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COMPARISON OF THE ENTHALPY STATE OF VESICLES OF DIFFERENT SIZE BY THEIR INTERACTION WITH α-LACTALBUMIN

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The characteristics of small unilamellar, large unilamellar and large multilamellar vesicles of dimyristoylphosphatidylcholine and their interaction with α-lactalbumin are compared at pH 4. (1) By differential scanning calorimetry and from steady-state fluorescence anisotropy data of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene it is shown that the transition characteristics of the phospholipids in the large unilamellar vesicles resemble more those of the multilamellar vesicles than of the small unilamellar vesicles. (2) The size and composition of the lipid-protein complex formed with α-lactalbumin around the transition temperature of the lipid are independent of the vesicle type used. Fluorescence anisotropy data indicate that in this complex the motions of the lipid molecules are strongly restricted in the presence of α-lactalbumin. (3) The previous data and a comparison of the enthalpy changes, ΔH, of the interaction of the three vesicle types with α-lactalbumin allow us to derive that the enthalpy state of the small unilamellar vesicles just below 24°C is about 24 kJ/mol lipid higher than the enthalpy state of both large vesicle types at the same temperature. The abrupt transition from endothermic to exothermic ΔH values around 24°C for large vesicles approximates the transition enthalpy of the pure phospholipid

Introduction

Recent reviews [1–3] show that the use of vesicles is extremely widespread in studies on the fundamental role of phospholipids in the cell membrane and as carrier systems for targeting. In these studies liposomes were used in different forms: small unilamellar vesicles, obtained by sonication, large unilamellar vesicles, prepared by different methods [4,5] and large multilamellar vesicles obtained by mechanical shaking. Many different physical techniques were used to characterize the physical state of the small and large vesicles. Summarized the phospholipids in highly curved bilayers undergo a broad gel-to-liquid-crystalline transition [6–8] and show a smaller transition enthalpy due to a lower cooperativity of the phase transition [9]. It was further demonstrated that the phase-transition properties are sensitive to the size of the vesicle only in the 20–70 nm region [7,8]. Cornell et al. [10] suggested in a theoretical study that the highly curved bilayers in small vesicles are stable only if energy is fed into the system. Gruenewald et al. [11], in a differential scanning calorimetric study of unilamellar vesicles with diameters between 36 and 80 nm, found that the steepness of the lipid phase transition is predominantly determined by the transition enthalpy rather than by the cooperativity. They further suggested that below the transition temperature

the lipid molecules are in a higher energetic state in small vesicles than in larger aggregates.

Hitherto, as shown in the studies cited above, information on the energy content of vesicles of different size was obtained mainly by physical techniques. However, information on the energy content of vesicle systems can also be derived by comparing the interaction enthalpy of each vesicle type with the same protein obtained by batch calorimetry at constant temperature, if the three vesicle types form the same interaction product with this protein. It was shown previously [12–14] that α-lactalbumin, in its interaction with small unilamellar vesicles at pH 4, behaves in many different aspects like apolipoproteins. It destroys the vesicles at low lipid-to-protein molar ratios to form a stable lipid-protein complex of definite composition at 24°C. Although such interactions between α-lactalbumin and phospholipids at pH 4 have no physiological repercussion, they were used as a model system to study the role of amphipathic helices in lipid-protein associatoins. α-Lactalbumin is, according to Segrest and Jackson [15], a candidate for such associations.

In this paper we compare the enthalpies of interaction of α-lactalbumin with small unilamellar, large unilamellar and large multilamellar vesicles of dimyristoyl phosphatidylcholine. Since the lipid-protein complexes formed with the three vesicle types are identical, we are able to show that the greatest difference in energy content between the small vesicles and both large vesicle types is observed just below the transition temperature, 24°C, of the lipid.

Materials and Methods

Protein and vesicle solutions

All vesicle solutions were prepared with dimyristoylphosphatidylcholine. The origin and characteristics of α-lactalbumin and dimyristoylphosphatidylcholine were described before [12]. All protein and liposome solutions were 0.1 M in NaCl in the 10 mM acetate buffer (pH 4). Small unilamellar vesicles were prepared as described before [12]. Multilamellar vesicles or liposomes were prepared by shaking the buffer solution of the phospholipid for 10 min at room temperature. Large unilamellar vesicles were prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos [5]. The lipid (5 mg) was added to a mixture of 2 ml diethyl ether and 0.6 ml buffer solution. This solution was sonicated for 10 min in a bath-type sonicator until a stable emulsion was obtained. Then the mixture was placed on the rotary evaporator at 30°C where the diethyl ether was removed under reduced pressure. A viscous gel phase was formed at this stage which was mechanically shaken for 10 s and to which 2 ml thermostatically controlled buffer solution was added. From this aqueous suspension the last traces of diethyl ether were removed by an additional evaporation. With this preparatoin, large unilamellar vesicle suspensions with concentratoins of phospholipid up to 2 mg/ml could be obtained. These solutions were optically much clearer than normal liposome solutions with the same concentration. The stability of these preparations was checked by electron microscopy (Results section). For the differential scanning calorimetry concentrated large unilamellar vesicles (19 mg/ml) were prepared by evaporating the diethyl ether from a dispersion containing 20 mg phospholipid, 3 ml diethyl ether and 1 ml buffer solution. Large unilamellar vesicles were also prepared by the diethyl ether injection method of Deamer and Bangham [4]. Although we obtained large unilamellar vesicles, their concentration (0.3 mg/ml) was too low to be useful in batch microcalorimetry. In fluorimetry, in gel chromatography and electron microscopy, large unilamellar vesicles of both preparation methods gave comparable results.

Phospholipid concentrations were determined as described before [12]. For the determination of α-lactalbumin in the different gel chromatographic samples, another method was used. α-Lactalbumin in the absence of phosphatidylcholine is easily determined spectrophotometrically with a Beckman D-spectrophotometer at 280 nm using a value of $E^{1\%} = 20.1$ [16]. However, in the presence of phosphatidylcholine this method cannot be used. Therefore, α-lactalbumin was determined by the fluorescamine method of Böhlen et al. [17]: 0.5 ml of a solution of 30 mg fluorescamine in 100 ml dioxane is added to 1.75 ml buffer solution containing 0.25 ml of an α-lactalbumin solution in the 0.005–0.1 mg/ml range. The highly fluorescent

reaction product is measured at 475 nm (excitation at 390 nm). Before each series of protein concentration determinations, the fluorescence of the reaction product was standardized against the tryptophan absorption at 280 nm for pure α-lactalbumin solutions of known concentration. In this method it is found that the presence of phosphatidylcholines (even in circumstances where a lipid-protein complex was formed) did not alter the fluorescence intensity of fluorescamine.

Methods

The procedures for gel chromatography, batch microcalorimetry, electron microscopy and steady-state fluorescence polarization were previously described in detail [12–14]. Differential scanning calorimetric measurements were performed with a Perkin Elmer DSC-2C, equipped with a Thermal Analysis Data Station (TADS). Large-volume liquid sample pans were used. They can contain up to 75 μl and were filled with about 50 mg aqueous lipid suspension or complex solution. The lipid concentration was about 19 mg/ml for the vesicle suspensions and about 40 mg/ml for the lipid-α-lactalbumin complex. The calorimeter was calibrated with a benzene standard. The accuracy of these measurements was about 0.3°C. A scan rate of 1.25 $K \cdot min^{-1}$ or 5 $K \cdot min^{-1}$ was used. The determination of a transition temperature with this dynamic technique depends on the type of endotherm. For narrow endotherms, characteristic for a well-defined first-order transition, the transition temperature corresponds to its onset temperature. The same procedure is followed for the temperature calibration of the differential scanning calorimeter. When the transition is smeared out over a broad temperature range, no single transition temperature can be attributed. The temperature at the maximum of the endotherm is taken as the average transition temperature.

Results

Characterization of the large unilamellar vesicles

Electron microscopy and differential scanning calorimetry

Electron micrographs of small unilamellar and large multilamellar vesicles are not shown, since they are identical to the known pictures in the literature. The small vesicles are about 30–40 nm in diameter. The large unilamellar vesicles obtained by the reverse-phase evaporation method are shown in Fig. 1. They have a mean diameter of 150 nm. Electron micrographs taken of these samples after 24 h at room temperature did not show a transformation to multilamellar vesicles. However, some of the large vesicles formed aggregates of unilamellar species. Small unilamellar and large multilamellar vesicles have been carefully studied in the past by differential scanning calorimetry by many different authors. Since to our knowledge no calorimetric scans are available on the large unilamellar vesicles, prepared under these conditions, Fig. 2 compares such scans for large multi- and unilamellar vesicles. The large unilamellar vesicles always show for the main transition a lower onset,

Fig. 1. Electron micrograph of negatively stained large unilamellar vesicles. Total magnification is ×38250.

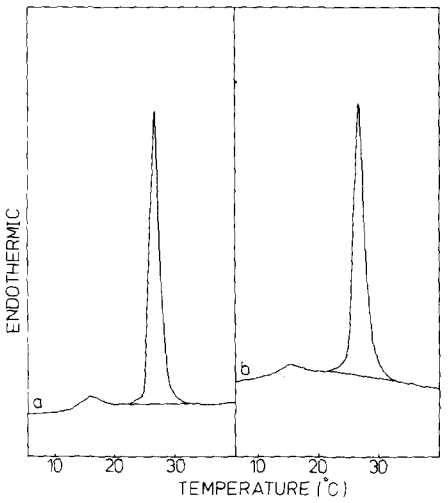

Fig. 2. Differential scanning calorimetric tracings for multilamellar vesicles (a) (60 μl containing 1.34 mg lipid in solution) and for large unilamellar vesicles (b) (60 μl containing 1.24 mg lipid) at a scan rate of 5 K/min.

a somewhat broader transition and a lower transition enthalpy. For instance, at a scan rate of 1.25 $K \cdot min^{-1}$, the onset temperature is 24.1°C with a ΔH of 18.9 $kJ \cdot mol^{-1}$ for the large unilamellar vesicles, compared with 24.5°C and a ΔH of 20.9 $kJ \cdot mol^{-1}$ for the multilamellar vesicles. Kantor et al. [18] found for the latter vesicles 23.9°C and 23 $kJ \cdot mol^{-1}$. The pretransition is also present in the scan of the large unilamellar vesicles, but always smaller than for the multilamellar vesicles.

Fluorescence anisotropy

In Fig. 3A,B steady-state fluorescence anisotropy data, r_s, of diphenylhexatriene incorporated into the hydrocarbon region of the vesicles, are plotted as a function of temperature. These data were calculated from steady-state fluorescence polarization values P, according to the relation

$$r_s = \frac{2P}{3-P} \tag{1}$$

On theoretical grounds [19,20] as well as by experiments using time-resolved fluorescence depolarization measurements [21–23] it is shown that the steady-state fluorescence anisotropy can be written as:

$$r_s = r_d + r_\infty \tag{2}$$

The first component, r_d, is a dynamic component. It is related to the rate of molecular reorientation of the probe. The second component, r_∞, is the static contribution to r_s. It is related to the range of rotational motion of the probe. In the wobbling-in-cone model of Kinosita et al. [19] it is assumed that the major axis of the rod-shaped diphenylhexatriene molecule wobbles uniformly within a cone of semiangle θ_c. The residual anisot-

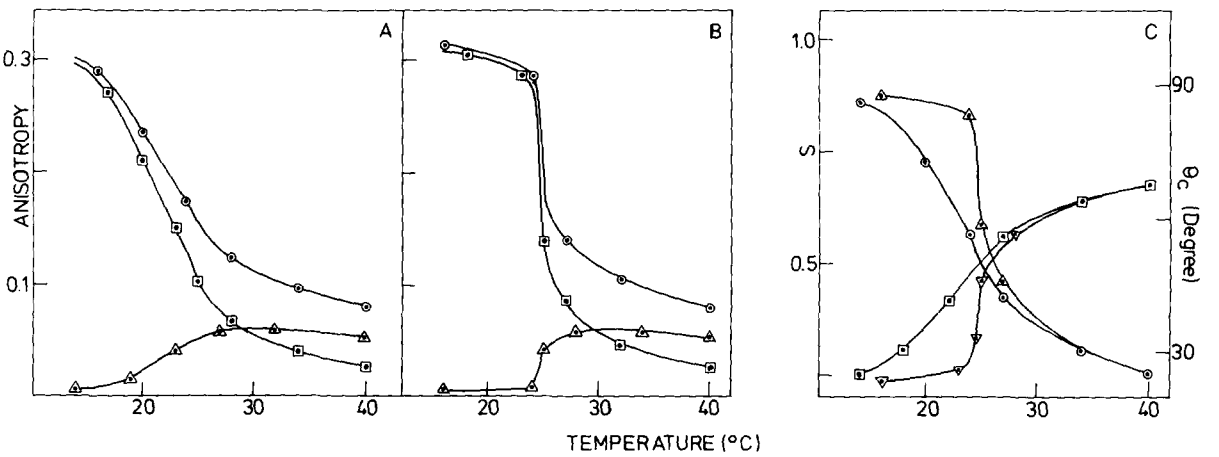

Fig. 3. Diphenylhexatriene fluorescence anisotropy data (⊙, r_s; ⊡, r_∞; △, r_d) for small unilamellar (A) and for large unilamellar vesicles (B). The order parameter, S, (⊙, small unilamellar vesicles; △, large vesicles) and the cone angle, θ_c, (⊡, small unilamellar vesicles; ▽, large vesicles) are also shown (C).

ropy is then related to this cone angle by

$$r_\infty / r_0 = [1/2 \cos\theta_c(1+\cos\theta_c)]^2 \quad (3)$$

or to the order parameter, S, by

$$r_\infty / r_0 = S^2 \quad (4)$$

From Eqns. 3 and 4 it is clear that a higher r_∞ implies a higher order parameter and a smaller cone angle.

Recently, Van Blitterswijk et al. [24] have suggested that there should exist a unique relationship between r_s, r_d and r_∞ for artificial and biological membranes in the sense that if two membranes have the same r_s they should have the same r_∞ and therefore the same order parameter. This relationship is shown in Fig. 2 of their paper. In the region $0.13 < r_s < 0.28$ this relationship is linear and given by

$$r_\infty = \tfrac{4}{3} r_s - 0.10 \qquad 0.13 < r_s < 0.28 \quad (5)$$

However, we wish to warn for the fact that probably no unique relationship exists between r_s, r_d and r_∞ for all kinds of membranes, as will be seen further. Nevertheless, for dimyristoylphosphatidylcholine this relationship is a good one [24] and we used it to separate r_s into its two components, as is shown on Fig. 3A,B where r_s, r_d and r_∞ are depicted for the different vesicle types. The dependence of anisotropy on temperature for both large vesicle types is the same as shown in Fig. 3B. It differs from the behavior of the small vesicles (Fig. 3A). Below the transition temperature of the phospholipid, r_∞ is considerably more important than r_d which is small and at low temperatures practically vanishing. Above the transition temperature the dynamic component predominates. Furthermore, in contrast with the broad transition range for the small vesicles, a sharp transition at 24.5°C occurs for the large vesicles. Those data are in agreement with the Raman spectroscopic data of Van Dael et al. [25]. The small temperature range of the phase transitions for the large unilamellar vesicles follows the scattering data of Van Dijck et al. [7] who found that when the diameter of the vesicles increases from 50 to 80 nm, the pattern of the phase transition resembled more that of multilayered liposomes. Since our large unilamellar vesicles have a much larger diameter than 80 nm, it is expected that their transition pattern very closely approaches the pattern of multilayered vesicles.

On Fig. 3C the cone angle and the order parameter calculated from Eqns. 3 and 4 are depicted as a function of temperature. In these calculations and following Van Blitterswijk et al. [24] and Heyn et al. [26] we used the theoretical value of 0.4 for r_0. The main conclusion from 3C is that the order parameter, S, and the cone angle, θ_c, are nearly the same for small and large vesicles above the transition temperature, but the most important difference is observed in the region just below this temperature. For all vesicles the cone angle is about 25° at 15°C and 70° above the transition temperature. Both values are in agreement with the data of Kinosita et al. [27] for the same phospholipid.

Vesicle-protein interactions

Influence of the vesicle type on the interaction

As stated in the introduction, comparison of the energy content of the three vesicle types is possible only if the same lipid-protein complex is formed with the protein. Therefore, experiments were carried out to investigate the nature of the complex formed after incubation of α-lactalbumin with each vesicle system in the transition temperature region (23 and 25°C).

Gel chromatography. The different types of vesicle at various lipid concentrations ranging from 0.3 to 2 mg/ml were mixed with α-lactalbumin at a molar ratio 40 : 1. The mixtures were incubated at 25 or 23°C for 2–5 h before chromatography on a Sepharose 6B column was carried out. The analysis of the eluted fraction gives similar results for the different starting mixtures at both temperatures (Fig. 4): no isolated vesicles are observed, but all the phospholipid elutes in an intermediate region together with an important part of the α-lactalbumin at an elution volume which is independent of the original vesicle size. The remaining α-lactalbumin elutes at the specific volume for the free protein. When the incubation is carried out with multilamellar vesicles (Fig. 4D) or large unilamellar vesicles (data not shown) at 23°C, a tem-

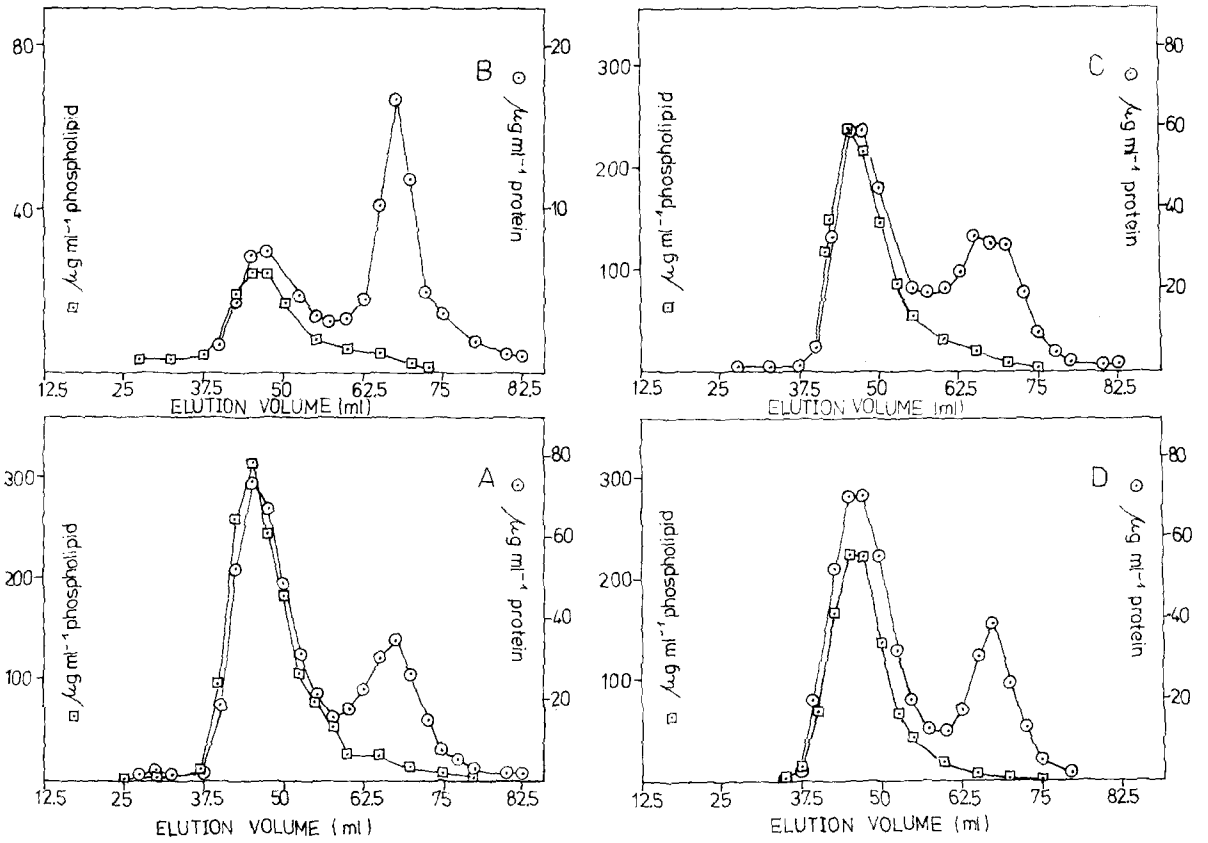

Fig. 4. Elution profiles of the lipid-protein complex on a Sepharose 6B column after an incubation time of 2–5 h. (A) Complex formed by incubating 1.5 mg phospholipid/ml as small unilamellar vesicles with 0.68 mg α-lactalbumin at 25°C (molar ratio, 45). (B) Complex formed by incubating 0.3 mg phospholipid/ml as large unilamellar vesicles with 0.18 mg α-lactalbumin at 25°C (molar ratio, 35). (C) Complex formed by incubating 1.5 mg phospholipid/ml as multilamellar vesicles with 0.68 mg α-lactalbumin at 25°C (molar ratio, 45). (D) As C but at 23°C.

perature at which endothermic ΔH values are obtained, the same elution pattern is observed but the longer incubation time (5 h) is needed to eliminate the peak of free liposomes.

The results indicate that around the transition temperature, the binding of α-lactalbumin to the different vesicles induces a breakdown of the vesicular structures to give significantly smaller complexes. By the sedimentation method it is confirmed that the fraction which elutes in the intermediate region is uniform in size (Hanssens, I., Herreman, W., Van Ceunebroeck, J.-C., Dangreau, H., Gielens, C., Preaux, G. and Van Cauwelaert, F., unpublished results). From phosphate and protein analysis it is calculated that the lipid-to-protein molar ratio within these aggregates is independent of the vesicle type and is approximately equal to 70 : 1.

Electron microscopy. Data obtained by gel chromatography are partially controlled by electron microscopy. Pure liposomes and liposome/α-lactalbumin mixtures of a molar ratio 40 : 1 are prepared and negatively stained with uranyl acetate. The electron microscopic pictures of the lipid-protein complexes (the intermediate peak of the gel chromatography), whether taken with small unilamellar, large unilamellar or multilamellar vesicles after the interaction of α-lactalbumin, always show the same bar-shaped entities which were formed as shown previously in Fig. 9 of Ref. 12. Since they also elute at the same elution volume, and their molar ratio is the same, we can conclude

that the interaction products for all vesicle types are the same.

Fluorescence anisotropy. As already remarked, the interesting relationship between r_s, r_d and r_∞ as proposed by Van Blitterswijk et al. [24] probably has no general validity. Recent data [28] for erythrocyte membranes, and roughly also sarcoplasmic reticulum and mitochondrial membranes, satisfy the relationship. However, significant deviations exist for dioleoylphosphatidylcholine [29], for purple membranes [28], for the interaction of cholesterol with di-(dihydrosterculoyl)phosphatidylcholine [30] and for the interaction of cytochrome oxidase with dimyristoylphosphatidylcholine [27]. As a consequence, the relationship between r_s, r_d and r_∞ of Van Blitterswijk et al. [24] must be handled with great care. In order to check if the relationship is valid for a given membrane, time-resolved fluorescence depolarization measurements are necessary. Therefore, such experiments were carried out for the interaction of α-lactalbumin with dimyristoylphosphatidylcholine vesicles [31]. From these experiments it followed that the empirical relation between r_s, r_d and r_∞ as proposed in Ref. 24 is applicable to this system [31].

In Fig. 5 we compare for the three vesicle systems the anisotropy data as a function of temperature for different conditions of incubation with α-lactalbumin after 2 h.

In Fig. 5A the interaction is shown with small unilamellar vesicles. Only r_d and r_∞ values are depicted. The changes in these two quantities in comparison with their value in the pure phospholipid are independent of the incubation temperature. On the figure it is seen that the protein induces a drastic increase in the static component, r_∞, and a decrease in the dynamic component, r_d.

With both types of large vesicle, no measurable interaction occurs after an incubation for 2 h at 20°C, as is shown in Fig. 5B,C where only r_∞ values are given. When the mixtures are scanned from 20°C to higher temperatures, an increase in r_∞ occurs which starts around 25°C. By raising the temperature, a complex is formed in the neighbourhood of the transition temperature. It is remarkable that in the upward scan of the large unilamellar vesicles after incubation at 20°C a maximum always appears around 28°C.

We also show in Fig. 5B,C a downward scan after α-lactalbumin has interacted with the large vesicle types at 35°C for 2 h. A shift to higher r_∞ values is observed. Only for the large unilamellar

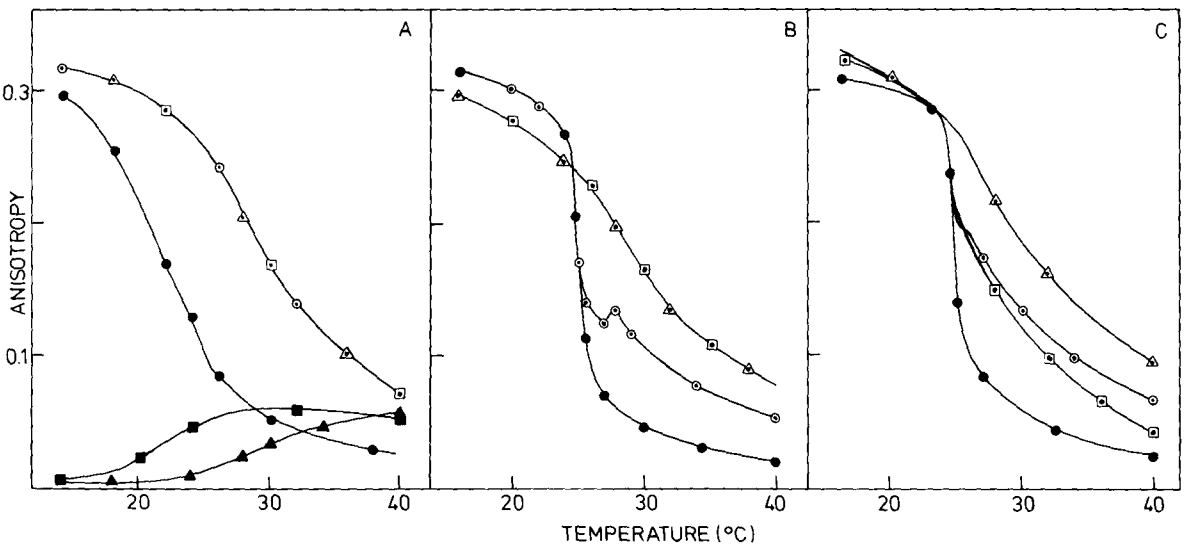

Fig. 5. Fluorescence anisotropy r_∞ temperature scans after an interaction of 2 h of α-lactalbumin with each vesicle type at different incubation temperatures (⊙, 20°C upward scan; ⧋, 25°C upward and downward scan; ⊡, 35°C downward scan) for small unilamellar vesicles (A), large unilamellar vesicles (B) and large multilamellar vesicles (C) in comparison with the pure phospholipid (●). For small unilamellar vesicles also the dynamic component, r_d, is shown before (■) and after (▲) the interaction with the protein. The lipid-to-protein molar ratio was 8.

vesicles below the transition temperature does r_∞ decrease in comparison to the r_∞ values of the pure lipid. For the large multilamellar vesicles a sharp transition still occurs around 24.5°C, indicating that the interaction at 35°C after 2 h was not as complete as was the case for the other vesicles and that some liposomes remained intact.

Most important, however, is the fact that all three vesicle types, after incubation near the transition temperature in the same molar ratio, show nearly the same equilibrium r_∞ values, which is only possible if the same kind of complex is formed in the three cases.

Characterization of the complex

Differential scanning calorimetry. A calorimetric scan of the lipid-protein complex, separated by gel chromatography, was performed under the same conditions as used for the pure vesicles. The result is shown in Fig. 6. The total enthalpy of transition is 21.4 kJ/mol lipid. The thermal transition starts at 21°C and is spread over a broad temperature region to 35°C, with a maximum around 26°C. DSC scans of complexes of apolipoprotein A-II with the same phospholipid were obtained by Massey et al. [32]: they found that these complexes have lower enthalpies of melting, a broader melting range and a higher transition temperature than the pure phospholipid. The enthalpy of melting

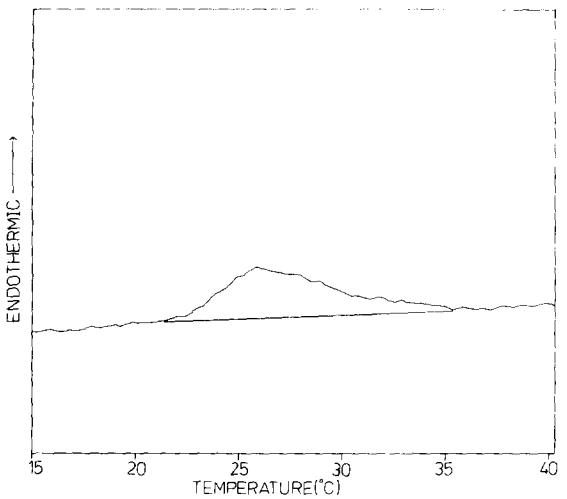

Fig. 6. Differential scanning calorimetric tracing for the lipid-protein complex: 60 μl from a solution prepared by incubation 0.6 ml α-lactalbumin (50 mg/ml) with 2 ml vesicles (50 mg/ml).

depended on the molar ratio. A similar broad scan and a value of 21 kJ/mol was also found by Epand et al. [33] for the same lipid in the presence of glucagon, although Epand and Sturtevant [34] found later with a Privalov calorimeter that the transition curve was composed of two components, a major one centred around 26.1°C and a smaller one around 21°C, with a total transition enthalpy of 13.4 kJ/mol lipid.

Fluorescence anisotropy. The results of Fig. 5 show that the complex formed with the three vesicle types has higher r_∞ values than the pure lipid. For small unilamellar vesicles, for instance, at 25°C r_∞ in the pure phospholipid equals 0.102, corresponding to an order parameter, S, of 0.505 (Eqn. 4). Assuming the wobbling-in-cone model of Kinosita et al. [19] this corresponds with a cone angle $\theta_c = 52°$ (Eqn. 3). After the interaction with α-lactalbumin at this temperature, $r_\infty = 0.256$, which leads to a value of 0.800 for S corresponding to a cone angle $\theta_c = 31°$. Therefore, the molecular motions of the probe molecules are strongly restricted in the presence of α-lactalbumin, which means that the order of the phospholipids is strongly increased.

Thermodynamics of the interaction

Enthalpy changes of the interaction of α-lactalbumin with dimyristoylphosphatidylcholine for different vesicle systems can only be compared if at all temperatures the interaction in the batch microcalorimeter is finished within a reasonable time, i.e., about 30 min. However, as has been shown previously [12,35,36], the interaction of proteins with phospholipids is strongly dependent on the transition temperature of the phospholipid.

For the small unilamellar vesicles, the interaction was always exothermic (Fig. 7) in the 20–30°C temperature range and ended in 30 min. A maximum heat exchange ($\Delta H = -1300$ kJ/mol α-lactalbumin) is observed around 23°C.

With the large multilamellar vesicles a sharp transition occurs from a maximal endothermic value ($\Delta H = +500$ kJ · mol^{-1}) at 23.8°C to a maximal exothermic value ($\Delta H = -600$ kJ · mol^{-1}) at 24°C. These results confirm our previous report [37] and resemble the data of Massey et al. [32] for the interaction of apolipoprotein A-II with the same phospholipid in liposome form. Our

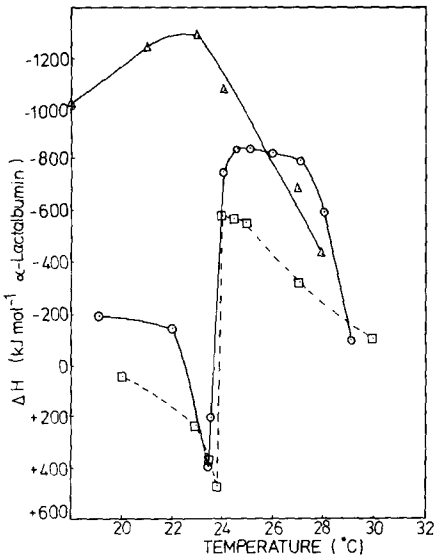

Fig. 7. Enthalpy change upon binding α-lactalbumin to dimyristoylphosphatidylcholine as a function of temperature at a lipid-to-protein molar ratio of 100: △, small unilamellar vesicles; ⊙, large unilamellar and ⊡, large multilamellar vesicles. As mentioned in the Results section, the latter do not fulfil equilibrium conditions.

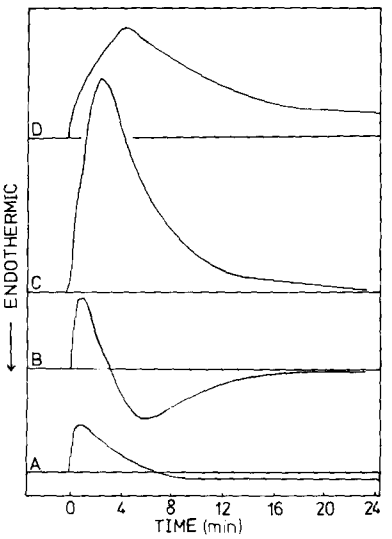

Fig. 8. Experimental tracings with large unilamellar vesicles from batch calorimetry at a lipid-to-protein molar ratio of 100 at different temperatures. A, 22.5°C; B, 23.3°C; C, 24.0°C; D, 27.0°C.

tracings from the batch calorimetry are similar to the results of their Fig. 5 [32]. However, our tracings did not return completely to the baseline after 30 min, indicating that equilibrium was not yet attained. The ΔH values for the multilamellar vesicles plotted in Fig. 7 are values calculated from the calorimetric scans stopped after 30 min.

For the large unilamellar vesicles the shape of the curve in Fig. 7 resembles more the one for the multilamellar than that of the small unilamellar vesicles: a sharp transition is observed around 24°C from $+400$ kJ · mol^{-1} to a plateau above 24°C at -850 kJ · mol^{-1}. The main difference with the results for the multilamellar vesicles lies, however, in the batch calorimetric tracings. In Fig. 8 we plot these tracings obtained for the interaction at different temperatures with the large unilamellar vesicles: below the transition temperature an initial exothermic effect is present which is never seen with the multilamellar vesicles. The exothermic effect is followed by an endothermic one, which is maximal at 23.5°C, and disappears at the transition temperature. Above the transition temperature the effect is always exothermic. As is shown in Fig. 8, above 26°C the heat effect does not return to the baseline within 30 min and therefore the enthalpy values for the large unilamellar vesicles above 26°C are not equilibrium values. From Fig. 7 we can also conclude that the physical adsorption of protein at temperatures no greater than 20°C in the case of large unilamellar vesicles amounts to -200 kJ/mol protein but for multilamellar vesicles ΔH approximates to zero.

Discussion

Our results from differential scanning calorimetry (Fig. 2), fluorescence polarization (Fig. 3 and 5) and batch calorimetry (Fig. 7) permit a first conclusion: large unilamellar vesicles, prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos [5], have more characteristics in common with the multilamellar vesicles than with the small vesicles. The gel-to-liquid-crystalline transition occurs in a narrow temperature region, an indication for a high cooperativity in the phase transitions. Although the small unilamellar vesicles have the same order parameter, S, at 15°C as the large unilamellar and multilamellar

vesicles (Fig. 3C), S decreases gradually between 15 and 24°C for the small vesicles, but remains nearly constant for the large vesicles. This indicates that the large vesicles, due to their small curvature in the bilayer, maintain a highly ordered packing of the lipids, even just below the transition temperature. These data are in agreement with previous results of studies on the influence of the curvature on the phase transition parameters [6–8,10]. This difference in transition behavior is also responsible for the different shape of the ΔH vs. t data for the small and large vesicles. While the small vesicles, with their highly curved bilayer and disordered packing arrangements of the lipid molecules, form complexes very quickly and exothermically with protein molecules in the 20–30°C region, the same occurs for both large vesicle types only in the 22–26°C region. Below 22°C the large vesicles only adsorb α-lactalbumin to the outer surface, while above 26°C the complex formation occurs much more slowly, as can be deduced from the batch calorimetric scans.

A second conclusion is that the kind of complex formed in the 23.5–25°C region is independent of the vesicle preparation, i.e., the lipid-protein composition, the shape and the size of the complex are identical as determined by gel chromatography (Fig. 4) and electron microscopy (data not shown). The fluorescence anisotropy r_∞ values vs. t of the probe in the complex (Fig. 5) obtained after incubation at 25°C are independent of the starting vesicle type. Furthermore, the r_∞ values of the probe in the complex particles above 24°C are higher than when the probe is present in the pure phospholipids. As shown in Eqns. 3 and 4, higher r_∞ values are related to a higher order parameter and a lower cone angle for the movement of the diphenylhexatriene molecules. We must conclude, therefore, that α-lactalbumin in the complex particle strongly restricts the motion of the phospholipids, and raises the gel-to-liquid-crystalline transition temperature. This conclusion is confirmed by the calorimetric scan (Fig. 6) of the complex particles, which shows an endothermic heat transition over a broad temperature range. However, such a broad range of endothermic heat transitions for the complex particle may have a second cause: the instability of the complex, which can lead to an endothermic dissociation at higher temperatures. A breakdown of the lipid-protein complex with molar ratio 70 : 1, formed at 23°C, into lipid-protein complexes of lower molar ratio (i.e., 15 : 1 at 33°C) indeed occurs at a rate which is higher than the rate of the calorimetric scan of Fig. 6 (Hanssens, I., Herreman, W., Van Ceunebroeck, J.-C., Dangreau, H., Gielen, C., Preaux, G. and Van Cauwelaert, F., unpublished results). It was further proved by the fact that polarization values of diphenylhexatriene measured at a scan rate of 1 $K \cdot min^{-1}$ coincide with the polarization values obtained under equilibrium conditions at different temperatures. Therefore, heat effects due to the breakdown of the complex particle and rearrangements of the phospholipid are certainly present in the calorimetric scan of Fig. 6. In this respect α-lactalbumin at pH 4 differs from apolipoprotein A-II [32] which forms stable complexes with the same phospholipid and from glucagon-dimyristoylphosphatidylcholine, complexes which dissociate slowly [33]. For both these systems, the relative contributions, to the differential scanning calorimetric endotherm, of the phospholipids in different environments were quantitatively evaluated [32,34] and used, together with data of heat exchanges due to α-helicity changes in the protein, to explain quantitatively ΔH vs. t data of the type shown in our Fig. 7. Since our calorimetric scan in Fig. 6 is the sum of two processes, a quantitative decomposition of ΔH is therefore not possible in our case.

In a third conclusion, we believe that our enthalpy data of Fig. 7 permit comparison of the enthalpy states of the different vesicle systems around 24°C. To our knowledge, such calculations have never been obtained from the experimental data. We can write for the interaction of α-lactalbumin with each vesicle type at 23.5°C:

$$\Delta H = H_c - (H_{SUV} + H_{\alpha\text{-}LA})$$

$$\Delta H = H_c - (H_{LUV} + H_{\alpha\text{-}LA})$$

$$\Delta H = H_c - (H_{MLV} + H_{\alpha\text{-}LA})$$

where H_c, H_{SUV}, H_{LUV}, H_{MLV} and $H_{\alpha\text{-LA}}$ are the enthalpy states of, respectively, the complex, the small and large unilamellar vesicles, the multilamellar vesicles and the protein. Since after interaction at 23.5°C the complex formed is identical for the three vesicle types, and since the possible

enthalpy contributions from α-helicity changes in the protein are also identical for the three cases, ΔH of the interactions is a measure of the difference in enthalpy of the different types of vesicle. Therefore, we can conclude from Fig. 7 that at 23.5°C the small unilamellar vesicles are 1700 kJ/mol protein or 24 kJ/mol lipid higher in energy than both large vesicle types which have about the same enthalpy content (the conversion is done by dividing the first number by 70, since the molar ratio in the complex is 70 : 1). This difference not only results from the difference in physical state between both types of vesicle. The transition from the gel-to-liquid-crystalline state is about 21 kJ · mol^{-1}. At this temperature the major part of the small unilamellar vesicles is in the liquid-crystalline state and most of the large vesicles are still in the gel state. This would result in a difference in enthalpy smaller than 21 kJ · mol^{-1}. The higher experimental value must therefore be ascribed to the higher enthalpy of the small vesicles in their liquid-crystalline state compared to the enthalpy of the large unilamellar vesicles also in their liquid state. This may result from the difference in curvature due to the input of surface energy by sonication.

A fourth conclusion that can be drawn from our enthalpy data in Fig. 7 is that the size of the abrupt enthalpy change around 24°C observed for both large vesicle types must be of the order of 21 kJ/mol lipid, the difference in enthalpy of the phospholipid below and above the transition temperature. Indeed, as above, we may write:

at 23.5°C $\quad \Delta H = H_c - (H_{LUV} + H_{\alpha\text{-}LA})$

at 25.0°C $\quad \Delta H' = H'_c - (H'_{LUV} + H'_{\alpha\text{-}LA})$

$\Delta H = 400$ kJ/mol protein or 5.7 kJ/mol lipid and $\Delta H' = -850$ kJ/mol protein or -12.1 kJ/mol lipid. The abrupt change is therefore approx. 18 kJ/mol lipid. The difference in the transition enthalpy can be partly due to the fact that we assume H_c and H'_c and $H_{\alpha\text{-La}}$ and $H'_{\alpha\text{-LA}}$ to be the same. Also, we suppose that at 23.5°C the phospholipid is completely in the gel state and at 25°C in the liquid-crystalline state. The same calculations for the large multilamellar vesicles were not carried out since, as mentioned in the batch calorimetric data section, the interaction did not reach equilibrium. Therefore the ΔH data are smaller than those for the large unilamellar vesicles.

An almost identical temperature-dependent enthalpy change was obtained by Massey et al. [32] for the interaction of liposomes with apolipoprotein A-II and by Epand and Sturtevant [34] for the interaction with glucagon. For apolipoprotein A-II we calculate from their figure an abrupt change of 1460 kJ/mol protein or 19.5 kJ/mol lipid (the molar ratio in the complex is 75). For glucagon the difference between both is 957 kJ/mol protein or 19.1 kJ/mol lipid (the molar ratio in the complex is 50). Both numbers are, as expected, very close to 21 kJ · mol^{-1} found for the pure lipid transition.

Finally, the consecutive steps in the interaction of α-lactalbumin with dimyristoylphosphatidylcholine can be envisaged as follows. In a first step the protein adsorbs to the outer surface of the vesicles. For the large unilamellar vesicles at 20°C this adsorptoin is observed (Figs. 7,8) and an estimation of the exchanged heat can be made: it amounts to -200 kJ/mol protein (Fig. 8). For the large multilamellar vesicles with a low outer surface area, the heat exchange approximates zero in our case as in the case of glucagon [34]. The exothermic nature of these interactions probably promotes the transition of a part of the phospholipids from the gel to the liquid-crystalline state. The formation of boundary regions between gel and liquid state of the phospholipids facilitates the penetration of the protein. This is accompanied by a change in its conformation (tryptophan fluorescence changes [12]) and hydrophobic binding to the acyl chains of the phospholipid (monitored by the polarization changes of diphenylhexatriene). The total thermal effect below the transition temperature is the sum of the exothermic binding of the protein, the endothermic gel-to-liquid-crystalline transition, the change in conformation of the protein and the rearrangement of the lipid-protein aggregate. When the experiment is carried out between 24 and 26°C the endothermic step is omitted since the large vesicles are in the liquid state. The fact that ΔH becomes less exothermic above 27°C for the three vesicle systems is related both to the formation of a complex which has a lower lipid-to-protein molar ratio and to the fact that equilibrium is not attained after 30 min.

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