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The effect of α-lactalbumin on the thermotropic phase behaviour of phosphatidylcholine bilayers, studied by fluorescence polarization, differential scanning calorimetry and Raman spectroscopy

Herman Van Dael and Frans Van Cauwelaert

Interdisciplinair Research Centrum, K.U. Leuven Campus Kortrijk, Kortrijk (Belgium)

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The effects of bovine α-lactalbumin on the thermotropic properties of dimyristoylphosphatidylcholine liposomes are studied by Raman spectroscopy, fluorescence polarization and differential scanning calorimetry. The Raman spectrum reveals the drastic effects of the protein on the phospholipid structure. The transition temperature shifts downwards and the inter- and intrachain order in the lipid matrix progressively diminish with increasing protein concentration. Up to a lipid to protein molar ratio $R = 25$, the bilayer structure however is maintained. From fluorescence polarization data we conclude that the protein restricts the mobility of the DPH probe. In view of the Raman results, the lower probe mobility obviously cannot be associated with a more rigid lipid matrix. Nevertheless the transition temperatures of the α-lactalbumin-phospholipid complex increases. DSC measurements give no decisive way out for this discrepancy. These results confirm that different types of lipid order are involved in lipid–protein interactions. Compared to the free protein, the α-helicity of the protein has increased in the complex.

Introduction

Phospholipid bilayers have evoked great scientific interest as models for biological membranes. They are widely used in the study of the structural and dynamical properties of membranes. Especially they are suitable for investigations about the interactions between lipids and proteins.

Depending on their nature, proteins are either embedded in the hydrophobic interior of the bilayer as intrinsic or integral proteins, or they are attached to the outside of the bilayer as extrinsic or peripheral proteins. Poly(L-lysine), which is commonly used as a model for extrinsic membrane proteins because of its basic character, has a pronounced stabilizing effect on dipalmitoylglycerol bilayers [1,2] and on model membranes of cardiolipin [3]. Intrinsic membrane proteins like cytochrome *c* oxidase [4] and chlorophyllase [5] also stabilize the bilayer structure in model membranes. The myelin basic protein on the other hand has a perturbing effect on DMPA and DMPS bilayers in both the gel and the liquid state [6]. Polymixin [7,8], melittin [9] and most of the amphipathic proteins and apolipoproteins also de-

Abbreviations: BLA, bovine α-lactalbumin; DMPC, dimyristoylphosphatidylcholine; T_m, gel to liquid-crystal transition temperature; R, lipid to protein molar ratio; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry.

Correspondence: H. Van Dael, Interdisciplinair Research Centrum, K.U. Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium.

stabilize the bilayer structure. It is therefore clear that lipid–protein interactions are not governed by a simple general rule. To get a systematic view on the way in which proteins affect lipid structures, a lot of experiments on different systems is required in a variety of physicochemical conditions and with different physical techniques.

In this contribution, we propose a Raman spectroscopic, DSC and fluorescence polarization study of the interaction of bovine α-lactalbumin with DMPC liposomes. Bovine α-lactalbumin is a small water-soluble protein that undergoes partial conformational changes in an acidic medium and in the binding process of the Ca^{2+} or Na^{+} ions [10–13]. Upon decalcification a hydrophobic surface on the protein becomes more exposed to the solvent or to any interacting species [14,15]. The interaction of bovine α-lactalbumin with phosphatidylcholine vesicles is strongly pH-dependent [16]. Above the isoelectric point (pH = 5), the electrostatic interaction induces a conformational change by which an apolar site of the protein interacts hydrophobically with the lipid bilayer. Below the isoelectric point discrete micellar complexes are formed whereby amphipathic regions of the protein interact with the lipid bilayer. Recently it was also found [17] that at low pH bovine α-lactalbumin induces fusion of phosphatidylserine/phosphatidylethanolamine vesicles. In that process a segment of the protein is inserted into the bilayer. These authors also suggest that the penetration of this bovine α-lactalbumin loop into the bilayer is responsible for the fusion.

Materials and Methods

Materials

DMPC and bovine α-lactalbumin were purchased from Sigma Chemical Co. and were used as supplied. 1,6-Diphenyl-1,3,5-hexatriene was obtained from Eastman Kodak Co. All lipid dispersions and protein solutions were 0.1 M in NaCl and 0.01 M in acetate buffer at pH = 4.

Multilamellar dispersions of DMPC bilayers were prepared by vortexing a buffer solution with lipid concentration of 20% (w/w) for 10 min at room temperature. Appropriate amounts of bovine α-lactalbumin were subsequently added and the mixture was incubated during 1 h at the main transition temperature. It was indeed shown by batch calorimetry [18] that incubation below 22°C or above 26°C leads to an incomplete or to a slow interaction of bovine α-lactalbumin with DMPC.

Raman spectroscopy

For Raman spectroscopy measurements the lipid dispersion was transferred in a Kimax glass capillary tube. After the capillary was spun in a bench-top clinical centrifuge for 10 min, the sample was allowed to equilibrate in the laser beam for 2 h at the highest temperature. Succeeding experiments at descending temperature were performed after minimum 20 min equilibrium at each temperature.

The sample temperature was kept constant by fitting the capillary tube in a copper block that is thermostated by a cryostatic circulator. The temperature measured by a copper-constantan thermocouple placed very near the sample, was corrected by 1.7 C° to account for laser heating.

Spectra were obtained in a Coderg Raman spectrometer after excitation of the sample with the 514.5 nm line of a Coherent Radiation CR-3 argon ion laser. The laser power at the sample was approx. 250 mW and the spectral width 5 cm^{-1}. The monochromator gratings are driven by an Apple II desk computer interfaced to the spectrometer at a scan rate of 1 cm^{-1}/s. The photon counting data collected at each wavenumber were stored in the same computer. The spectra in the C-H region were obtained as an average over 5 scans, while in the C-C region for each spectrum 10 scans are accumulated. All data were seven point smoothed by the Savitsky-Golay method [19].

Fluorescence polarisation

To obtain the final lipid concentration of 0.1 mg/ml, that is required for a maximum sensitivity of the Elscint MV-1A microviscosimeter for steady state fluorescence polarisation measurements, the Raman samples were diluted 2000× with the same buffer as used for the preparation of the original samples.

The labelling of the liposomes with DPH and the further experimental procedure is already described before [20].

Differential scanning calorimetry

Differential scanning calorimetric measurements were performed with a Perkin-Elmer DSC-2C calorimeter equipped with his own data analysis station. Sample pans of 70 μl were filled with a lipid suspension in 0.01 M acetate buffer and 0.1 M NaCl at pH = 4. The lipid concentration was 25 mg/ml. To obtain the samples with different DPMC/BLA molar ratio, the appropriate amounts of bovine α-lactalbumin (BLA) were added to this lipid solution. A scan rate of 2 C°/min was always used.

Results

(a) C-H stretching mode region

In the C-H stretching region (2750–3050 cm^{-1}), the two dominant Raman features at 2845 and 2880 cm^{-1} are assigned, respectively, to the symmetric and the asymmetric C-H stretching modes for the coupled methylene moieties of the hydrocarbon chains in the bilayer interior. Upon melting, the 2880 cm^{-1} band decreases in intensity relative to the 2845 cm^{-1} band and it is subject to broadening and shift to higher frequency. A greater relative peak-height intensity ratio h_{2880}/h_{2845} reflects stronger lateral interchain interactions in the bilayer [21]. This intensity ratio is thus a sensitive probe for the intermolecular coupling and for the lateral packing of the acyl chains.

The band around 2925 cm^{-1} mainly arises from the symmetric C-H stretching mode of terminal methyl groups in the hydrophobic center of the bilayer. As the lipid chain symmetry is lowered, an underlying infrared active methylene asymmetric stretching mode becomes also Raman active and superimposes itself on the main contribution [22]. This effect contributes to the increasing intensity of the 2925 cm^{-1} band as the temperature increases. The relative peak-height intensity ratio h_{2925}/h_{2880} is therefore indicative of both the lattice packing order and the intrachain conformational order. It is an index of the overall disorder of the lipid acyl chain matrix.

In Fig. 1, the C-H stretch spectrum of the bovine α-lactalbumin containing samples is compared with the pure DMPC spectrum (curve A) below (7°C) and above (36°C) the mean transition temperature (23.5°C).

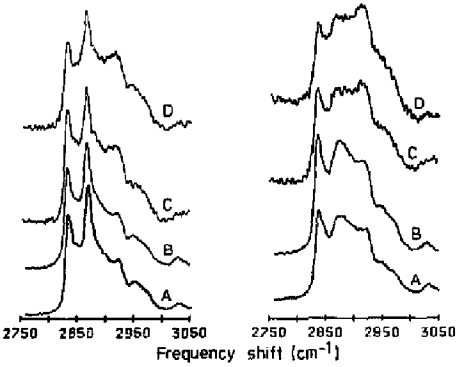

Fig. 1. Effect of α-lactalbumin on the C-H stretching vibrations of DMPC dispersions at 7°C (left panel) and 36°C (right panel). A, control spectra of pure DMPC bilayers; B, lipid to protein molar ratio of 140:1; C, 35:1; D, 25:1. Intensities have arbitrary units.

α-Lactalbumin exhibits observable effects on the normal phase transition of DMPC at a molar ratio R as low as 140 : 1 lipid to protein. Although at this low protein level, the spectra (Fig. 1B) resemble the pure ones at low and at high temperature, the real protein influence can be clearly observed from the complete temperature profiles on Fig. 2A and B. The shape of the transition curve is maintained but both spectral indices indicate a downward shift for T_m over 5 C°. At this concentration, the internal bilayer structure and the intermolecular coupling is only indirectly affected and the entire liposome is more fluidized by the protein.

The markedly broadened transition at $R = 70$ on the contrary points to a smaller degree of cooperativity in the bilayer induced by protein perturbation. This temperature profile approximates that of pure small unilamellar vesicles [23] where cooperativity has decreased by the curvature of the lipid particles. The midpoint of the transition at $R = 70$ however, is returned to the transition temperature of the pure lipid sample.

A drastic change in the lipid order is established at a lipid to protein concentration of 35 : 1. The sigmoidal-shaped curve, characteristic of a phase transition, is still present in the ratio h_{2925}/h_{2880} but has nearly disappeared in the ratio

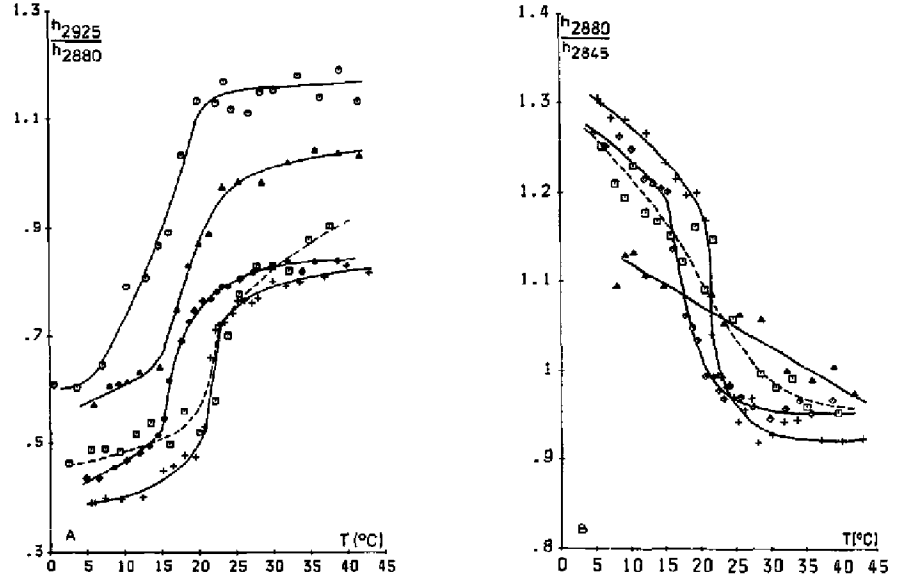

Fig. 2. Temperature profiles derived from the Raman spectral h_{2925}/h_{2880} (A) and h_{2880}/h_{2845} (B) peak-height intensity ratios. (+) pure DMPC; (◇) lipid to protein molar ratio of 140:1; (□) 70:1; (△) 35:1; (○) 25:1. (The dashed line for $R = 70$ is used for clarity.)

h_{2880}/h_{2845}. In this DMPC-BLA system, cooperativity and intermolecular order have considerably decreased, while the intrachain order and the order of the terminal methyl groups in the hydrophobic center are less affected. The perturbing effect nevertheless can be clearly observed even in the ratio h_{2925}/h_{2880} that has augmented up to 0.57 in the gel and up to 1.04 in the liquid state. At $R = 25$, the enhancement of the 2925 cm^{-1} peak continues in both states so that the ratio h_{2925}/h_{2880} varies

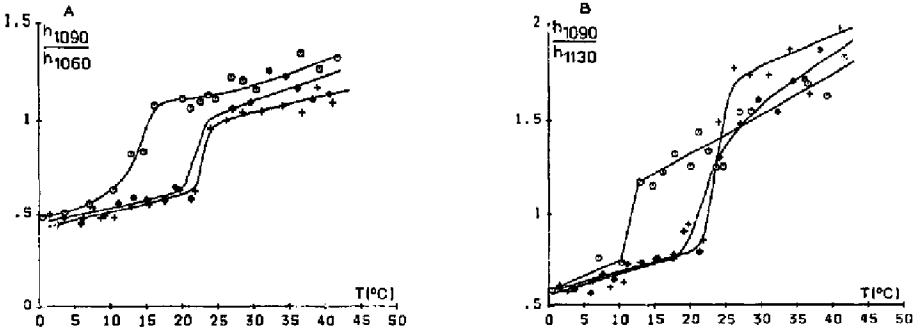

Fig. 3. Temperature profiles derived from the Raman spectra h_{1090}/h_{1060} (A) and h_{1090}/h_{1130} (B) peak-height intensity ratios. (+) pure DMPC; (◇) $R = 140$; (○) $R = 25$.

from 0.6 to 1.2. As can be seen on Fig. 1 C and D, the sharp and isolated peak at 2880 cm^{-1} at low temperature, shades off into a broad band that reaches up to the 2925 cm^{-1} peak when the transition to the liquid state is effectuated.

(b) The C-C stretching region

In the skeletal optical mode region (1000–1200 cm^{-1}), the Raman spectrum of DMPC is dominated by bands originating from vibrations of the hydrocarbon chains. The C-C stretching vibrations of long portions of the acyl chains give rise to three characteristic peaks.

If the temperature is raised, the intensity of the 1060 cm^{-1} and 1130 cm^{-1} bands decreases. At the same time, the 1090 cm^{-1} intensity increases, reflecting the growing population of *gauche* conformers. The 1090/1130 cm^{-1} and 1090/1060 cm^{-1} intensity ratios therefore are widely used as probes for the *gauche/trans* population and for the intrachain disorder.

These general features can be found back in the transition curves, constructed from the peak height ratio's h_{1090}/h_{1060} (Fig. 3A) and h_{1090}/h_{1130} (Fig. 3B) at different temperatures.

The effect of α-lactalbumin on the C-C stretching vibrations is not so pronounced as on the C-H stretch. Fig. 3 shows that the intrachain order of the lipids both in the gel and in the liquid crystal phase as well as the transition temperature are only slightly modified by low bovine α-lactalbumin contents. A further increase of the protein content to $R = 25$ shifts T_m downwards to about 12°C, completely in agreement with the T_m value gathered from the C-H stretch measurements.

In the high protein concentration spectra, the presence of α-lactalbumin appears by a supplementary peak just above 1000 cm^{-1}. This is a well known and strong peak in many protein spectra [24]. The mean contribution is due to the breathing vibration of the benzene ring in the phenylalanine side chains and the high frequency shoulder arises from an indole-ring vibration of the tryptophan groups. We must remark here that in this high protein concentration spectra, phenylalanine vibrations contribute also to the spectrum at 1130 cm^{-1}. Therefore the measured value for h_{1090}/h_{1130} (Fig. 3B) is an underestimation of the real value. This explains why on Fig. 3B the curve for $R = 25$ lies beneath the transition curve of the pure DMPC sample in the liquid state at high temperature.

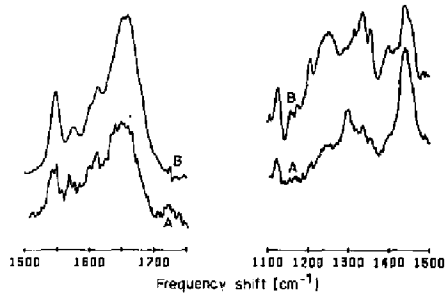

Fig. 4. Spectra in the amide I (left) and the amide III (right) region for (A) DMPC-BLA complex with $R = 25$ (B) pure BLA. These spectra are recorded at room temperature. Intensities are given in arbitrary units. BLA, bovine α-lactalbumin.

(c) The protein structure

As mentioned before, Raman peaks due to the protein can also be distinguished in the spectra of complexes with high protein concentration. This offers an interesting tool to compare the structure of α-lactalbumin in the complex with the free protein conformation. As spectral indices for the protein structure, the amide I (1640-1680 cm^{-1}) and the amide III (1240–1300 cm^{-1}) bands can be used.

In the complex, the amide I band undergoes a net shift to lower wavenumbers (Fig. 4). This means that the relative amount of the different protein conformations has changed so that the α-helix contribution has augmented. Hanssens et al. [16] drew the same conclusion from their circular dichroism experiments. At pH = 4, they calculated a helical content of $30 \pm 3\%$ for α-lactalbumin in the absence of DMPC vesicles, while in contact with the vesicles a helical content of $50 \pm 3\%$ is reached.

In the amide III region, random coil and β-sheet contributions are situated around 1250 cm^{-1} while the helix conformation can be distinguished at higher wavenumbers. In the DMPC-BLA complex, the latter contribution interferes with the strong CH_2 twisting vibration of DMPC at 1298

cm^{-1}. We can, however, show that in the complex, the intensity of this peak has increased. Compared to the intensity of the C-N symmetric stretching vibration at 718 cm^{-1}, that is assumed to be independent of the bilayer conformation [25] and that can be used as internal standard [26], the intensity of the 1300 cm^{-1} peak, h_{1300} grows from 1.1 in the pure sample to 1.4 in the complex with $R = 25$. This value remains the same below and above the main transition temperature and suggests an increased α-helicity in the complex.

(d) Fluorescence polarization

The steady-state fluorescence polarization P is defined as

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

in which $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities parallel and perpendicular to the incident light direction. Considering recent attempts to interprete fluorescence data in terms of order parameters of the lipid matrix [27] and to correlate them with the rotational diffusion of the probe in an anisotropic environment [28], it has however more physical sense to use the steady-state anisotropy r_s as relevant fluorescence parameter. It is related to the polarization in a simple way

$$r_s = \frac{2P}{3 - P}$$

In Fig. 5, the anisotropy data of DPH incorporated into DMPC liposomes with an increasing amount of bovine α-lactalbumin are plotted as a function of temperature. In the gel phase the influence of bovine α-lactalbumin is negligible but in the liquid phase bovine α-lactalbumin does rise both T_m and r_s. This effect was earlier already established for a sample with a higher protein content ($R = 8$) [18].

The anisotropy r_s can also be written [29] as the sum of two components: r_d, the dynamic component related to the molecular reorientation rate of the probe and r_∞, the static component. Van Blitterswijk et al. [30] have proposed an empirical relationship between r_s that can be derived directly from experimental results and r_∞ that can be associated with the lipid order parameter S.

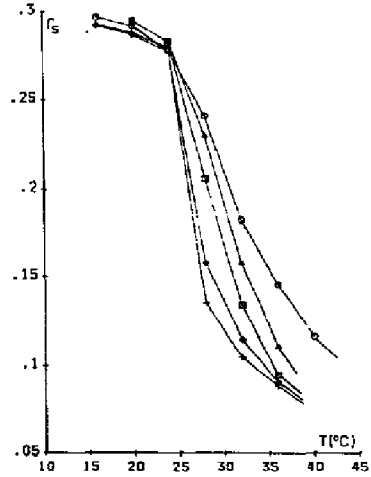

Fig. 5. Diphenylhexatriene fluorescence anisotropy data for pure DMPC bilayers (+) and for the DMPC-BLA complex with $R = 140$ (◇), $R = 70$ (□), $R = 35$ (△) and $R = 25$ (○). Incubation temperature 24.5 °C. Samples were prepared from the batches for the Raman experiment after diluting 2000× with the same buffer. BLA, bovine α-lactalbumin.

$$r_\infty = \tfrac{8}{3} r_s - 0.10$$

Although this equation is not valid for all membrane systems (see discussion on p. 277 in Ref. 18), time-resolved experiments [31] have proven the applicability to the DMPC/BLA system.

In the wobbling-in-cone model of Kinosita et al. [32], that is valid for cylindrical probe molecules aligned between the acyl chains of the lipid, the symmetry axis of the probe wobbles within a cone of total aperture $2\theta_c$. The order parameter then is defined and related to θ_c by

$$S = \left[\frac{r_\infty}{r_o}\right]^{1/2} = \tfrac{1}{2}\cos\theta_c(1 + \cos\theta_c)$$

The fluorescence anisotropy in the absence of any rotational motion of the probe, r_o, can be determined as 0.4 for DPH [29,30].

TABLE I

THE ORDER PARAMETER S AND THE CONE SEMIANGLE θ_c AS A FUNCTION OF TEMPERATURE FOR DIFFERENT BOVINE α-LACTALBUMIN CONCENTRATIONS IN DMPC LIPOSOMES

Incubation conditions for the samples were 2 h at 24.5°C.

T (°C)	$R=\infty$		$R=140$		$R=70$		$R=35$		$R=25$	
	S	θ_c(°)	S	θ_c(°)	S	θ_c(°)	S	θ_c(°)	S	θ_c(°)
16	0.852	26	0.850	26					0.860	25
20	0.843	27	0.841	27	0.856	26	0.856	26	0.847	27
24	0.827	28	0.823	29	0.833	28	0.833	28	0.823	29
28	0.447	58	0.526	50	0.661	41	0.719	36	0.744	35
32	0.316	63	0.365	61	0.443	56	0.526	50	0.600	45
36							0.346	62	0.486	53

The data for S and θ_c at various temperatures are conveniently arranged in Table I. It is clear that S and θ_c, independent of the protein concentration, are hardly affected in the gel phase but just above 24°C, both they undergo a drastic change that strongly depends on the bovine α-lactalbumin content. In the presence of bovine α-lactalbumin, the motion of the probe molecule is severely restricted. In terms of lipid matrix properties, this is usually related to a more rigid and more ordered phospholipid chain assembly.

It is also useful to emphasize here the role of incubation temperature in the DMPC–BLA interaction in a separate set of experiments. After an incubation period of 2 h at 24.5°C, the DMPC–BLA interaction seems to have reached an equilibrium at any protein concentration used, because the upward temperature scans totally coincide with the downward ones. After an incubation period of 2 h at 4°C, however, no trace of lipid-protein interaction is found. At ascending temperature the polarization data of a mixture even with $R=27$, hardly diverge from the pure lipid sample data. Incubation for the same period at 15°C, leads to intermediate anisotropy values.

(e) DSC

The variation of excess specific heat with temperature for the simple two-state, first order, endothermic gel to liquid crystalline phase transition, exhibited by the pure phospholipid dispersion ($R=\infty$) is depicted in Fig. 6. In this thermogram, the peak going from 24°C to 30°C has his maximum at 25°C.

By adding bovine α-lactalbumin, the transition region is broadened and an additional maximum appears at 27.2°C. Such a two-component curve

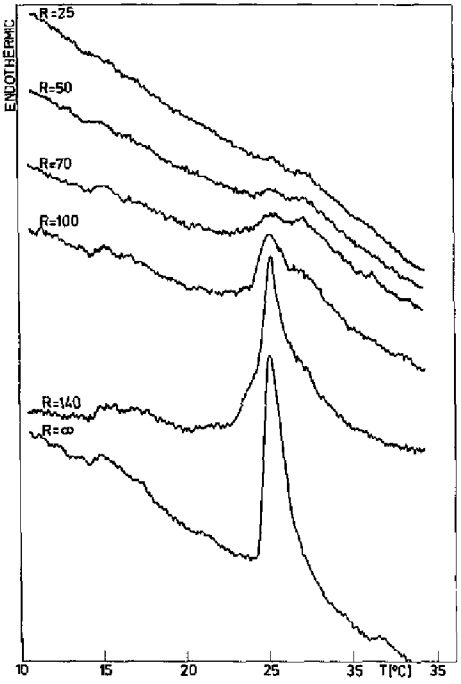

Fig. 6. Calorimetric scan of DMPC vesicles with different concentrations of bovine α-lactalbumin. R is the lipid to protein molar ratio.

indicates the presence of two different physical states or differently ordered environments in the lipid bilayer. The first maximum remains at the same temperature and refers to the transition of the unperturbed pure DMPC bilayer. The relative intensity of the second component increases as a function of concentration. Then more acyl chains are situated in a bovine α-lactalbumin perturbed domain and will be rearranged in a DMPC-BLA complex. For $R < 70$, the relative strength of the latter peak to the former shows no further increase. The protein itself shows no phase transition in the examined temperature region.

Discussion

The interaction of α-lactalbumin with DMPC was already studied by other techniques. The microcalorimetric and tryptophan fluorescence studies on sonicated vesicles [33] have indicated that at pH = 4 the original vesicles break down after interaction with α-lactalbumin to form a lipid-protein complex with a molar ratio of about 80, while at pH = 7 the interaction of bovine α-lactalbumin with DMPC is rather weak. These results have been confirmed by fluorescence polarization measurements of Herreman et al. [20]. They have also found that compared to the pure phospholipid, the transition temperature of the complex has increased with 3–4.5 C° depending on the molar ratio. Gel chromatography and light scattering experiments [34] have completed the investigation of the bovine α-lactalbumin interaction with sonicated vesicles. The comparison of the enthalpy state of vesicles of different size after their interaction with bovine α-lactalbumin [18] has led to the conclusion that the size and the composition of the lipid-protein complex formed with bovine α-lactalbumin around the lipid transition temperature at pH = 4, is independent of the vesicle type used. Interaction with small unilamellar, large unilamellar and large multilamellar vesicles gives rise to a complex of the same size and with the same lipid to protein molar ratio. The mode of interaction is not determined to a great extent by the demetallization of the protein [16]. In our case where 0.30 Ca^{2+} is bound per protein molecule at pH = 7, it is showed by Permyakov et al. [11] that due to the competition of Ca^{2+} and protons for the same site at pH = 4 only a few bound Ca ions are left at the protein. Furthermore the effect of 0.1 M NaCl on the protein conformation is very small at this pH [41]. However, lipid solubilization and complex formation are favoured by the unfolding of the tertiary structure of bovine α-lactalbumin [16].

As our Raman results contrast with the fluorescence results cited above [20], especially so far as the transition temperature behaviour is concerned, we repeated the fluorescence experiments on diluted Raman samples. As mentioned in the Results section (d), the increase of the transition temperature found in earlier measurements [20] is confirmed and contrasts with the Raman results where a net decrease of the transition temperature is found when bovine α-lactalbumin is added to the lipid. Additional Raman experiments (not shown) on the interaction of bovine α-lactalbumin on dipalmitoylphosphatidylcholine liposomes also exhibit a similar decrease of T_m and confirm the perturbation of the lipid bilayer effectuated by the protein. Such discrepancy in the transition behavior of a lipid-protein system derived from fluorescence and Raman data is not new and has already been described for a cardiotoxin-DMPA [36] and for the melittin-DMPC system [9]. These authors suggest several reasons that could be responsible for such an apparent discrepancy. The time scale used for obtaining Raman results (10^{-13} s) is very different from the time scale in fluorescence polarization experiments (10^{-8} s). Raman spectroscopy gives direct information on the C-H and C-C vibrational states while fluorescence polarization is a typical probe method. Exact location of DPH in the lipid bilayer can be questioned and the information obtained with this technique corresponds only with the lipid state in the neighbourhood of the probe molecule.

Studying the effects of bee venom melittin on the order and the dynamics of DMPC multilamellar vesicles, Bradrick et al. [37] recently also discovered a disparity between their fluorescence polarization experiments with a DPH probe and their Raman results. In the fluorescence polarization experiments, the protein induces a decrease in the lipid order parameter only at the transition temperature and below and above T_m, the order parameter is barely affected. On the other hand,

the Raman data detect a decrease in the inter- and intramolecular order of the acyl chains of multilamellar vesicles below T_m and a decrease of only the intermolecular order in the liquid-crystalline phase. All these data were gathered with samples at a lipid to protein molar ratio = 60. This disparity between the Raman and the DPH data also is discussed in terms of differences in time scale of the two techniques and in the state of aggregation of the lipid-bound protein.

In addition to these reasons, the intrinsic difference at the molecular level between the measured 'orders' seems important. The examples cited above [9,36,37] and our measurements on the DMPC–BLA interaction are a conformation of the statement of Jähnig et al. [38] "that the experimental data on lipid–protein interaction cannot be interpreted in terms of an alteration of a single-type of lipid order". According to these authors the orientational order of the phospholipid chains is a superposition of their conformational order and their rigid-body order. Petersen and Chan [39] proposed the product

$$S = S_{\text{conform}} \cdot S_{\text{rigid-body}}$$

where S is the total orientational order. The DPH experiments predominantly sense the rigid-body of the lipid chains i.e. the rocky fluctuation of the entire lipid chains. In Raman spectroscopy, however, the average conformational order of all chains is measured. If this theoretical model is applied to our results, the conformational order of the phospholipid chains has decreased over the entire temperature range while the rigid-body order remains fairly constant below the transition temperature but increases at and above the transition temperature.

In that perspective we compared the earlier Raman spectroscopic data [40] and the very recent calorimetric data of Liddle and Tu [41] on the effect of myotoxin a on the thermotropic phase behaviour of model lipid membranes. They stated that the main gel to liquid-crystal phase transition temperature of DMPC was decreased by myotoxin a in their Raman experiments [40]. Their DSC measurements on the contrary [41] show an increase of T_m from 23°C to 32–35°C. This shift is concentration dependent. A further analysis of their measurements also shows that the transition is completely abolished at a molar ratio 20:1 in the Raman experiments while the calorimetric effects persist even at $R = 10$.

From our own calorimetric data, it can be concluded that in the DMPC–BLA complex at least two different transitions take place. Even at the highest bovine α-lactalbumin concentration a part of the DMPC bilayer melts like the pure one. On the other hand, addition of bovine α-lactalbumin leads also to a stabilization of a phospholipid fraction that melts at higher temperatures.

From this discussion, it is clear that protein–lipid interactions studies can lead to different results when they are carried out by different techniques. As DSC experiments at their turn introduce disparities, they cannot remove the manifest discrepancy between our Raman and fluorescence polarization results.

Another problem to be discussed is the way in which bovine α-lactalbumin interacts with the lipid bilayer and contributes to the disorder in the acyl chains. That process is influenced by the molar ratio in a significant way.

At low concentrations ($R = 140$) the amphiphatic helices of bovine α-lactalbumin can associate with the phospholipid headgroups on the bilayer surface. The geometrical effect of such an association is an increase in surface area of the lipid [42]. That leads to a reduced intermolecular interaction between the lipid chains and to higher values for the h_{2925}/h_{2880} intensity ratio, reflecting the positional disorder with more spacing between the chains. The melting curve is not significantly smoothed by this low bovine α-lactalbumin concentration. Thus bovine α-lactalbumin behaves here as an extrinsic protein without an extensive penetration in the bilayer. This type of interaction has earlier be found in the polyglycine-DMPC complex [43]. From this interaction mechanism, it is also clear that the thermotropic properties derived from C-C vibrations (Fig. 3) in the lipid chain itself are much less affected than the C-H vibrations. At this protein concentration a stable vesicular complex is formed.

Increasing the bovine α-lactalbumin concentration broadens the transition curve; the complex becomes unstable and breaks up to form disc-like

micellar complexes. From $R = 35$ on, the conformational order is strongly disturbed in the gel phase suggesting a lower symmetry arising from an increased mobility of the acyl chains. The decrease of transition temperature appears also in the C-C stretching region due to the greater amount of *gauche* conformers. At this point, the earlier statement [18] that fluorescence anisotropy data indicate that the motion of lipid molecules is strongly restricted in the presence of bovine α-lactalbumin must be reinterpreted. From our Raman results it is clear that bovine α-lactalbumin on the contrary enhances internal motion of the acyl chains. Our fluorescence data (Table I), however, confirm that in the liquid state the rigid body fluctuations of the DPH probe are restricted by the protein while in the gel phase the probe mobility is unaffected.

Upon complex formation the helical content of bovine α-lactalbumin strongly increases due to the interaction between the amphipatic helix and the polar and apolar phases of the phospholipid bilayer [16]. In such a situation it is not excluded that due to the uneven surface of the α-helix the conformational order of the lipid chain decreases while the DPH anisotropy increases.

Finally we can give support to the theory of Kim and Kim [17] that suggested that only a segment of bovine α-lactalbumin penetrates into the bilayer, forming a loop in the hydrophobic interior while the terminal segments are situated at the outside of the bilayer. Our experiments cannot predict what portion of the protein goes inside the bilayer but the suggestion that the sequence from amino acid 85 to amino acid 106 penetrates, is not in contradiction with the calculated data [16] of the mean hydrophobicity $\langle \mathcal{H} \rangle$ and the mean helical hydrophobic moment $\langle \mu_H \rangle$ of helical segments. That segment can fold to form a loop with the hydrophobic sites facing the acyl chains of the phospholipid. The increased helicity observed by adding bovine α-lactalbumin to DMFC (Ref. 16 and this work) could point in that direction.

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