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INTERACTION OF α-LACTALBUMIN WITH DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES

II. A FLUORESCENCE POLARIZATION STUDY

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Summary

The interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles was studied as a function of temperature, pH and the molar ratio of phospholipid to protein. The method consisted of measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene used as a probe embedded in the vesicles.

After incubation of the protein with the phospholipid for 2 h at 23°C, the polarization of the light emitted by this probe shifted to higher values; the shift was greater at acidic pH than at neutral pH. After incubation at 37°C, no shift in polarization was found at pH 7, 6 and 5 while a strong increase occurred at pH 4. Lowering the temperature, after incubation at 37°C, had little effect on the polarization at neutral pH. At pH 5, however, and in the transition range of the phospholipid, the polarization increased greatly.

A kinetic study of the interaction carried out around the transition temperature of dimyristoyl phosphatidylcholine as a function of pH shows that the speed of complex formation between α-lactalbumin and the lipid increases from neutral to acidic pH.

From the present results and in agreement with our earlier calorimetric and fluorescence data (Hanssens, I., Houthuys, C., Herreman, W. and van Cauwelaert, F.H. (1980) Biochim. Biophys. Acta 602, 539—557), it is concluded that at

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Abbreviation: Mes, 4-morpholineethanesulphonic acid.

neutral pH the interaction mechanism is probably different from that at acidic pH. At neutral pH and at all temperatures, α-lactalbumin is mainly adsorbed electrostatically to the outer surface of the vesicle with little or no influence on the transition temperature of the phospholipid. At this pH, only around the transition temperature is penetration possible. At pH 4, however, the protein is able to penetrate the vesicle at all temperatures and to interact hydrophobically with the phospholipid fatty acid chains. As a result of this interaction, the transition temperature is increased by about 4°C. This different behaviour changes progressively upon acidification: at pH 5, penetration seems to be impossible at temperatures far above the transition temperature but occurs rapidly around the transition temperature.

Introduction

As mentioned by Lee [1] in a review article dealing with lipid phase transition and phase diagrams, few studies of the influence of proteins on lipid phase transitions have been done.

In the preceding paper [2], a microcalorimetric, gel-chromatographic and fluorescence study was reported on the interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. Whereas most proteins in all circumstances show either integral or peripheral characteristics, α-lactalbumin presents an interesting case, since it was shown [2] that above its iso-ionic point at pH 5 it behaves like a peripheral protein, mainly adsorbing to the outer surface of the vesicles. However, at and below pH 5, α-lactalbumin penetrates the vesicles, just like an integral protein, with greatest ease around the transition temperature. It was suggested that a conformational change of the protein, induced by acidification, is the cause of this behaviour.

While it was clearly demonstrated in the previous study that the protein conformation was altered in a different way by the interaction with the lipid at pH 7 and 4, we wanted to know how the protein in the same circumstances influences the lipid bilayer. Therefore, the former study is extended with fluorescence polarization data. According to the technique of Shinitzky and coworkers [3,4], a fluorescent dye incorporated into the hydrocarbon region of the vesicles is used to monitor the change in fluidity that occurs in the hydrophobic part of the bilayer. In our experiments, 1,6-diphenyl-1,3,5-hexatriene, which is now the most frequently employed fluorescence polarization probe, was used. This probe has many applications. It is used in the study of the thermotropic behaviour of phospholipid dispersions and their mixture in water [5—7], of lipid fluidity properties of human and bovine serum lipoproteins [8,9] and of lipid dynamics in biological membranes [10—13]. More details on the characteristics of this probe and of its applications are to be found in a survey article by Shinitzky and Barenholz [14].

In the first part, our study consists of measurements of the fluorescence polarization of the light emitted by the probe after α-lactalbumin was added during 2 h to the vesicles under neutral and acidic conditions at different lipid-to-protein molar ratios and different temperatures. This allowed us to estimate: (1) the conditions under which α-lactalbumin behaves either like a peripheral

protein or like an integral protein; (2) its influence on the transition temperature after its interaction with the phospholipid.

In the second part, the kinetics of interaction of the protein with the phospholipid around its transition temperature were studied as a function of pH. Therefore, the fluorescence polarization was followed as a function of time immediately after α-lactalbumin was added to the vesicles. The rate of complex formation increased upon acidification.

The conclusions from our earlier microcalorimetric and fluorescence data [2] are confirmed.

Materials and Methods

Steady-state emission polarization

Materials. α-Lactalbumin from bovine milk and L-α-dimyristoyl phosphatidylcholine were obtained from Sigma and used without further purification. The fluorescent probe was purchased from Eastman Kodak Co.

Solutions. All protein and lipid solutions were 0.1 M in NaCl and 0.01 M in the appropriate buffer: Tris buffer at pH 7, Mes buffer at pH 6 and acetate buffer at pH 5 and 4. Protein and lipid concentrations were determined as described before [2].

Preparation of vesicles. Single-shelled vesicles were prepared by sonication at 50 W using a Branson 50 sonifier equipped with a microtip; 5 mg dimyristoyl phosphatidylcholine were sonicated in 2 ml buffer for 30 min at 40°C. The sonicated solution was then filtered through a Millipore filter (1.2 μm) to remove some titanium particles.

Fluorescence labelling of vesicles. For labelling, $2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran was diluted 1000-fold with vigorously stirred buffer for 30 min at 25°C. A clear and stable aqueous dispersion of $2 \cdot 10^{-6}$ M diphenylhexatriene was obtained which is practically devoid of fluorescence [4,15]. 100 μl of vesicles were incubated with 0.9 ml of this dispersion for 30 min at 37°C [5]. The incorporation of the probe into the hydrocarbon region of the vesicles was demonstrated by a sharp increase in fluorescence intensity.

Fluorescence polarization measurements. The complex formation of dimyristoyl phosphatidylcholine vesicles with α-lactalbumin at different molar ratios was followed after adding α-lactalbumin during 2 h to the labelled vesicles. The incubations were performed at 23 and 37°C. Measurements of fluorescence polarization were performed with an Elscint MV-1A microviscosimeter. Diphenylhexatriene was excited at 366 nm with plane-polarized light and its fluorescence detected at 430 nm. The microviscosimeter yields directly the value of the polarization (P) as defined by:

$$P = \frac{I_{/\!/} - I_{\perp}}{I_{/\!/} + I_{\perp}}$$

where $I_{/\!/}$ and $I_{\perp}$ are the fluorescence intensities oriented parallel and perpendicular, respectively, to the direction of the polarized incident light. Fluorescence polarization and temperature measurements were made continuously during cooling at a scan rate of 1°C/min. The temperature of the sample was measured

by means of a thermistor located inside the cuvette. The temperature and fluorescence polarization were recorded on an x-y recorder.

Kinetics

The kinetic measurements around the transition temperature of the phospholipid as a function of pH were carried out as follows. Starting from the polarization value of the light emitted from the probe in the pure lipid, the polarization was followed as a function of time immediately after α-lactalbumin was added to the vesicles. In order to do this and to have about the same molar ratio as in the case of steady-state emission polarization measurements, a concentrated aqueous solution of protein was made. Vesicles were prepared by sonicating a solution of 1.1 mg phospholipid in 10 ml buffer; 2.3 ml of these vesicles were then incubated for 30 min at 37°C with 0.1 ml of the same diphenylhexatriene dispersion as already mentioned. Subsequently, this solution was placed in the microviscosimeter at a temperature of 23.5°C. After the temperature was stabilised, 100-μl of the concentrated α-lactalbumin solution at the same temperature were injected into the vesicles solution and after 10 s the polarization values could be determined and followed further.

Results

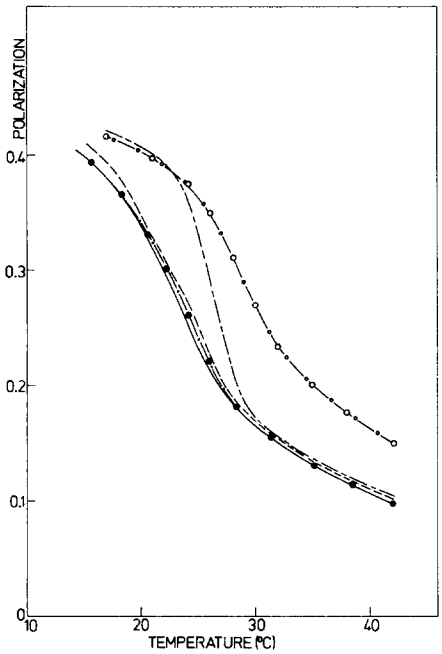
Steady-state emission polarization as a function of pH

These series of measurements were carried out at a constant lipid-to-protein molar ratio of 8.50 : 100-μl vesicles at a concentration of 2.5 mg/ml were added to 0.78 ml of a 0.8 mg/ml α-lactalbumin solution. The incubation of the protein with the phospholipid was performed at 37 and 23°C. The latter temperature is near the gel-to-liquid-crystalline phase transition of dimyristoyl phosphatidylcholine.

pH 7 and 6. In Fig. 1 it can be seen that at pH 7 as well as at pH 6, the incubation of α-lactalbumin with the vesicles at 37°C does not influence the polarization of the fluorescent light of the probe. During cooling, small differences occur between these curves and that of the pure phospholipid. At first sight, it is difficult to say whether these differences are caused by the scan procedure or if they are real, as the shifts are not significant enough to account for the last possibility. However, the kinetic measurements showed that it was indeed a real effect.

After the incubation at 23°C, the sample was brought immediately to 42°C and scanned. As seen in Fig. 2, a change in the polarization of the emitted light from the probe is perceived towards higher values. Furthermore, the change in polarization at this incubation temperature is greater at pH 6 than at neutral pH 7.

pH 5. The study of the interaction of α-lactalbumin with dimyristoyl phosphatidylcholine at this pH can be very interesting, since pH 5 is the iso-ionic point of this protein. The following conclusions can be drawn from Figs. 1 and 2. The incubation at 37°C has no influence on the polarization. During cooling, however, and from about 30°C, there is a drastic increase in the polarization especially in the transition range of the phospholipid. This indicates that at this pH, after incubation at 37°C, the type of interaction changes during cooling.



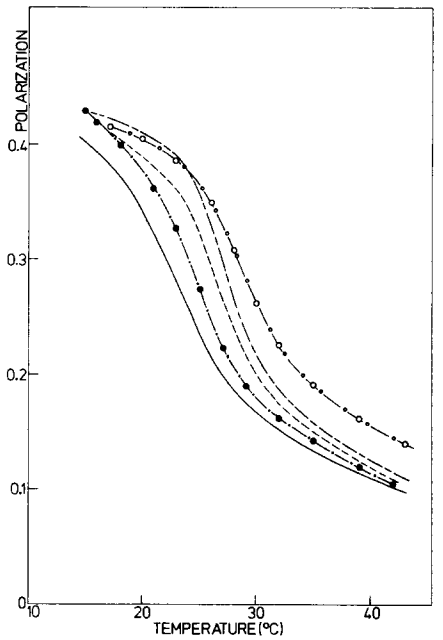


Fig. 1. Polarization as a function of temperature after incubation at 37°C at a lipid-to-protein molar ratio of 8.5 : 1 at different pH values: pH 7 (●——●——●). pH 6 (- - - - -), pH 5 (— — —) and pH 4 (○——○——○). The solid line corresponds to the pure phospholipid.

Fig. 2. Polarization of the light emitted from the probe as a function of temperature after incubation at 23°C under the same conditions as described in Fig. 1 (symbols as in Fig. 1).

This change must be very fast at pH 5, in contrast to the behaviour at pH 6 and 7. After incubation at 23°C and bringing the sample to 37°C the polarization has increased greatly compared to the data obtained at 37°C, indicating that at 37°C no further change occurred in addition to those at 23°C (Fig. 2). The data on the polarization after incubation at 37 and 23°C and at pH 5 are summarized in Fig. 3 to demonstrate the critical influence of the transition temperature of dimyristoyl phosphatidylcholine (23°C) on the behaviour of α-lactalbumin. In Fig. 2, it is also seen that the increase in polarization after the incubation of α-lactalbumin with the phospholipid at 23°C is greater at pH 5 than at pH 6 and 7.

pH 4. From the first two figures it is concluded that, in contrast to the other pH values, the incubation temperature has no significant influence. Incubation after 2 h, at both 37 and 23°C, gives the same result. Therefore, it is supposed that the complex formed at high temperatures must be the same as that formed around the transition temperature.

The effect on the transition temperature, T_t, after binding of α-lactalbumin to the phospholipid at 23°C is given in Table I. The transition temperature was calculated using a Tektronix 4051 computer graphic system as follows. The experimental curves were placed on a Tektronix 4662 plotter connected with the computer. Polarization values were taken at intervals of 0.5°C. Absolute values of dP/dT were calculated by means of the three-point method; $|dP/dT|$

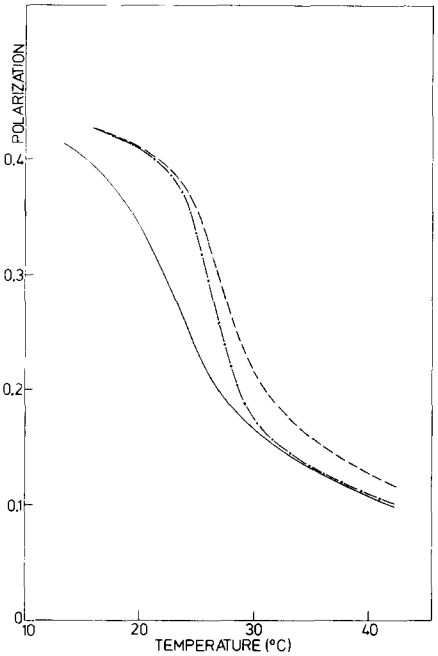


Fig. 3. Temperature scan of the polarization at pH 5 after the incubation of α-lactalbumin with the vesicles at 23°C (- - - - -) and at 37°C (●———●); N = 8.5 : 1.

was plotted as well as the original experimental curve. The maximum value of $|dP/dT|$ gives an estimate of T_t. Using this procedure, the accuracy with which T_t is obtained is no better than 0.5°C. The transition temperature of pure dimyristoyl phosphatidylcholine calculated with this method is 24.5 ± 0.5°C, which corresponds with the value of 24.4 ± 0.5°C reported by Lentz and co-workers [5] who used the same experimental technique. From the table it is concluded that at neutral pH there is only a small increase in T_t. At acidic pH the shift of T_t to higher temperatures is about 4°C.

Polarization as a function of the lipid-to-protein molar ratio

In order to obtain different molar ratios, 100 μl of vesicles of a 2.5 mg/ml solution were added to different quantities of a 0.8 mg/ml α-lactalbumin solution.

TABLE I

TRANSITION TEMPERATURE AS A FUNCTION OF pH AFTER INCUBATION AT 23°C AND AT A LIPID-TO-PROTEIN MOLAR RATIO OF 8.50 : 1

pH	T_t (°C)
7.0	25.0 ± 0.5
6.0	25.5 ± 0.5
5.0	28.0 ± 0.5
4.0	28.5 ± 0.5

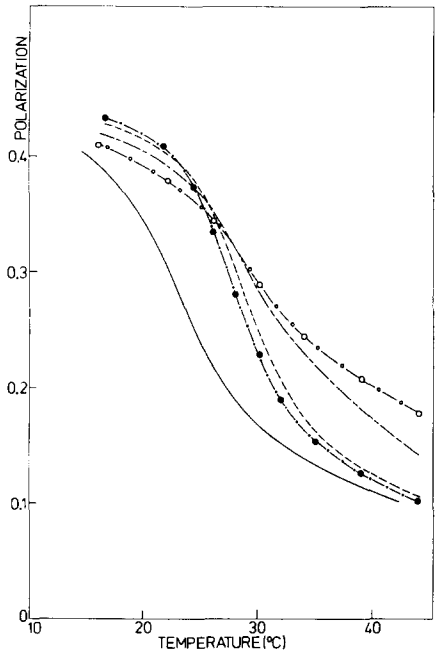


Fig. 4. Polarization of the emitted light at pH 4 after incubation at 23°C at different lipid-to-protein molar ratios: $N = 42.5 : 1$ (●——●——●), $N = 21.3 : 1$ (- - - - -), $N = 8.5 : 1$ (— - —) and $N = 4.25 : 1$ (○——○——○). The solid line corresponds to the pure phospholipid.

The influence of the lipid-to-protein molar ratio (N) on the binding of α-lactalbumin to the phospholipid was only measured at pH 4, at which the incubation temperature had no effect on the polarization for $N = 8.50 : 1$. Also, at other molar ratios, the polarization was independent of the reaction temperature.

The influence of the molar ratio is shown in Fig. 4. The influence on the polarization after adding more α-lactalbumin to the vesicles is especially strong above the transition temperature: the more α-lactalbumin is added the more the polarization increases. Below the transition temperature the polarization values are less dependent on concentration.

Table II shows the influence of the concentration of α-lactalbumin on the

TABLE II

PHASE TRANSITION OF DIMYRISTOYL PHOSPHATIDYLCHOLINE-α-LACTALBUMIN COMPLEXES AT pH 4 AND AT DIFFERENT LIPID-TO-PROTEIN MOLAR RATIOS

N, molar ratio of dimyristoyl phosphatidylcholine to α-lactalbumin.

Complex	T_t (°C)
Lipid	24.5 ± 0.5
$N = 42.5 : 1$	27.5 ± 0.5
$N = 21.3 : 1$	28.0 ± 0.5
$N = 8.50 : 1$	28.5 ± 0.5
$N = 4.25 : 1$	29.0 ± 0.5

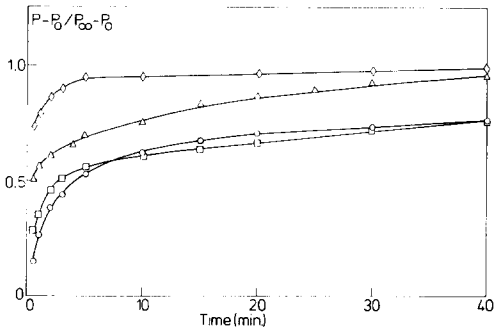


Fig. 5. Rate of complex formation of α-lactalbumin with dimyristoyl phosphatidylcholine at 23°C at different pH values: pH 7 (○), pH 6 (□), pH 5 (△) and pH 4 (◇). $P - P_0/P_\infty - P_0$ is the relative quantity of the total complex.

transition temperature of the phospholipid. In the presence of increasing quantities of α-lactalbumin, the phospholipid transition range is broadened and T_t shifts to higher temperatures.

Kinetics

The particular result obtained at pH 5 during the cooling process after the incubation at 37°C indicates that at this pH, and of course also at more acidic pH, the complex formation of α-lactalbumin with dimyristoyl phosphatidylcholine must be fast in contrast to that at more neutral pH. Therefore, a kinetic study was carried out as a function of pH around the transition temperature of the phospholipid. The method has been already discussed in Materials and Methods. A constant lipid-to-protein molar ratio of 5.3 was used: 100 μl of an α-lactalbumin solution of 10 mg/ml in water were injected into 2.3 ml of vesicles at a concentration of 1.1 mg/10 ml buffer. The results are shown in Fig. 5 in terms of $P - P_0/P_\infty - P_0$, where P_0 is the polarization in the pure lipid, P the polarization after injecting α-lactalbumin and P_∞ the polarization 2 h after the protein was injected. $P - P_0$ represents the change in polarization caused by the reaction of α-lactalbumin with the vesicles and $P - P_0/P_\infty - P_0$ represents the relative quantity of the complex formed. The reaction is not a first-order reaction nor a linear combination of two first-order reactions as has been suggested. Therefore, we determined the rate constant $k_{1/2} = 1/\tau_{1/2}$, where $\tau_{1/2}$ represents the time required for formation of 50% of the final complex. The rate constant has values of 0.25 min^{-1} at pH 7, 0.33 min^{-1} at pH 6, 2 min^{-1} at pH 5 and 8.6 min^{-1} at pH 4. These results clearly show that the complex formation is a function of pH as already supposed from the steady-state emission polarization measurements.

Discussion

We wish to point out that our data will be interpreted on the basis of polarization only, rather than extrapolating them to 'microviscosity' as has been done by many authors who assumed that the Perrin equation for an isotropic solution is also valid for anisotropic lipid bilayers [3,16].

Recent investigations, however, have shown that the extrapolation from polarization data to absolute microviscosity is not valid [17—19]. Andrich and Vanderkooi [20] already warned against this type of extrapolation, besides in many cases it is not necessary.

As shown by other authors (e.g., the review by Lee [1]), lipid-protein interactions depend also on the polarity of the protein molecule as well as on the physical state of the phospholipid molecules. Polar proteins, which contain a high percentage of hydrophilic amino acids, will rather adsorb to the outer surface of the membrane with little or no partial penetration. Proteins with more hydrophobic amino acids at their surface tend to penetrate the phospholipid bilayer. This is followed by an interaction with the fatty acid chains with a concommitant change in the transition temperature of the lipid phase. The influence on the transition temperature of binding of a protein to a phospholipid has been studied by Novosad et al. [21], Jonas et al. [22] and Rosseneu [23], using different apolipoproteins, by Verkleij et al. [24] and Papahadjopoulos et al. [25], using the myelin basic protein (A1 protein) and by Grant et al. [26], using glycophorin. In the extended work of Papahadjopoulos et al. [25], various proteins have been studied for their ability to alter the thermotropic properties of phospholipid bilayer membranes. However, not only the polarity of the protein is important, but also the fluidity of the bilayer and the size (single bilayer or multibilayer) of the liposomes play an important role [27—33].

In view of these remarks, we expected to see different behaviour of α-lactalbumin towards dimyristoyl phosphatidylcholine depending on the incubation temperature and on the pH. From our observations, the affinity of α-lactalbumin for the phospholipid at neutral pH seems to be different from that observed at acidic pH. We suggest that two alternative interaction mechanisms occur during the complex formation.

(1) Adsorption, which is ionic in nature. The protein remains at the outer surface of the vesicle: it behaves like a peripheral protein, without influence on the mobility of the fatty acid chains of the phospholipid molecules. In this case, the polarization of the emitted fluorescent light of diphenylhexatriene is unaltered.

(2) Adsorption, followed by penetration into the vesicles: the protein behaves like an integral protein. The mobility of the fatty acid chains decreases, as a result of which the fluorescence polarization increases. The binding of α-lactalbumin to the phospholipid in this case reduces the acyl chain mobility of the lipid molecules and increases the transition temperature.

pH 7 and 6. For a low lipid-to-protein molar ratio, no effect on the fluidity of the bilayer is observed when the incubation temperature is 37°C. During the temperature scan, a small effect is detected when the mixture passes through the transition temperature of the phospholipid. After incubation around the transition temperature of the phospholipid, a shift of the whole curve to higher values is observed. The kinetic study confirms these observations: as the reaction rate around the transition temperature is small at these pH values, the shift at 23°C during the scan after incubation at 37°C must be much smaller than that after incubation at 23°C.

Our earlier chromatographic data [2] show that after incubation at pH 7,

only a small fraction of α-lactalbumin elutes with the vesicles: the vesicles maintain their size and shape, indicating that α-lactalbumin is mainly adsorbed to the outer surface.

This adsorption, eventually followed by a slight penetration, occurs mainly around the transition temperature, although at this pH value no maximum was found in the ΔH vs. temperature plot as reported in Ref. 2. This is probably due to a kinetic effect: the data of Fig. 5 indicate that the interaction occurs in two steps: a fast step that is finished after 4—5 min, and a slower step which takes at least about 2 h. ΔH vs. temperature data [2] as well as the scan after 2 h of incubation at 37°C (Fig. 1) only reflect the instantaneous interaction (the first step), while the scan after an incubation of 2 h at 23°C also measures the second step. The same conclusions can be drawn at pH 6. The effect, however, is more pronounced at this pH for the same molar ratio.

pH 5. Incubating α-lactalbumin and dimyristoyl phosphatidylcholine at pH 5 either at 37 or at 23°C results in remarkably different behaviour. No shift in the polarization is found after incubation above 30°C. This indicates that although α-lactalbumin is already more apolar than at pH 7 and 6, even a liquid crystalline bilayer does not allow it to penetrate. However, at and around the transition temperature, favourable packing on the bilayer allows fast and easy penetration resulting in a rapid and large increase in polarization. The kinetic study confirms that the apolar interaction of α-lactalbumin with dimyristoyl phosphatidylcholine is fast at 23°C and at this pH. This is in agreement with earlier chromatographic data [2] which show that after incubation at 23°C at pH 5, a lipid-protein complex is formed, the size of which differs from the size of the original vesicles. Furthermore, a maximum in the ΔH vs. temperature curve was also obtained in the microcalorimetric data, indicating rapid formation of the complex at about 23°C.

After incubation at 23°C and scanning from 42 to 15°C, a shift to higher polarization values is also observed for the 30—40°C region: this means that the α-lactalbumin-lipid complex which is formed remains stable at higher temperatures. This is in agreement with our earlier report [2] that the complex is resistant to changes in pH, temperature and the effect of urea and guanidine-chloride.

pH 4. In contrast to pH 5, at which the protein-lipid interaction is strongly enhanced around the transition temperature, this is no longer the case at pH 4. The expansion of the protein molecule due to this pH [2] makes its surface even less polar, and has the effect that incubation of α-lactalbumin and the phospholipid for 2 h at a molar ratio of 8.5 : 1 at 23 or 37°C yields similar polarization curves (Figs. 1 and 2). Therefore, the protein-lipid interaction is much less dependent on the transition temperature. However, in Fig. 4, it can be seen that the increase in polarization over the whole temperature region, but mainly above 30°C, is strongly dependent on the molar ratio.

In this very stable complex, α-lactalbumin increases the transition temperature of the phospholipid by 3—4.5°C, depending on the molar ratio. In this respect, α-lactalbumin at pH 4 behaves like the different apolipoproteins at neutral pH in their interaction with dimyristoyl phosphatidylcholine [21—23] and like the myelin basic protein [24].

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