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Binding of bovine seminal plasma protein BSP-A1/-A2 to model membranes: Lipid specificity and effect of the temperature

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Abstract

Bovine seminal plasma (BSP) contains a family of phospholipid-binding proteins. The affinity of the protein BSP-A1/-A2 for lipid membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and POPC containing 30% (mol/mol) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or cholesterol, has been investigated by the isothermal titration calorimetry (ITC). This study confirms the association of these proteins to lipid bilayers, and provides a direct characterization of this exothermic process, at 37 °C. The measurements indicate that the protein affinity for lipid bilayers is modulated by the lipid composition, the lipid/protein ratio, and the temperature. The saturation lipid/protein ratio was increased in the presence of cholesterol and, to a lesser extent, of phosphatidylethanolamine, suggesting that it is modulated by the lipid acyl chain order. For all the investigated systems, the binding of BSP-A1/-A2 could not be modeled using a simple partitioning of the proteins between the aqueous and lipid phases. The existence of "binding sites", and lipid phase separations is discussed. The decrease of temperature, from 37 to 10 °C, converts the exothermic association of the proteins to the POPC bilayers to an endothermic process. A complementary 1-D and 2-D infrared spectroscopy study excludes the thermal denaturation of BSP-A1/-A2 as a contributor in the temperature dependence of the protein affinity for lipid bilayers. The reported findings suggest that changes in the affinity of BSP-A1/-A2 for lipid bilayers could be involved in modulating the association of these proteins to sperm membranes as a function of space and time; this would consequently modulate the extent of lipid extraction, including cholesterol, at a given place and given time.
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The bovine seminal plasma (BSP) contains a family of acidic, phospholipid-binding proteins (BSP-A1/-A2, BSP-A3 and BSP-30-kDa), collectively called BSP proteins. BSP-A1/-A2 has the same amino acid sequence and differs only in their glycosylation level. BSP-A1/-A2 and BSP-A3 have an average mass of about 15 kDa [1]. These proteins, products of the seminal vesicles [2–4], bind to sperm membranes upon ejaculation and induce lipid efflux from bovine sperm [5–8]. A similar efflux was observed with human fibroblasts [9]. The characterization of the resulting particles revealed the presence of phosphatidylcholine (PC) and cholesterol (chol) [10]. Cholesterol efflux and the regulation of the cholesterol/phospholipid ratio of the sperm plasma membrane are suggested to be important processes to trigger the acrosome reaction, allowing the sperm to fertilize the egg [11–13]. Therefore, BSP-A1/-A2–lipid interactions are proposed to play an important role in the capacitation process of sperm in the female reproductive tract [8,14]. These phenomena become even more significant because it appears that protein homologs may be ubiquitous in mammals [15].

The amino acid sequence of BSP-A1/-A2 includes a portion similar to the gelatine-binding domain of fibronectin [3,16], consisting of two antiparallel β-sheet structures arranged in tandem [17–19]. These strands, along with two irregular loops, suggest the existence of a partially exposed hydrophobic surface that could act as lipid binding site [17,19,20]. Similar to

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fibronectin, BSP-A1/-A2 possesses two distinct binding sites per molecule, both located on the same face of the protein [17,20,21]. The binding to choline affects the structure of the protein in solution. An infrared (IR) study reported an increase of the turn content, at the expense of the disordered segments, when BSP-A1/-A2 bound to choline [22]. Size-exclusion chromatography showed that, at room temperature, BSP-A1/-A2 is aggregated, and polydispersed. However, when bound to choline, they are proposed to form dimers, suggesting a dissociation of protein multimers upon binding [22]. The dimer structure, including the phosphorylcholine binding sites, has been determined by X-ray crystallography [20,23]. Differential scanning calorimetry (DSC), and IR spectroscopy showed that free BSP-A1/-A2 in solution undergoes two transitions upon heating [22,24]. The first transition, at 36 °C, is associated to the dissociation of the protein multimers. The dissociation into monomers and dimers has also been reported at this temperature from a heparin-binding study [25]. The second transition (55 °C) is associated to the thermal denaturation of the proteins. The binding to lipids bearing a choline group leads to the disappearance of the first transition, and to a shift of the second one towards a higher temperature (76 °C) [22,24]. The loss of the first transition in the presence of PC is consistent with the hypothesis that BSP-A1/-A2 multimers dissociate upon the binding. The shift of the second transition suggests a stabilizing effect of the lipid membrane on the structure of the protein.

The vital role of BSP-A1/-A2 in capacitation is associated to its interactions with membrane lipids, as recently reviewed in [8,14]. BSP-A1/-A2 is reported to display a specific affinity for the choline head group of phospholipids [21,26]. This specific affinity for PC has been also inferred from electron spin resonance (ESR) [27], and surface plasmon resonance (SPR) studies [28]. By ESR, spin-labelled phospholipids were added to a dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) matrix and the proportions of immobilized — on the ESR time scale — probe were determined. These studies indicate that BSP-A1/-A2 restricts the mobility of the acyl chains of lipids upon binding, and the lipid immobilization was exploited as probe for the association [27,29]. The chain stiffening in the fluid phase reported by ESR did not lead to a change in the transition temperature from the gel-to-liquid crystalline of DMPC, but a decrease in the molar enthalpy associated to this transition was observed [24]. ESR and fluorescence studies have shown that BSP-A1/-A2 binds to PC membranes with a saturation molar ratio of 10–12 lipids per protein [24,27,30]. It was found that the proportion of motionally restricted spin-labelled phospholipids with head groups other than PC was smaller than that measured with labelled PC when one BSP-A1/-A2 per about 10 lipids was added [27]. A reduced affinity for phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylserine (PS) was concluded from these measurements. A study by SPR [28] also indicated a binding specificity for the PC lipids, as well as a reduced binding of BSP-A1/-A2 to membranes that included phospholipids bearing a negative charge (PG, phosphatidic acid), or zwitterionic PE. It must be pointed out that all the investigated membranes in that later study contained 20 (w/w)% cholesterol. In the absence of cholesterol, surface plasmon resonance experiments indicated the lipid efflux from PC membranes caused by the proteins.

The impact of cholesterol on BSP-A1/-A2 binding has been also examined and, at this point, no unified view has emerged. Cholesterol does not seem to interact directly with the proteins [10,26,31]. However, an increased proportion of spin-labelled lipids immobilized by BSP-A1/-A2 was observed when DMPC bilayers contained 20 mol% cholesterol [32], the specificity for the phospholipid head group being maintained. Spin-labelled cholestane was practically not immobilized in the presence of protein when incorporated in DMPC bilayers at 28 °C [27], whereas about 30% was found motionally restricted in eggPC liposomes at 4 °C [31]. When the DMPC bilayers contained 20 mol% cholesterol, a considerable fraction of spin-labelled cholestane was immobilized upon BSP-A1/-A2 binding [32]. An enhanced binding induced by cholesterol was proposed from these measurements. Using the intrinsic fluorescence of BSP-A1/-A2, it was shown that the fluorescence intensity increases observed upon titrations of the protein were similar for PC bilayers and PC bilayers containing up to 40 mol% cholesterol, discounting an effect of the sterol on the protein binding [24]. Alternatively, cholesterol was shown to hinder the ability of the protein to induce the efflux of encapsulated materials in vesicles [24], as well as the solubilization of the lipid membranes themselves, as inferred from light scattering [27], suggesting a reduction of the interactions.

In the present work, we have examined the thermodynamics of the association of the BSP-A1/-A2 with a number of lipid model membranes using isothermal titration calorimetry (ITC). ITC is well-suited for studying peptide–membrane interactions [33,34] and provides a complementary approach to examine the protein binding to lipids, using unsupported bilayers. We have characterized the association thermodynamics of the proteins with lipid membranes formed with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) to provide fluid membranes. A significant proportion of various lipids was included in the membranes to assess their influence on BSP-A1/-A2 binding. We have examined especially the influence of PE, PG, and cholesterol, via their modulation of the bilayer physicochemical properties. During the investigation, we have found that the BSP-A1/-A2–lipid interactions are strongly dependent on temperature, a feature that has not been reported previously. The present study also includes 2D-infrared experiments to assess the structure changes of the proteins as a function of the temperature, in order to provide insights into the influence of this parameter on the ITC results. It has been shown that 2D-IR spectroscopy can increase the spectral resolution and permits the observation of limited changes in protein structure that are not detectable otherwise [35].

1. Materials and methods

1.1. Chemicals

BSP-A1/-A2 was isolated as described elsewhere [1,2]. POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and DMPC were purchased from Avanti Polar Lipids (Birmingham, AL). 3-[*N*-Morpholino]propanesulfonic acid

(MOPS) and cholesterol were obtained from Sigma (St-Louis, MO). They were used without any further purification.

1.2. Isothermal titration calorimetry

In order to prepare the lipid mixtures, appropriate amounts of individual lipids were dissolved in a benzene/methanol (95/5 (v/v)) solution. These solutions were mixed and then lyophilized. The resulting lipid powders were hydrated with a MOPS buffer (10 mM, pH 7.4) and submitted to 5 freeze-and-thaw cycles (from liquid nitrogen to ~35 °C). Large unilamellar vesicles (LUVs) were prepared by extruding the lipid suspensions 10 times through polycarbonate filters with 100 nm-pore size, using a commercial extruder (Avestin, Ottawa, ON). Exact phospholipid concentrations were determined by the Fiske–SubbaRow phosphorus assay [36]. BSP-A1/-A2 was solubilized in the same buffer. The pH of the protein solutions was measured and re-adjusted if necessary with a dilute NaOH solution.

ITC experiments were performed on a MicroCal VP-ITC calorimeter (MicroCal Inc., Northhampton, MA). Typically, the calorimeter cell was filled with lipid LUV suspension (0.5–20 mM) while a BSP-A1/-A2 solution (1.5 to 3 mg/ml) was introduced in the injection syringe. The volume of the cell was 1.4527 ml. The protein solution was injected in the calorimeter cell (typically 7 injections of 40 µl each) under constant stirring (300 RPM). Data were analyzed using the Origin™ software (version 5.0) provided by MicroCal. The reported values and the standard deviations are obtained from triplicates. Controls showed that the dilution of the proteins upon their injection in the cell filled with buffer lipid did not have any significant contribution in the measured heat. The dilution of the LUVs caused by the addition of buffer led to a small enthalpic contribution; this contribution was measured on every LUV population and was subtracted from the enthalpies measured during the titration.

1.3. Infrared spectroscopy

Infrared spectroscopy experiments were carried on a Bio-Rad FTS-25 spectrometer (Bio-Rad Laboratories, Cambridge, MA) equipped with a water-cooled globar source, and a deuterated triglycine sulphate detector. BSP-A1/-A2 was dissolved in D_2O to allow proton–deuterium exchange, lyophilized and re-dissolved in the MOPS buffer (10 mM, pH 7.4) prepared in D_2O. The final protein concentration was 3 (w/w)%. An aliquot of ~10 µl was placed between two CaF_2 windows and the sample was inserted in a homemade temperature controller. For DMPC/BSP-A1/-A2 samples, the lipid powder was hydrated with the D_2O-based MOPS buffer (10 mM) and submitted to 5 freeze-and-thaw cycles (from liquid nitrogen to 40 °C). An appropriate volume of BSP-A1/-A2 solution was added to obtain the desired lipid/protein ratio. The mixtures were submitted to 5 additional freeze-and-thaw cycles (from liquid nitrogen to ~37 °C; the latter was selected to prevent protein denaturation). The final protein concentration was 3 (w/w)%. An aliquot of ~10 µl was analyzed.

The spectra were recorded between 20 and 70 °C, with increasing temperature. A 4-minute equilibration period was set prior to the data acquisition. For each spectrum, 100 scans were co-added. A background spectrum was recorded at each temperature. Data were analyzed with the Grams/32 software (Thermo Galactic, Salem, NH, USA). For the Amide I' band analysis, a cubic polynomial baseline was drawn in the region between 1605 and 1705 cm^{-1}. 2D infrared maps were generated by the Bio-Rad Win-IR Pro software (version 2.97). The calculations were performed on the difference spectra, which were highlighting the Amide I' changes. These spectra were obtained by subtracting the spectrum of a sample at the lowest temperature from those obtained at different temperatures. The maps were normalized in z so the absolute values are transferable from one map to another.

2. Results

2.1. Isothermal titration calorimetry

The isothermal calorimetric titration of POPC LUVs with BSP-A1/-A2 was performed at 10, 20, and 37 °C (Fig. 1). At 37 °C, the titration profile shows practically only exothermic peaks. The area of the peaks decreases progressively upon the

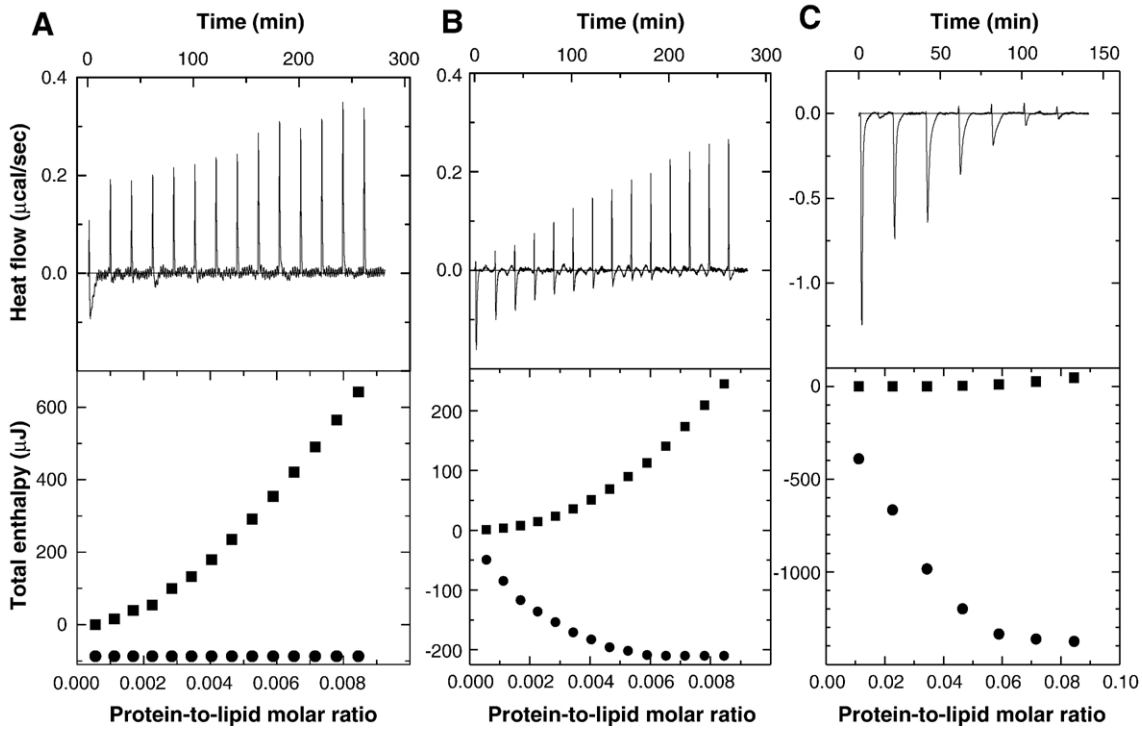

Fig. 1. Isothermal calorimetric titration of POPC LUVs with BSP-A1/-A2 at A) 10 °C (POPC 5 mM; BSP-A1/-A2 0.2 mM — 14 injections of 20 µl), B) 20 °C (POPC 5 mM; BSP-A1/-A2 0.2 mM — 14 injections of 20 µl) and, C) 37 °C (POPC 0.5 mM; BSP-A1/-A2 0.2 mM — 7 injections of 40 µl). The upper panels show the heat flow changes resulting from the injections. The lower panels show the calculated total binding enthalpy. The endo-(■) and exo-(●) thermic contributions are displayed separately.

Table 1
Membrane saturation ratio (lipids/bound BSP-A1/-A2) and molar binding enthalpy, calculated from the titrations carried out at 37 °C

System	ΔH_{mol} (kJ/mol)	Saturation* (lipids/bound protein)
POPC	−70±2	16±1
POPC/POPE (7/3)	−72±1	26±2
POPC/POPG (7/3)	−87±7	11±1
POPC/chol (7/3)	−58±1	172±15

*The ratios are reported taking into account only the lipids of the external leaflet (mean value±standard deviation, $n=3$).

titration. Therefore, the recorded peaks are associated to the binding of the proteins to the lipid vesicles and the decrease of their area occurred as the lipid membranes become saturated with proteins. A similar titration was performed using a large excess of lipids in the cell, where 5 µl of a 0.1 mM BSP-A1/-A2 solution was injected to a lipid suspension of 6 mM (data not shown). In these conditions, the injections lead to a series of exothermic peaks with a similar area, suggesting that the injected BSP-A1/-A2 is practically complete bound to the lipid membranes. From these values, the molar enthalpy variation (ΔH_{mol}) related to the binding of BSP-A1/-A2 to POPC vesicles (i.e. the transfer of the protein, from the aqueous environment to the lipid bilayers) is calculated. This value includes the enthalpic contributions associated to changes in the protein as well as the lipid structure. The ΔH_{mol} of BSP-A1/-A2 to POPC LUV is −70±2 kJ/mol (Table 1). The lower panel of Fig. 1 shows the total binding enthalpy measured upon the titration (ΔH_i), i.e. $\Delta H_i = \sum_i \Delta h_i$ where Δh_i is the enthalpy variation associated to the ith injection. The total enthalpy reaches a maximum of about −1400 µJ after 5 injections, when the protein/lipid molar ratio in the cell reaches 0.06. Using the ΔH_{mol} value, it is found that, at the end of the titration, the lipid/bound BSP-A1/-A2 ratio reached about 32±2. This corresponds to 16 lipids per bound protein if one considers only the lipids of the external leaflet, supposing no translocation of the protein (Table 1).

At 20 °C, two different phenomena are observed up to the 9th injection. At the beginning of the titration, an exothermic signal is predominant. It is accompanied by a sharp endothermic contribution. The exothermic component decreases to nothing upon titration while the endothermic peak grows significantly. The endothermic signal seems to be detected prior to the exothermic contribution, indicating that the events related to these different signals do not happen with the same kinetics. The lower panel shows again the total binding enthalpy measured during the titration. The two components, observed for a single injection, were integrated separately by manually selecting the integration limits. The energy involved in the exothermic peaks is considerably smaller than those measured at 37 °C, by a factor of about 7. It must be noted that the lipid concentration in the cell was 10 times that used at 37 °C in order to obtain a titration, based on the exothermic peaks, over a reasonable number of injections. The observed differences between the two titration profiles suggest that temperature affects the binding of BSP-A1/-A2 to POPC vesicles. In order to define more precisely the influence of the temperature, ITC titrations were also carried out at 10 °C. The results (Fig. 1A) indicate that the low temperature amplifies the endothermic signal observed at 20 °C. Only a small exothermic peak during the first injection is observed at 10 °C.

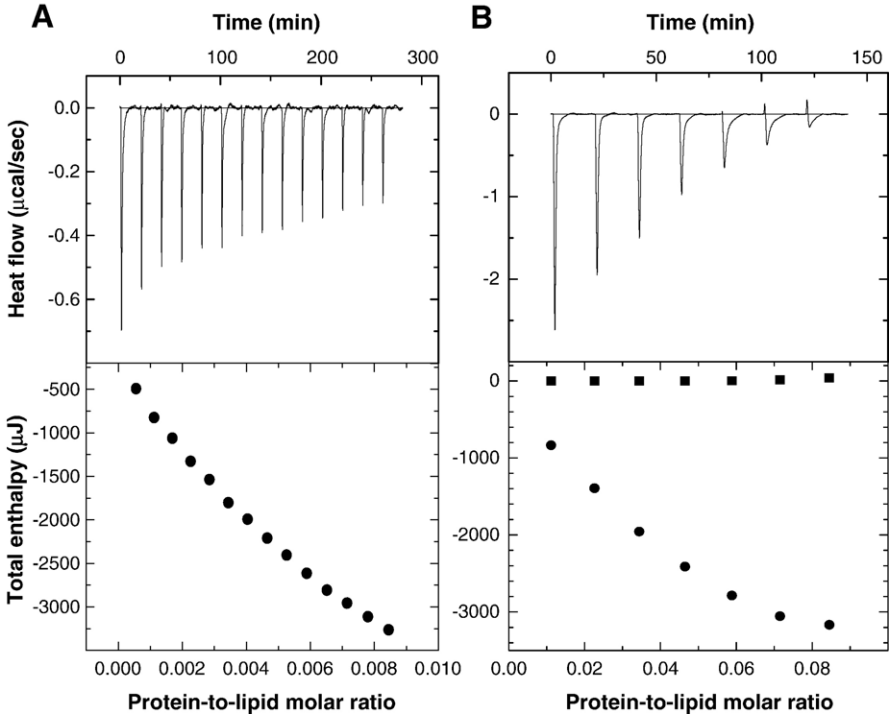

Fig. 2. Isothermal calorimetric titration of POPC/POPG (7/3) LUVs with BSP-A1/-A2 at A) 20 °C (lipids 5 mM; BSP-A1/-A2 0.2 mM — 14 injections of 20 µl) and, B) 37 °C (lipids 0.5 mM; BSP-A1/-A2 0.2 mM — 7 injections of 40 µl). Only the exothermic component can be seen in the presence of the negatively charged POPG. The endo-(■) and exo-(●) thermic contributions are displayed separately.

Therefore, we conclude that the endothermic signal is associated with a phenomenon promoted by low temperatures whereas the exothermic one is associated to a phenomenon mainly occurring at higher temperatures. These two phenomena can coexist, or compete one with another, as exemplified by the data acquired at 20 °C. Interestingly, a change in temperature from 37 to 20 °C, between two successive injections during the titration, causes a change from exothermic to endothermic peaks (data not shown). This change is reversible when the temperature is brought back to 37 °C.

The isothermal titrations of LUVs made of POPC/POPE (7/3) (data not shown) were qualitatively similar to those observed for pure POPC; only exothermic peaks were observed at 37 °C, while both endothermic and exothermic components were detected at 20 °C. At 37 °C, for POPC/POPE membranes, the lipid/bound protein ratio at the end of the titration was estimated to be 52±4 (or 26 lipids of the external leaflet), and the ΔH_{mol} was −72±1 kJ/mol (Table 1).

The binding of BSP-A1/-A2 to negatively charged membranes was assessed using POPC/POPG (7/3) vesicles (Fig. 2). At 37 °C, the titration profile is similar to the one with pure POPC LUVs. The titration shows a series of exothermic peaks whose area decreases upon titration. As for the previous systems, the lipid/bound protein ratio at the end of the titration, and ΔH_{mol} of BSP-A1/-A2 binding to the POPC/POPG (7/3) vesicles were calculated and their values are 22±2, and −87± 7 kJ/mol, respectively; the saturation ratio becomes 11 if one considers only the external lipid leaflet (Table 1). At 20 °C, only the exothermic peak can be observed upon the titration of POPG-containing LUVs with BSP-A1/-A2. It must be noted that at 20 °C, the lipid concentration for the titrations was 5 mM, as in the case of POPC titrations, in order to facilitate the comparison. In these conditions, the titration was incomplete but the results clearly show that the presence of negatively charged lipids inhibited the process associated to the endothermic signal.

For cholesterol-containing LUVs (POPC/chol (7/3)) (Fig. 3), the ITC experiments at 37 °C only show an exothermic signal, similar to the experiments performed at this temperature with the other lipid systems. In this case, the lipid concentration is increased to 5 mM to obtain an appropriate titration upon the addition of the same quantity of protein. This phenomenon indicates in a straightforward manner that the lipid/bound protein ratio at the end of the titration is considerably larger than for the previously investigated lipid systems. In the case of the cholesterol-containing bilayers, 40-µl aliquots of 0.1 mM BSP-A1/-A2 solution were added to 10 mM lipid suspensions to determine ΔH_{mol}, estimated to −58±1 kJ/mol. The POPC/bound BSP-A1/-A2 ratio for POPC/chol (7/3) vesicles is 344± 30 (or 172 phospholipids of the external lipids) (Table 1). At 20 °C, the titration with BSP-A1/-A2 leads to only endothermic signals and their area increases during the titration.

2.2. Binding isotherms

The binding or partitioning isotherms can be defined by the variation of X_b, the molar fraction of bound protein to the membranes, as a function of C_f, the concentration of protein free in solution. X_b is described as:

$$X_b = \frac{n_{P,b}}{n_{P,b} + n_L^0} \quad (1)$$

where $n_{P,b}$ is the number of moles of bound peptide, and n_L^0 is the number of moles of lipids in the calorimeter cell. X_b^* refers

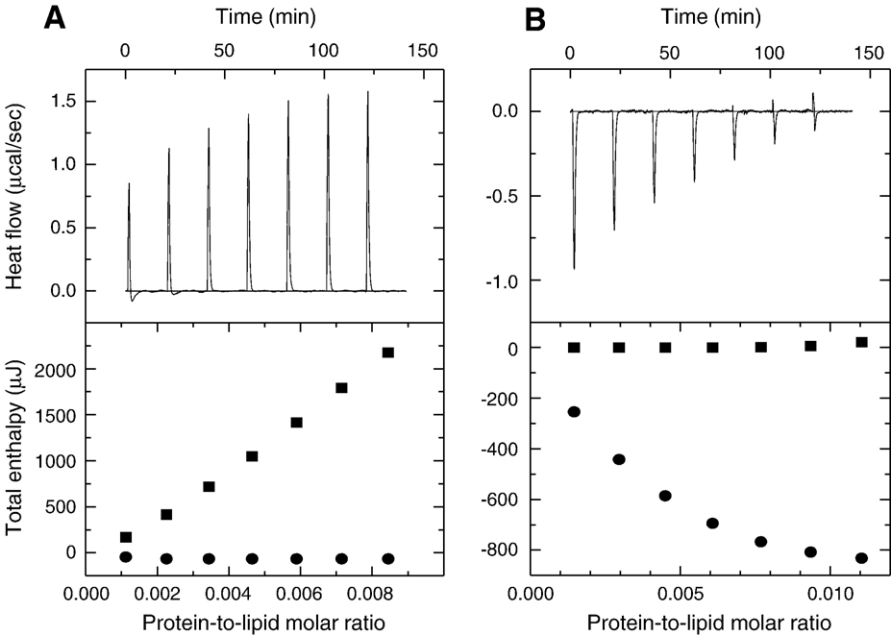

Fig. 3. Isothermal calorimetric titration of POPC/chol (7/3) LUVs with BSP-A1/-A2 (lipids 5 mM; BSP-A1/-A2 0.2 mM — 7 injections of 40 µl) at A) 20 °C and, B) 37 °C. The components are all endothermic at low temperature and all exothermic at high temperature. The endo-(■) and exo-(●) thermic contributions are displayed separately.

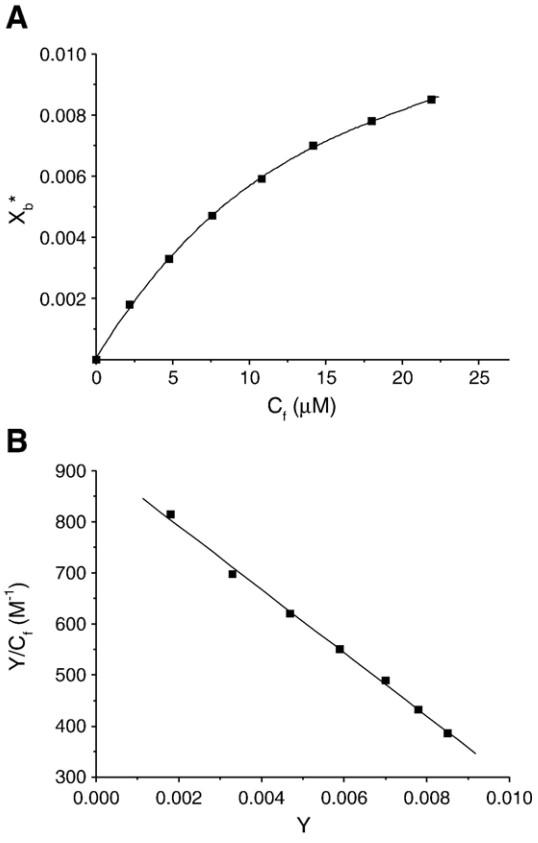

Fig. 4. A) Isotherm of BSP-A1/-A2 (0.2 mM) binding to 7/3 POPC/chol vesicles (5 mM) (■). The mole fractions of bound proteins are expressed taking into account only the outer lipid leaflet as discussed in the text; this parameter is represented by X_b^*. B) Scatchard plot for the same system. The solid line is the linear fit.

to the molar fraction but taking into account only the lipid external leaflet. The $n_{P,b}$ can be obtained directly from the ITC data using:

$$n_{P,b} = \frac{\sum_i \Delta h_i}{\Delta H_{mol}} \quad (2)$$

In this approach, ΔH_{mol} is considered constant over the complete titration. Knowing the quantity of bound protein after each injection, the concentration of protein free in solution, C_f, can be calculated for the conditions prevailing after each injection. The binding isotherm is plotted for POPC/chol system in Fig. 4A. This isotherm, as well as all those associated to the other investigated systems, did not show a linear variation. BSP-A1/-A2 binding to lipid membranes could be simulated by Scatchard plots. This approach has been recently used to analyze the SPR data for lipid–BSP-A1/-A2 systems [28]. In this case, it is assumed that the lipid vesicles provide n_{site} independent and equivalent binding sites to the proteins, and the sites are characterized by an intrinsic dissociation constant, K. This approach corresponds to a Langmuir isotherm and the curvature is associated with the progressive crowding of the surface (or progressive occupation of the sites) by BSP-A1/-A2 already adsorbed. In this approach, Y is defined as the ratio of the bound BSP-A1/-A2 over the external leaflet lipid concentration, and C_f is the free protein concentration. The data provided good linear fits for Scatchard plots and a typical example is presented in Fig. 4B. The resulting K and n values are presented in Table 2. The number of sites is expressed in terms of lipid/bound BSP-A1/-A2 ratio, $n_{L/P}$ (i.e. $1/n_{site}$). The data points for which ≥90% of the proteins were bound were not included in the fits because the uncertainties on these small C_f values led to large variations of the Y/C_f ratios.

The binding of BSP-A1/-A2 to lipid membranes could also be described by a partitioning between lipid-bound and free proteins, taking into account the charge density at the membrane interface [33,34,37] (see Supplementary material). Despite the different ansatz, the extracted affinities showed similar trends to those derived from the Scatchard/Langmuir model.

2.3. Two-dimension infrared spectroscopy

Fig. 5 shows the influence of the temperature on the Amide I' band of the IR spectra of free BSP-A1/-A2 (A) and 15/1 DMPC/BSP-A1/-A2 mixtures (B). At 20 °C, the Amide I' band of the free BSP-A1/-A2 shows a broad contour, with a maximum close to 1646 cm^{-1} and with a shoulder near 1680 cm^{-1}. Upon heating, the band is shifted towards higher frequencies, its maximum reaching 1651 cm^{-1} at 70 °C. This behavior is in agreement with a previous study [22]. The variation of the position of the maximum as a function of temperature is reported in Fig. 6. The frequency variation is fairly linear between 20 and 45 °C. Subsequently, the shift becomes more abrupt between 45 and 65 °C. This shift illustrates a change in the secondary structure of the protein. It was interpreted as a thermal denaturation of BSP-A1/-A2, as assessed previously by CD and by DSC [22]. The presence of DMPC does not lead to a significant effect on the Amide I' band of BSP-A1/-A2 at low temperatures (Fig. 5). However upon heating up to 70 °C, the band showed a small variation with temperature (Figs. 5 and 6), in agreement with the literature [22]. The gel-to-liquid crystalline transition of DMPC (T=23 °C) does not appear to affect the protein secondary structure, as no significant changes were observed around this temperature. This absence of temperature-induced change of the Amide I' band in the presence of lipids indicates that the protein is bound to the lipids and this association leads to the stabilisation of the protein structure.

In order to get more details about the temperature-induced changes of the BSP-A1/-A2 secondary structure, we have examined the spectra series using 2D-IR analysis. Two temperature

Table 2
Intrinsic dissociation constants (K) and number of lipids per bound BSP-A1/-A2 obtained with Scatchard model at 37 °C

System	K (mM^{-1})	$n_{L/P}^*$ lipids/protein
POPC	103±21	10±1
POPC/POPE (7/3)	463±173	23±2
POPC/POPG (7/3)	148±49	7±2
POPC/chol (7/3)	66±20	92±21

*The ratios are reported taking into account only the lipids of the external leaflet (mean value±standard deviation, n=3).

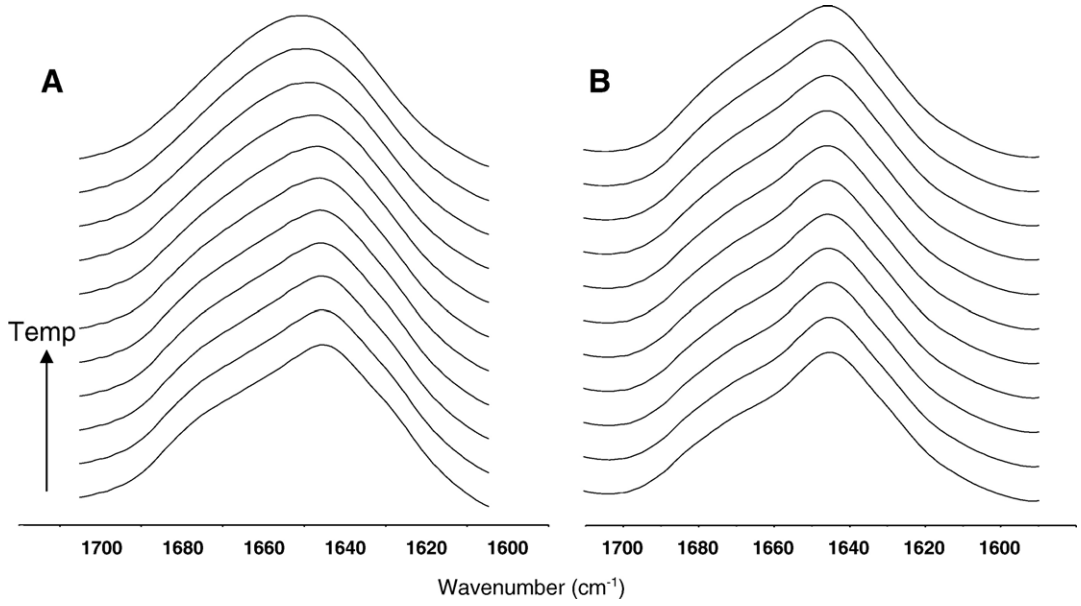

Fig. 5. Temperature dependence (between 20 and 70 °C, 5 °C step) of the Amide I' band of BSP-A1/-A2 A) free in solution and B) at a 15/1 DMPC/BSP-A1/-A2 molar ratio.

intervals are defined on the basis of the regimes of the shift of the Amide I' band observed for free BSP-A1/-A2 (Fig. 6). The first interval is set from 20 to 45 °C, a range where the Amide I' band shift is gradual and limited to 1 cm^{-1}. The second temperature interval is between 45 and 70 °C, where the band is shifted more abruptly by about 4 cm^{-1}. Synchronous 2D-IR maps detail the major changes of the secondary structure of free (Fig. 7A) and bound (Fig. 7B) BSP-A1/-A2, as a function of temperature. The left panels are representative of the changes between 25 and 45 °C, whereas the right panels are obtained from the variations 45 and 70 °C. As guides, the difference spectra, obtained from the subtraction of the spectra at the lowest temperature from the mean spectra (over the whole temperature range), are displayed on the sides of the 2D-maps. These illustrate clearly the increase of the

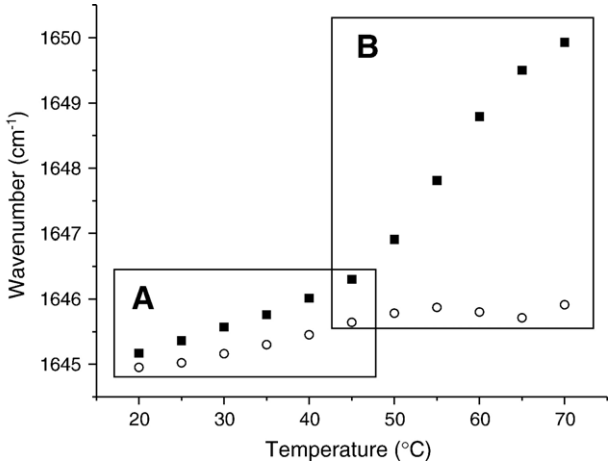

Fig. 6. Frequency shift of the Amide I' band maximum with temperature, for BSP-A1/-A2 free in solution (■) and for the DMPC/BSP-A1/-A2 complex with a molar ratio of 15/1 (○). Domains A and B (denaturation) have been defined for the 2D-IR analysis.

1659 cm^{-1} component and the decrease of the 1627 cm^{-1} and 1682 cm^{-1} components. These changes are in agreement with previous IR results, using band decomposition and deconvolution [24] and are interpreted as a loss of β-sheet structure converting into α-helix segments and solvent-shielded loops [22]. The 2D-IR maps indicate that all the spectral changes occur in a concomitant way. On the synchronous maps, negative cross peaks are observed for 1627 cm^{-1}/1659 cm^{-1}, and 1688 cm^{-1}/1659 cm^{-1}, while a positive cross peak is located at 1627 cm^{-1}/1688 cm^{-1}. Asynchronous maps (not shown) showed no significant correlation, indicating that the Amide I' band changes are synchronous upon a temperature increase. Fig. 7B shows synchronous maps associated to the temperature variation, in the Amide I' band region, for a DMPC/BSP-A1/-A2 15/1 sample. The maps illustrate the more limited variations observed in the case of the lipid-bound protein, compared to its aqueous free form, especially over the 45 to 70 °C interval. It has to be noted, however, that these changes, even though much less pronounced, occur at very similar positions. These changes may be associated to a small fraction of unbound proteins that would undergo a structural transition upon heating.

3. Discussion

The thermodynamics of BSP-A1/-A2 association are clearly dependent on the composition of bilayers. First of all, we should start with the discussion associated to the molar binding enthalpy of BSP-A1/-A2 to vesicles (ΔH_{mol}), and the lipid/protein saturation ratios (Table 1) as these parameters are practically derived directly from ITC measurements, considering ΔH_{mol} constant during the titration. These two parameters are modulated by the lipid composition of bilayers. The binding of BSP-A1/-A2 to lipid membranes at 37 °C is an exothermic process for all bilayer systems investigated in the present work. The measured ΔH_{mol} values for pure POPC, and POPC/POPE

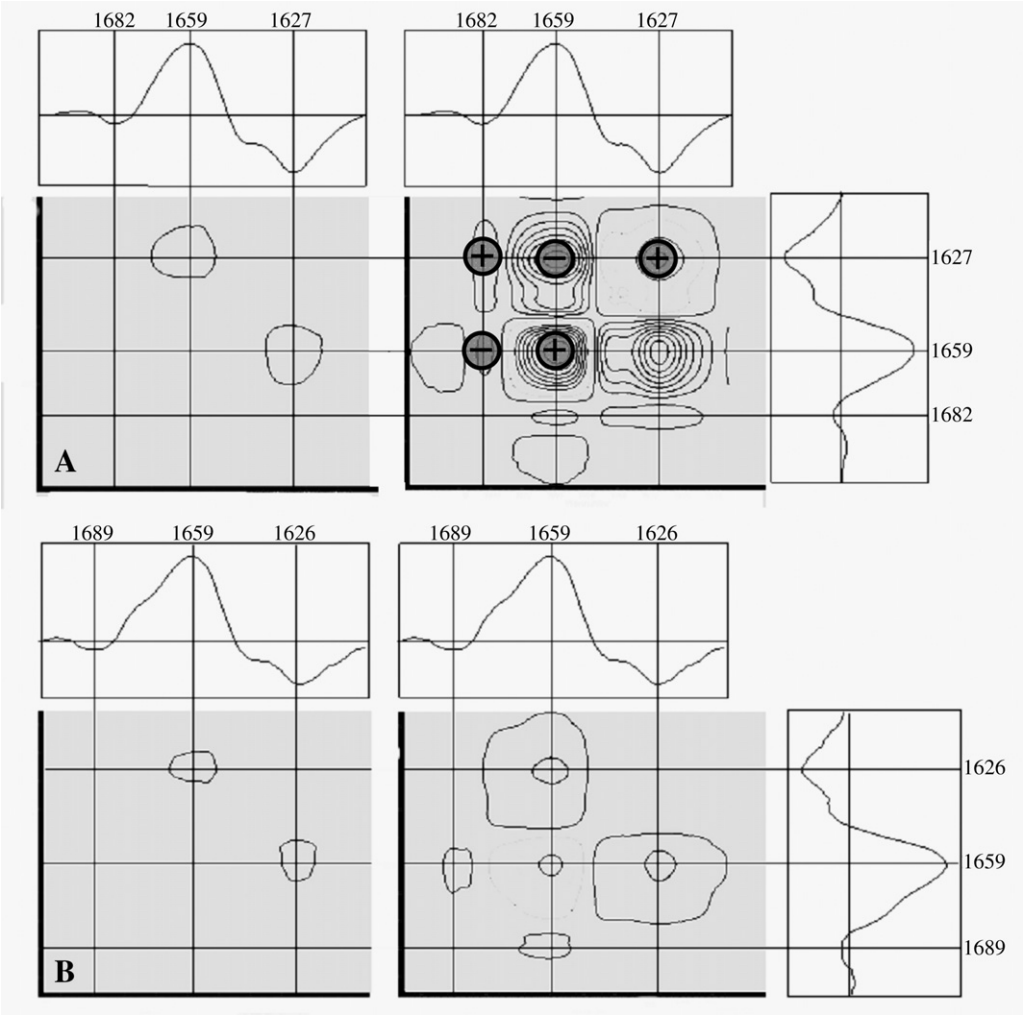

Fig. 7. 2D-IR synchronous maps of the Amide I' band of BSP-A1/-A2 A) free in solution and B) at a 15/1 DMPC/BSP-A1/-A2 molar ratio. Left panels describe the spectral variations between 25, and 45 °C whereas the right ones describe those between 45, and 70 °C. Spectra on top and side are the mean difference spectra of the series recorded as a function of temperature. The *x* and *y* scales are expressed in wavenumber (cm^{-1}).

mixtures are comparable, suggesting that the lipid–protein interactions are of similar nature in these two systems. The binding of BSP-A1/-A2 to POPC/POPG vesicles yielded to a slightly higher ΔH_{mol}. This difference could be due to electrostatic interactions, and suggests that the presence of negative charges at the membrane interface promotes, from an enthalpic point of view, the binding of these proteins. Conversely, the presence of 30 mol% cholesterol in POPC matrices reduced the ΔH_{mol} value by about 17%. These are, to our knowledge, the first reported ΔH_{mol} values for this protein obtained directly from calorimetry. Previously, the binding enthalpies of BSP-A1/-A2 to lipids have been derived from the temperature dependence of the association free energy [28,38]. The ΔH_{mol} of BSP-A1/-A2 association with lysophosphatidylcholine micelles was extracted from the affinity constant obtained by uv-vis spectroscopy between 18 and 35 °C [38]. This was found to be −73 kJ/mol, a value similar to those measured here. This parameter was also derived from SPR measurements assessing the affinity of BSP-A1/-A2 to cholesterol-containing bilayers [28]. In that study, the temperature was varied between 5 and 21 °C, and the ΔH_{mol} for membranes formed with dimyristoylphosphocholine (DMPC) and cholesterol was calculated to be +7 kJ/mol, inferring an endothermic process. As discussed below, the discrepancy between the magnitudes of ΔH_{mol} is likely related to the temperature dependence of this parameter. Our calorimetric results establish that the binding of BSP-A1/-A2 to lipid assemblies at 37 °C is favored from an enthalpic point of view. It is slightly increased by the presence of 30 mol % POPG and decreased by the presence of 30 mol% cholesterol.

The membrane saturation POPC/BSP-A1/-A2 ratio, calculated from the ITC results (Table 1), is 32, considering all the lipids of the vesicles, and 16 if one considers only the lipids of the outer leaflet of LUVs. This value is consistent with a maximal binding of 10–12 DMPC per BSP-A1/-A2 obtained from turbidimetry, ESR spectroscopy and fluorescence [24,27,30]. In the turbidimetry and ESR experiments, all the lipids were exposed to BSP-A1/-A2 because the lipids were hydrated directly with a protein solution or the mixtures were

frozen-and-thawed to ensure their homogeneity. As previously indicated, a lipid/BSP-A1/-A2 ratio around 12 corresponds to the complete coverage of the lipid membrane surface [27]. A similar saturation ratio is obtained for the POPC/POPG system. Higher ratios are measured when the bilayers include 30 mol% POPE (26 lipids per BSP-A1/-A2, outer leaflet only) and cholesterol (172 phospholipids per BSP-A1/-A2, considering only POPC molecules, outer leaflet only). These higher values are a clear indication of the limited capacity of these membranes to adsorb BSP-A1/-A2. Using a Scatchard plot approach for the analysis of the ITC data, the values of $n_{L/P}$ are consistent with the membrane saturation ratios (Table 2), even though the exact meaning of these "lipid sites" is not clearly defined. The capacity of BSP-A1/-A2 binding to a lipid membrane is unambiguously dependent on its composition. The binding capacity of bilayers decreases from POPC ≈ POPC/POPG > POPC/POPE > POPC/chol.

The presence of cholesterol, and to a lesser extent POPE, modulates the stoichiometry of the overall binding and therefore leads to an apparent reduction of the binding capacity. At the end of the titrations with these two systems, the lipid vesicles are clearly not completely covered with BSP-A1/-A2. These 2 lipid species are well known to modulate the properties of the membranes. They reduce the acyl chain disorder of the lipid, the effect being more pronounced for cholesterol than for POPE [39,40]. Cholesterol is known to increase the order of fluid membranes [41–43], an effect also observed on sperm membranes [44]. This phenomenon is also illustrated by the reduction of surface compressibility, and the increase of cohesive strength observed in cholesterol-containing membranes [45]. In fact, cholesterol leads to the formation of a distinct lipid phase, the liquid ordered phase, which displays high lipid chain order while remaining in a fluid state [45]. These lipids also change the curvature properties of membranes. POPE has a pronounced tendency to form an inverted hexagonal (H_{II}) phase [46]. Cholesterol, up to 30 mol%, was found to favor the H_{II} phase in phosphatidylethanolamine [43,47]. These changes in bilayer properties may be at the origin of the different binding capacity. It has been shown that BSP-A1/-A2 has limited direct interactions with cholesterol [10,26,27,31]. Similarly, it was shown that the protein environment in a phosphatidylcholine matrix included preferentially PC over PE [27,28,31]. As a consequence, the binding of the proteins could induce local heterogeneities. The proteins may be excluded from cholesterol- or PE-rich domains, leaving a more limited lipid area to interact with. The binding of BSP-A1/-A2 to lipid membranes may also induce a local curvature of the lipid bilayer. The presence of cholesterol and POPE in these curved regions may be unfavorable and thus may limit the capacity of the bilayer to bind BSP proteins. Controlled binding by the micromechanical properties of the bilayers has been recently demonstrated in the case of melittin [48]. Moreover it was shown recently that lipid plane curvature can lead to lipid sorting [49–51]. The saturation ratios observed for BSP-A1/-A2 would be consistent with such phenomenon and this aspect must be examined carefully.

The isotherm binding or partitioning of the BSP-A1/-A2 to lipid membranes at 37 °C cannot be described, in our conditions, by a single equilibrium constant associated to the transfer of the protein from the aqueous environment to the lipid phase. For all the investigated systems, a deviation from a linear simple isotherm is observed. The curvature of the binding isotherms can have different origins. In the Scatchard/Langmuir isotherm model, it is taken into account by introducing the number of lipids per independent binding sites. The more pronounced curvature of the binding isotherms obtained for POPE-, and cholesterol-containing systems led to larger numbers of lipids per binding site. Indeed other phenomena could be at the origin of the non-linear isotherms. The putative long-range electrostatic interactions between the charged proteins and the membrane interface could lead to such behavior. It is also possible that, as mentioned above, the adsorption of the proteins leads to lipid phase separation and, in a context where the affinity appears to be dependent on the lipid composition, there could be a continuous evolution of the overall affinity constant during the titrations. In addition, as BSP-A1/-A2 is a mixture of isoforms with different degrees of glycosylation [1], these may have different binding capacities and may be also a contribution to the dependence of the binding constant with the degree of binding.

The ITC data analysis (Table 2) indicates that BSP-A1/-A2 displays a definite affinity for lipid membranes. The examined bilayers were formed in majority by phosphatidylcholine, a phospholipid for which BSP-A1/-A2 affinity is well established [24,27,28,30]. The basis for the putative specific interaction between the proteins and PC is the binding of phosphocholine groups to fibronectin domains, as inferred from the X-ray diffraction study [20]. The presence of negatively charged POPG in POPC bilayers does not affect the affinity of BSP-A1/-A2 for the membranes while the presence of 30 mol% of POPE in POPC bilayers is shown to increase the affinity of BSP-A1/-A2 for the LUVs. Because the enthalpic contributions are similar for POPC and POPC/POPE bilayers, the increased affinity should be mainly associated with an entropic factor. The presence of PE in the membrane seems to initially facilitate the binding of BSP-A1/-A2, although ultimately less BSP-A1/-A2 can adsorb on such vesicles. This non-linear binding could be due to several rationales as discussed above. Previous fluorescence studies [30] indicated a reduced affinity of the protein for PC/PE bilayers when the content of PE was increased. These experiments were carried out in conditions leading to 6 lipids per protein, a ratio rather low when one considers the binding capacity of these bilayers. Under these conditions, the amount of protein exceeds significantly the lipid/protein saturation ratio of PE-containing bilayers that we have measured (see Table 1), and this limited protein adsorption leads to an apparent reduced binding. Therefore, the present results indicate that the protein affinity can actually be finely tuned by PE content in the membranes. It is interesting to note that sperm plasma membranes contain an appreciable amount of PE (14% of lipid composition, ram sperm [11]). Thus, PE could play a role, whether chemical or structural, in the modulation of BSP-A1/-A2 binding to membranes. The ITC results indicated a decreased affinity constant for POPC/chol bilayer relative to that for pure POPC. It is known that cholesterol does not interact

directly with the protein [10,26,31] but stabilizes the bilayer structure, as illustrated by the reduction of the solubilization assessed by light scattering [27], and the reduction of the BSP-A1/-A2-induced efflux of encapsulated probes in liposomes [24]. Therefore, a decreased binding could be the origin of these reduced impacts of the protein on the membranes. As mentioned above, the formation of the liquid ordered phase in the presence of cholesterol could be responsible for a weaker partition coefficient. The present findings, however, contrast with previous ESR studies proposing that the presence of cholesterol increased the association of BSP-A1/-A2 to different phospholipids [32]. Even spin-labelled cholesterol, for which the motionally restricted component was too small to be quantified for the DMPC/BSP-A1/-A2 systems [27], was significantly immobilized in the cholesterol-containing DMPC membranes. The origin of this discrepancy is unidentified at this point. It may be related to an enhanced ability for the protein to immobilize lipids, considering the restricted motions of the labelled lipids in cholesterol-containing bilayers.

The ITC experiments of the binding of BSP-A1/-A2 on lipid membranes clearly indicate that the thermodynamics of the association are very sensitive to temperature. The most striking aspect of this dependence is the transformation of the exothermic association at 37 °C to an endothermic process at 20 °C in the case of POPC bilayers. The one- and two-dimension infrared spectroscopy indicates that the free protein undergoes denaturation at about 55 °C, in agreement with previous IR and DSC studies [22,24]. In the presence of lipid bilayers, the BSP-A1/-A2 structure is stable upon heating up to 70 °C, an observation consistent with the previously reported shift of the denaturation to 76 °C [24]. This shift is associated to the binding of the proteins to a choline group, and indicates that most of the proteins are bound to the bilayers in the conditions used in this study. The structure of the protein is not influenced by the gel-to-liquid phase transition of DMPC (T_m=23 °C), in agreement with previous results [24]. These results indicate that the denaturation of BSP-A1/-A2 can be excluded as a rationale for the drastic dependence of ΔH_{mol} on temperature. The two distinct signals, one being endothermic and the other exothermic, can actually coexist during the same titration, under certain conditions. The assignment of each signal to a precise molecular event is speculative. However the two contributions strongly suggest different types of associations. BSP-A1/-A2 can exist in an aggregated and polydispersed form [22], and the equilibrium between monomeric/dimeric species and the aggregated form is influenced by temperature, and by the binding of phosphorylcholine and heparin [22,25]. The thermal aggregated-to-monomeric/dimeric transition was reported to be at ~36 °C [22]. It has been shown that multimeric BSP-A1/-A2 undergoes a structural transition upon substrate binding, leading to a form analogous to that of the dissociated form [22]. Therefore it is possible that the phenomena leading to the endo- and exothermic peaks correspond to the association of different quaternary structures of BSP-A1/-A2. It must be reminded that the binding enthalpy includes the changes from both the proteins and the lipids; at low temperatures, the signal would include the dissociation enthalpy of the BSP-A1/-A2 multimers. The different protein structures could also lead to different protein reorganization upon binding, different penetration depth in the lipid bilayers, and/or different orientation. This phenomenon could also be affected by the amount of membrane-bound protein as the intensity of the endothermic component increases during the titrations — probably even at 37 °C where small endothermic peaks are observed at the end of the titrations. These changes in the binding event seem also sensitive to the properties of the bilayers. First, the dependence of the relative contributions of these two components on lipid composition and the temperature suggests that the intensity of the endothermic ITC signal increases as a function of the membrane order. This contribution becomes greater when the temperature is decreased and when cholesterol is present in the bilayers. Second, the bilayer surface charge appears to be also a significant parameter, as the temperature effect is less pronounced in the case of the POPG-containing membranes. This trend is consistent with the previous SPR study [28]. The ΔH_{mol} associated to the BSP-A1/-A2 binding to cholesterol-containing DMPC bilayer, as inferred from the kinetics measured between 6 and 20 °C, was endothermic (+7 kJ/mol). This positive value is in qualitative agreement with the endothermic peak that we observed for cholesterol-containing POPC bilayers at 20 °C. The SPR measurements reported that, in this temperature range, the ΔH_{mol} became negative when negatively charged DMPG was included in the bilayer, a result also consistent with our ITC results, for which no endothermic component was observed for POPG–POPC vesicles, even at 20 °C. The process of association of BSP-A1/-A2 to membranes is modified by temperature, and caution should be used when comparing literature values obtained at different temperatures.

In conclusion, this work determines that the affinity of BSP-A1/-A2 for membranes is modulated by the lipid composition, the lipid/protein ratio, and temperature, suggesting a complex description of the association of these proteins with the membranes. It is well established that the sperm membranes undergo an important reconfiguration of its lipid composition during the capacitation process [8,12,52]. Moreover, it was recently shown that small lipid domains, associated with specific lipid compositions, are present in sperm membranes and are reorganized during the capacitation [53–55]. Therefore, the variations in the affinity of BSP-A1/-A2 for lipid bilayers presented here could be involved in modulating as a function of space and time the association of these proteins and, consequently the extent of lipid extraction, including cholesterol, at a given place and given time. The putative involvement of such modulated affinity should be examined in the global picture of sperm capacitation and preservation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.10.025.

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