reversible membrane interactions, Mol. Membr. Biol. 16 (1999) 217–235.
[6] S.H. White, W.C. Wimley, Membrane protein folding and stability: physical principles, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 319–365.
[7] D. Stopar, R.B. Spruijt, J.A.M. Wolfs, M.A. Hemminga, Protein–lipid interactions of bacteriophage M13 major coat protein, Biochim. Biophys. Acta 1611 (2003) 5–15.
[8] H. Lqari, J. Pedroche, J. Giron-Calle, J. Vioque, F. Millan, Interaction of lupinus angustifolius L. α and γ conglutins with 13-hydroperoxide-11,9-octadecadienoic acid, Food Chem. 80 (2003) 517–523.
[9] A.A. Frazier, C.R. Roller, J.J. Havelka, A. Hinderliter, D.S. Cafiso, Membrane-bound orientation and position of the synaptotagmin I C2A domain by site-directed spin labeling, Biochemistry 42 (2003) 96–105.
[10] O. Halskau, N.A. Froystein, A. Muga, A. Martinez, The membrane-bound conformation of α-lactalbumin studied by NMR-monitored 1H exchange, J. Mol. Biol. 321 (2002) 99–110.
[11] E.D. Chrysina, K. Brew, K.R. Acharya, Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2 angstrom resolution reveal an effect of calcium on inter-lobe interactions, J. Biol. Chem. 275 (2000) 37021–37029.
[12] S. Banuelos, A. Muga, Binding of molten globule like conformations to lipid bilayers, J. Biol. Chem. 270 (1995) 29910–29915.
[13] E.A. Permyakov, V.M. Grishchenko, L.P. Kalinichenko, N.Y. Orlov, K. Kuwajima, S. Sugai, Calcium-regulated interactions of human α-lactalbumin with bee venom melittin, Biophys. Chem. 39 (1991) 111–117.
[14] K.M. Cawthern, E.A. Permyakov, L.J. Berliner, Membrane-bound states of α-lactalbumin: implications for the protein stability and conformation, Protein Sci. 5 (1996) 1394–1405.
[15] L.J. Berliner, K. Koga, α-Lactalbumin binding to membranes: evidence for a partially buried protein, Biochemistry 26 (1987) 3006–3009.
[16] P. Puyol, M.D. Perez, J.M. Ena, M. Calvo, Interaction of bovine β-lactoglobulin and other bovine and human whey proteins with retinol and fatty acids, Agric. Biol. Chem. 55 (1991) 2515–2520.
[17] K.M. Cawthern, M. Narayan, D. Chaudhuri, E.A. Permyakov, L.J. Berliner, Interactions of α-lactalbumin with fatty acids and spin label analogs, J. Biol. Chem. 272 (1997) 30812–30816.
[18] M.B. Martel, R. Got, Galactose transfer in the membranes of human milk fat globules, Biochim. Biophys. Acta 436 (1976) 789–799.
[19] J.P. Kamat, T.P.A. Devasagayam, Methylene blue plus light-induced lipid peroxidation in rat liver microsomes: inhibition by nicotinamide (vitamin B_3) and other antioxidants, Chem.-Biol. Interact. 99 (1996) 1–16.
[20] H. Sies, Biochemistry of oxidative stress, Angew. Chem., Int. Engl. 25 (1986) 1058–1071.
[21] N. Kishore, B. Sabulal, Thermodynamics of the interactions of some chloro and fluoro substituted alcohols with bovine alpha-lactalbumin, J. Chem. Soc., Faraday Trans. 94 (1998) 905–911.
[22] W.H. Kirchhoff, EXAM, US Department of Energy, Thermodynamics Division, National Institute of Standards and Technology, Gaithersburg, MD, USA.
[23] A. Kundu, N. Kishore, Interaction of 2,2,2-trifluoroethanol with proteins: calorimetric, densimetric and surface tension approach, Biophys. Chemist. 109 (2004) 427–442.
[24] E.L. Kovrigin, S.A. Potekhin, Preferential salvation changes upon lysozyme heat denaturation in mixed solvents, Biochemistry 36 (1997) 9195–9199.
[25] S.J. Bae, S.K. Han, K.S. Im, N.H. Kim, A scanning calorimetric study of the effect of clover saponin on liposomal phospholipid membrane, Arch. Pharm. Res. 11 (1988) 181–184.
[26] H.J. Hinz, J.M. Sturtevants, Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins, J. Biol. Chem. 247 (1972) 6071–6075.
[27] J.H. Fendler, Membrane mimetic chemistry, Wiley Interscience Publications, New York, 1983, p. 134.
[28] P.P. Constantinides, L. Ghosaini, N. Inouchi, S. Kitamura, R. Seshadri, M. Israel, A.C. Sartorelli, J.M. Sturtevant, Interaction of *N*-alkylanthracyclines with lipid bilayers: correlations between partition coefficients, lipid phase distributions and thermotropic behavior, Chem. Phys. Lipids 5 (1989) 105–118.
[29] J.J.O. Leary, P.D. Ross, I.W. Levin, Effects of anesthetic and nonanesthetic steroids on dipalmitoylphosphatidylcholine liposomes: a calorimetric and raman spectroscopic investigation, Biochemistry 23 (1984) 4636–4641.
[30] J.M. Sturtevant, A scanning calorimetric study of small molecule–lipid bilayer mixtures, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 3963–3967.
[31] I. Hanssens, C. Houthuys, W. Herreman, F.V. Cauwelaert, Interaction of α-lactalbumin with dimyristoylphosphatidylcholine vesicles: I. A microcalorimetric and fluorescence study, Biochim. Biophys. Acta 602 (1980) 539–557.
[32] I. Hanssens, W. Herreman, J.V. Ceunebroeck, H. Dangreu, C. Gielens, G. Preaux, F.V. Cauwelaert, Interaction of α-lactalbumin with dimyristoylphosphaidylcholine vesicles: III. Influence of the temperature and the lipid to protein molar ratio of the complex formation, Biochim. Biophys. Acta 728 (1983) 293–304.
[33] J. Menetrey, G. Flatau, E.A. Stura, J. Charbonnier, F. Gas, J. Teulon, M. Le Du, M. Boquet, P. Andre, NAD binding induces conformational changes in Rho ADP ribosylating clostridium botulinum C3 exoenzyme, J. Biol. Chem. 277 (2002) 30950–30957.
[34] H. Zhang, T. Zhou, O. Kurnasov, S. Cheek, N.V. Grishin, A. Osterman, Crystal structures of *E. coli* nicotinate mononucleotide

adenylyltransferase and its complex with deamido-NAD, Structure 10 (2002) 69–79.
[35] J. Eyschen, B. Vitoux, M. Marraud, M.T. Cung, G. Branlant, Engineered glycolytic glyceraldehyde-3-phosphate dehydrogenase binds the anticonformation of NAD^+ nicotinamide but does not experience a-specific hydride transfer, Arch. Biochem. Biophys. 364 (1990) 219–227.
[36] V.I. Polshakova, R.R. Biekofsky, B. Birdsall, J. Feeney, Towards understanding the origins of the different specificities of binding the reduced (NADPH) and oxidised ($NADP^+$) forms of nicotinamide adenine dinucleotide phosphate coenzyme to dihydrofolate reductase, J. Mol. Struct. 602–603 (2002) 257–267.
[37] Z. Deng, A. Aliverti, G. Zanetti, A.K. Arakaki, J. Ottado, E.G. Orellano, N.B. Calcaterra, E.A. Ceccarelli, N. Carrillo, P.A. Karplus, A productive $NADP^+$ binding mode of ferredoxin-NADP+ reductase revealed by protein engineering and crystallographic studies, Nat. Struct. Biol. 6 (1999) 847–853.
[38] J.C. Lee, S.N. Timasheff, The stabilization of proteins by sucrose, J. Biol. Chem. 256 (1981) 7193–7201.
[39] F. Kawaizumi, N. Nishio, H. Nomura, Y. Miyahara, Heat capacity measurements of aqueous solutions of mono-, di-, and trisaccharides using an isoperibol twin calorimeter, J. Chem. Thermodyn. 13 (1981) 89–98.
[40] A. Kundu, N. Kishore, Apparent molar heat capacities and apparent molar volumes of aqueous nicotinamide at different temperatures, J. Solution Chem. 32 (2003) 703–717.