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INTERACTION OF α-LACTALBUMIN WITH DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES

I. A MICROCALORIMETRIC AND FLUORESCENCE STUDY

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Summary

α-Lactalbumin and dimyristoyl phosphatidylcholine were used as a prototype to study the influence of a protein conformational change, induced by the pH, on the interaction between that protein and a phospholipid.

The enthalpy changes associated with the interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles were measured as a function of the molar ratio of phospholipid to protein, pH and temperature. Gel-filtration, electron-microscopic and fluorescence data for the same experimental conditions were also obtained. At pH 4 and 5, the enthalpy changes (ΔH) are not only larger than at physiological pH, but also show a maximum at about 23°C in the ΔH vs. temperature graph. At pH 6 and 7, on the contrary, ΔH increases with decreasing temperature without a maximum in the curve. Gel-chromatographic and electron-microscopic data show that at pH 6 and 7, the morphological characteristics of the vesicles are unchanged upon addition of α-lactalbumin, while at pH 4 and 5 at 23°C an extra peak appears in the gel-filtration graphs between the pure vesicles and α-lactalbumin. The new fraction contains lipid-protein complexes. Electron micrographs show that bar-shaped entities are formed. A red shift at 23°C and a blue shift at 37°C, both to 336 nm, are observed for λ_{max} of the fluorescence emission spectra at pH 4 when α-lactalbumin is brought into contact with the phospholipid. At the same time, a strong increase in the fluorescence intensity is observed. The chromatographic

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Abbreviation: Mes,4-morpholineethanesulphonic acid.

and fluorescence data indicate that a lipid-protein complex with a molar ratio of approx. 80 is formed. At pH 7 and different temperatures, the emission maximum remains at the wavelength of pure α-lactalbumin, the change in the fluorescence intensity, however, indicates that interaction with the lipid occurs.

The results can be explained on the basis of an electrostatic interaction at pH 6 and 7, and a hydrophobic interaction at pH 4 and 5.

Introduction

Membrane proteins are divided into two classes depending on their interaction with respect to the lipids of the membrane framework. 'Extrinsic' or 'peripheral' proteins associate only with the membrane surface. 'Intrinsic' or 'integral' proteins penetrate the membrane surface and enter the lipid bilayer. Intrinsic proteins are expected to have a non-random spatial distribution of polar and nonpolar groups, in the way that the surface area of the molecule that enters the lipid bilayer will predominantly consist of nonpolar residues [1]. It has been stated by Lenaz [2], that even for relatively simple protein-lipid systems, different types of interaction may occur. From these points of view, it is not astonishing that a predominantly extrinsic or intrinsic behaviour of a protein may depend on its conformational state. However, few studies exist where the influence of a conformational change on such behaviour is demonstrated. In this paper, α-lactalbumin and dimyristoyl phosphatidylcholine vesicles were used as a prototype to study the influence of a protein conformational change, induced by the pH, on the interaction between that protein and a phospholipid. α-Lactalbumin has been studied very carefully by different authors [3—17]. At physiological pH, α-lactalbumin is known to be a peripheral protein. In an acidic medium, α-lactalbumin undergoes a conformational change. α-Lactalbumin and its integral counterpart, galactosyltransferase, are proteins produced in important quantities in the Golgi apparatus of mammary glands. In the presence of α-lactalbumin, galactosyltransferase catalyzes the reaction [18,19]:

UDP-galactose + glucose → lactose + UDP

Although numerous aspects of the α-lactalbumin-galactosyltransferase complex have been studied, the interactions of this protein complex system with the lipid environment have not yet been considered. We started with the investigation of the binding characteristics of the peripheral protein, α-lactalbumin, towards phospholipids by the monolayer technique [20]. It is shown that α-lactalbumin interacts more strongly with dipalmitoyl phosphatidylcholine and cardiolipin at acidic pH than at physiological pH. At pH 7.4, α-lactalbumin does not penetrate monolayers of both phospholipids above pressures of 30 dyne/cm. Enzymatic hydrolysis of these monolayers by phospholipase C at pH 7 is inhibited partially or totally when α-lactalbumin is injected into the subphase prior to the enzyme injection, indicating that

although α-lactalbumin does not penetrate the monolayers, it is adsorbed to it by electrostatic interaction. This monolayer study with dipalmitoyl phosphatidylcholine is now extended to the interaction between dimyristoyl phosphatidylcholine vesicles and α-lactalbumin by batch microcalorimetry, gel chromatography, electron microscopy and tryptophan fluorescence. From the combined results it can be concluded that at neutral pH, α-lactalbumin is mainly adsorbed to the outer surface of the vesicles, behaving like a typical peripheral protein. However, on decreasing the pH, α-lactalbumin penetrates the vesicles rapidly and easily, thereby forming a complex with a definite molar ratio.

Materials and Methods

Protein and lipid. α-Lactalbumin from bovine milk was obtained from Sigma. Its purity was checked on a Sephadex G-100 column: a small shoulder due to glyco-α-lactalbumin was present. The protein concentrations were determined spectrophotometrically with a Beckman D-spectrophotometer at 280 nm using a value of $E^{1\%} = 20.1$ for α-lactalbumin [21]. L-α-Dimyristoyl phosphatidylcholine was purchased from Sigma. Purity and composition were confirmed by thin-layer and gas-liquid chromatography.

Protein and vesicle solutions. All protein and vesicle solutions were 0.1 M in NaCl in the appropriate buffer: acetate buffer at pH 4 and 5, Mes buffer or phosphate buffer at pH 6 and Tris buffer or phosphate buffer at pH 7--8. Single bilayer vesicles were prepared by sonication for 25 min in an N_2 atmosphere of the phospholipid suspended in the buffer with an MSE 150 disintegrator at maximum output. The jacketed vessel was maintained at 40°C with a thermostat. After sonication, the metal particles were removed by centrifugation at $25\,000 \times g$. Phosphatidylcholine concentrations were calculated from the P_i content according to the method of Vaskowsky et al. [22]. Gel-filtration chromatography and electron microscopy showed that most vesicles were single lamellar, while a peak due to liposomes could be present.

Gel chromatography. The vesicle-protein complexes were separated from the free protein molecules by gel-filtration chromatography on a 35×1.6 cm Sepharose CL-4B column. 1.7 ml of the complex solution were added to the column after 2 h of incubation. Detection of the elution pattern was carried out by an ultraviolet absorptiometer Spectrochrom F254 (Gilson Medical Electronics). Fractions of 2.5 ml were collected for analysis. Several methods were tried in order to detect α-lactalbumin in the vesicle fraction: from the ultraviolet spectra between 350 and 250 nm, it was obvious that small amounts of α-lactalbumin could not be detected on the background of turbidity caused by the vesicles, and from experiments with ^{125}I-labelled α-lactalbumin, it was observed that the iodonated α-lactalbumin did not behave as the α-lactalbumin did itself. Therefore, the amount of α-lactalbumin in the complex with dimyristoyl phosphatidylcholine was calculated from the difference between the free α-lactalbumin ultraviolet absorption in a pure α-lactalbumin sample and that in a sample containing the same quantity of α-lactalbumin in the presence of vesicles.

Microcalorimetry. The enthalpy change upon binding α-lactalbumin and dimyristoyl phosphatidylcholine was measured in an LKB-batch microcalorimeter, type 2107, equiped with siliconized Pyrex glass vessels and thermostatically controlled to 0.001°C by means of an air thermostat regulated by a water thermostat. This microcalorimeter contains a measuring cell and a reference cell mounted in opposition. Each cell is divided into two compartments: a 4 ml and a 2 ml compartment. The measuring cell is filled with 2 ml of a vesicle solution and 4 ml of the α-lactalbumin solution, both in the same buffer solution. The reference cell can be filled with 4 ml of an α-lactalbumin solution of the same concentration as in the measuring cell and 2 ml buffer solution. This enables, upon mixing, the subtraction of the heat of dilution of the protein from the heat of reaction. If in the reference cell 2 ml vesicles and 4 ml buffer are mixed, the heat of dilution of the vesicles is subtracted from the heat of reaction. In the concentration range used, the heats of dilution of proteins and vesicles were negligible. Therefore, the two compartments of the reference cell are usually filled with buffer solution. The heat was detected on the 10—50 μV scale of the LKB Bromma 2210 integrating recorder. An LKB 2107 control unit and Lauda K2RD thermostat were used in conjunction with the calorimeter. The enthalpy changes were calculated in kJ/mol protein.

Electron microscopy. The vesicles and vesicle-protein complexes were characterized by electron microscopy with a Jeol 100 ES electron microscope. One drop of a solution containing the vesicles and the protein at an appropriate concentration was spread on a copper grid covered with a Forman filter. After drying, the complex film was stained with a 0.5% uranyl acetate solution and dried.

Fluorescence and light-scattering measurements. Fluorescence measurements were performed on an Aminco-Bowman spectrofluorimeter, with an excitation wavelength of 280 nm. The cell compartment was thermostatically controlled at the incubation temperature, and the separate solutions were also thermostatically controlled before mixing. The excitation and emission slit-widths were both 1 mm. Uncorrected protein fluorescence spectra were recorded. Blank samples with the vesicles were recorded to correct the area under the fluorescence spectra for the light scattered by the vesicles. The final concentration of the phospholipid was mostly 1.8 mg/ml and the α-lactalbumin concentration 0.38 mg/ml, which corresponds to a molar ratio of 100. Light-scattering measurements were performed on the same instrument and under identical conditions, however, the excitation and the emission wavelengths were 400 nm.

Results

Batch microcalorimetry

Enthalpy change of binding as a function of the molar ratio. In this type of experiment, 2 ml of dimyristoyl phosphatidylcholine vesicles in the concentration range 0.37—16 mg/ml were added to 4 ml of an α-lactalbumin solution at a concentration of approx. 0.5 mg/ml. At a constant temperature of 30°C, four series of experiments were carried out in which the influence of the ionic

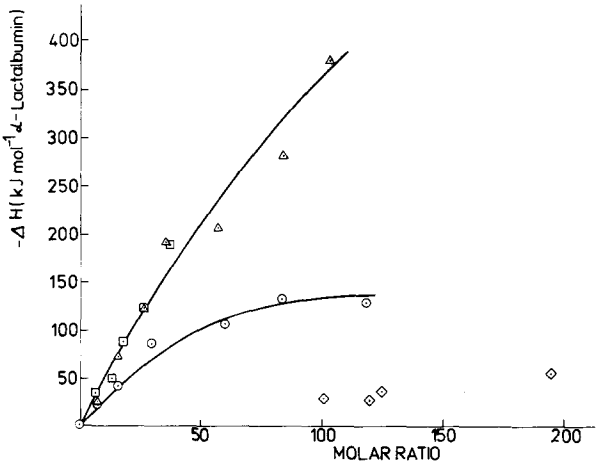

Fig. 1. Enthalpy change upon binding α-lactalbumin to dimyristoyl phosphatidylcholine vesicles as a function of the molar ratio at 30°C. At pH 4.6: ⊡, without NaCl; ⧋, 0.1 M NaCl. At pH 7.4: ⊙, without NaCl; ◈, 0.1 M NaCl.

strength and molar ratio on ΔH were studied. In Fig. 1, the results of plotting ΔH as a function of the molar ratio are shown. It is observed that at physiological pH, the enthalpy changes upon mixing the vesicles and the protein are small. Moreover, it is found that an increase in the ionic strength of the solution, by increasing the NaCl concentration to 0.1 M, decreases the ΔH values greatly. At acidic pH, increasingly larger values of ΔH are obtained with increasing molar ratio. Furthermore, ΔH is independent of the ionic strength of the solution and its values are much higher at acidic pH than at pH 7.

Enthalpy of binding as a function of pH. From the results of Fig. 1, it can already be concluded that pH plays an important role in the phospholipid-α-lactalbumin interaction. Therefore, experiments were carried out in which

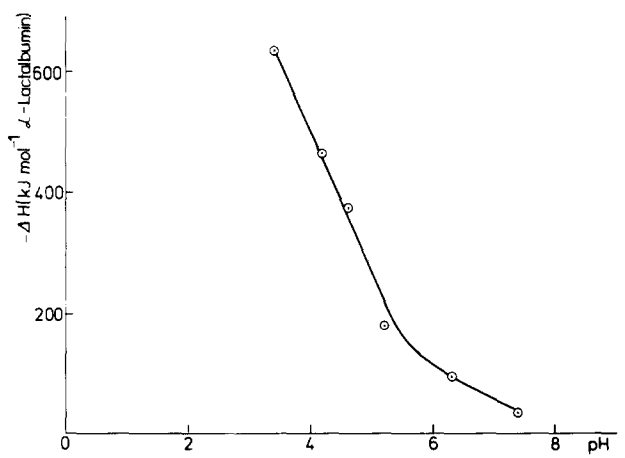

Fig. 2. Enthalpy change upon binding α-lactalbumin to dimyristoyl phosphatidylcholine vesicles as a function of pH at 30°C and at a molar ratio of 100.

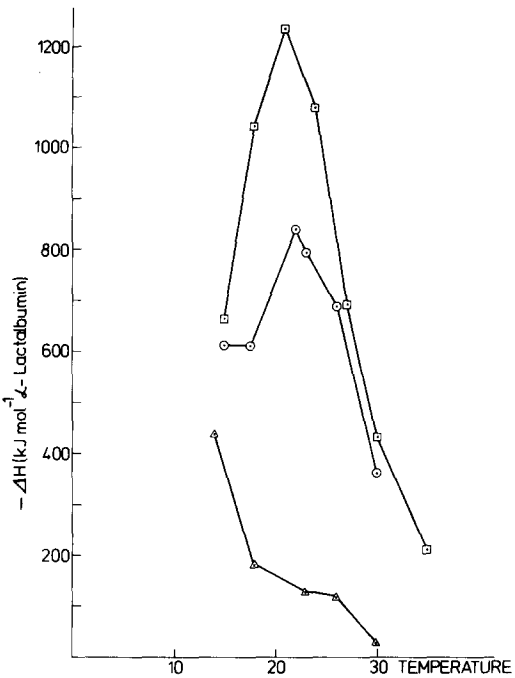

Fig. 3. Enthalpy change upon binding α-lactalbumin to dimyristoyl phosphatidylcholine vesicles as a function of temperature at a molar ratio of 100: pH 4 (□), pH 4.6 (○), pH 7.4 (△); NaCl, 0.1 M.

dimyristoyl phosphatidylcholine and α-lactalbumin were mixed at a constant molar ratio of approx. 100, at a constant temperature of 30°C but at pH values varying from 3.5 to 8. The results are plotted in Fig. 2. A drastic increase in heat release is observed when going from neutral to acidic pH.

Enthalpy of binding as a function of the reaction temperature. Previous work by others [23—26] has shown that temperature is an important factor in the regularity of the packing of the lipid molecules and therefore on the lipid-protein interactions. The enthalpy change of binding α-lactalbumin to the phospholipid vesicles was therefore measured between 30 and 15°C at pH 4, 4.6 and 7.4 for a molar ratio of approx. 100. In Fig. 3, it is seen that again the results differ at acidic and neutral pH. Whereas a maximum in ΔH is found in the acidic pH region around the transition temperature of dimyristoyl phosphatidylcholine, no such effect is observed at pH 7.4.

The microcalorimetric results at different pH and temperature lead to the following conclusions. (1) The ΔH values at acidic pH are much greater than at pH 7. (2) There is a maximum in the ΔH vs. temperature plot at pH 4, which is not present at pH 7. These results can be explained in two ways: either many more α-lactalbumin molecules are adsorbed at the outer surface of the vesicle with a maximum at about 24°C or the protein molecules penetrate the vesicle and a hydrophobic interaction between the protein and fatty acid chains of dimyristoyl phosphatidylcholine becomes possible. In order to clarify these posibilities, gel-filtration and electron-microscopic experiments were carried out.

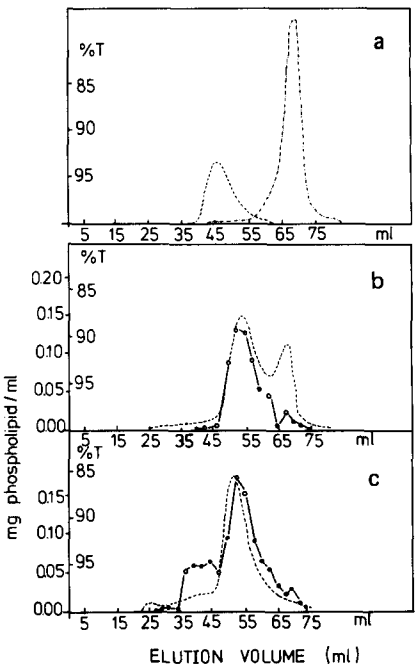

Fig. 4. Gel-filtration chromatographs of dimyristoyl phosphatidylcholine vesicles and α-lactalbumin. Experimental conditions: pH 4 (0.01 M acetate buffer in 0.1 M NaCl) and 23°C. (a) — — —, % *T* at 254 nm of pure vesicles (2.04 mg/ml); · — · — ·, % *T* at 254 nm of pure α-lactalbumin (0.38 mg/ml). (b) Molar ratio 51: - - - - - -, % *T* at 254 nm of a mixture of vesicles (0.91 mg/ml) and α-lactalbumin (0.38 mg/ml). ○, dimyristoyl phosphatidylcholine (mg/ml) determined by phosphate analysis. (c) Molar ratio 114: — — —, % *T* at 254 nm of a mixture of dimyristoyl phosphatidylcholine (2.04 mg/ml) and α-lactalbumin (0.38 mg/ml); ○, lipid (mg/ml) determined by phosphate analysis.

Gel-filtration chromatography

pH 7. The data obtained after incubating α-lactalbumin with dimyristoyl phosphatidylcholine vesicles show that only a small fraction of α-lactalbumin elutes with the vesicles as determined by extinction measurements of the free α-lactalbumin. Furthermore, the vesicles in contact with α-lactalbumin keep their original dimensions, since they elute at the same volume of pure vesicles (graph similar to Fig. 4a).

pH 4. Dimyristoyl phosphatidylcholine vesicles and α-lactalbumin, incubated at two different molar ratios at pH 4, show a totally different elution profile (Fig. 4) from that at pH 7. The pure vesicles elute between 40 and 55 ml, pure α-lactalbumin between 65 and 75 ml (Fig. 4a), while the interacting vesicles and α-lactalbumin molecules form a complex which elutes between 48 and 65 ml.

At a molar ratio of 114 (Fig. 4c), practically all the α-lactalbumin elutes between 48 and 65 ml and no absorption maximum is found at the elution volume of free α-lactalbumin. On analysis of phosphorus, it is found that about 25% of the phospholipid elutes before 48 ml, and about 75% elutes complexed to α-lactalbumin. From these values a molar ratio of 85 within the complex is calculated.

Similar conditions show that after incubation of vesicles and α-lactalbumin

at a molar ratio of 51 (Fig. 4b), the formed complex has a molar ratio of 76. At pH 5 and 23°C, this separated peak attributed to a complex between α-lactalbumin and phospholipid is also present, but not at pH 5 and 4 at 17°C. At 37°C, these experiments could not be carried out, since a precipitate was formed after 10 min.

Light-scattering measurements

An independent assessment of the change in vesicle structure was obtained from light-scattering measurements at 400 nm. For a dispersion with definite concentration, it can be stated that an increase in particle size or refractive index of the dispersed material will produce an increase in intensity of scattered light (I_θ) [27].

Fig. 5 is a plot of light scattering, measured at 400 nm and an angle of 90°, vs. time of vesicle/α-lactalbumin mixtures, respectively, at pH 4 and 7. It is apparent that at pH 7, the addition of α-lactalbumin to the vesicles has (apart from the dilution effect) no influence on the light scattering.

At pH 4, the effect on the light scattering is dependent on temperature. The mixing of α-lactalbumin and the vesicles at the phase transition temperature causes a marked decrease in light scattering. Gel-filtration chromatography (Fig. 4) shows that under these conditions of pH and temperature, the interaction between the protein and the lipid results in the formation of definite aggregates which differ from the original vesicles. Below the transition temperature, the mixture shows a limited decrease in light scattering, the formation of typical lipid-protein aggregate was not observed in that region. Above the phase transition temperature, the light scattering increases with time. This is due to a conglomeration of α-lactalbumin with the lipid material, which ultimately results in a visible turbidity and precipitation. The speed of the process increases with increasing temperature.

The light-scattering measurements at 400 nm and an angle of 90°, made on the vesicle/α-lactalbumin mixture (20 min after mixing at 30°C) as a function of decreasing temperature, are presented in Fig. 6. At pH 7, the light scattering

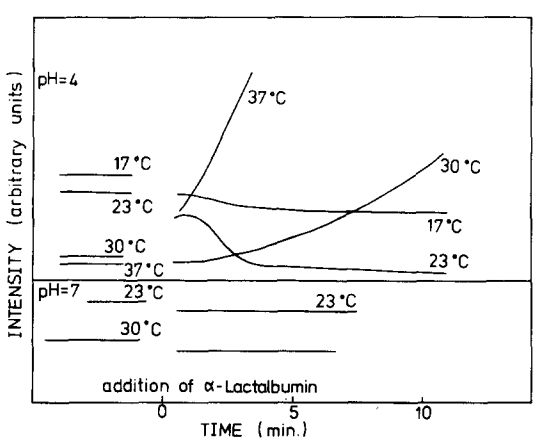

Fig. 5. Light scattering of dimyristoyl phosphatidylcholine vesicle/α-lactalbumin mixture as a function of time at 400 nm, pH 4 and 7, 0.1 M NaCl and different temperatures. Molar ratio 100.

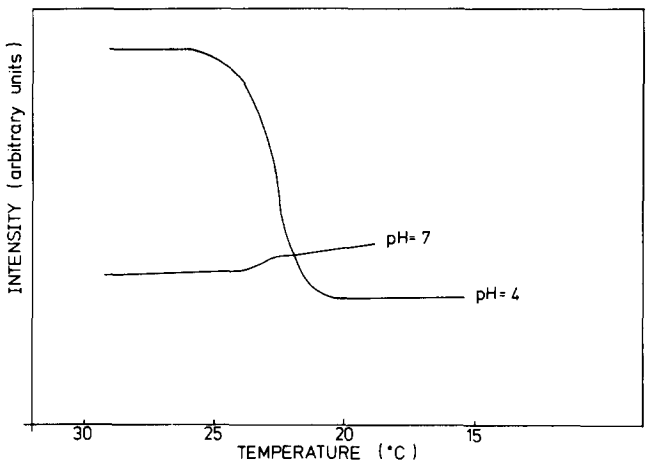

Fig. 6. Light scattering at 400 nm of dimyristoyl phosphatidylcholine/α-lactalbumin mixture as a function of decreasing the temperature at pH 7 and 4. The temperature scan started 20 min after mixing the lipid with α-lactalbumin at 30°C. Molar ratio 100.

of the vesicle/α-lactalbumin mixture behaves as a pure vesicle dispersion; at the transition temperature, light scattering increases, mainly due to an increase in the refractive index of the bilayer when the fatty acyl chains become quasi-crystalline. At pH 4 and 30°C, the scattering of the vesicle/α-lactalbumin mixture increased with time but the mixture was still optically clear after 20 min. On lowering the temperature, the light scattering decreased spectacularly at the transition temperatures and finally it reached the intensity that was obtained by direct mixing of the vesicles and α-lactalbumin below 23°C. This observation illustrates the direct connection between the formation of definite protein-lipid aggregates and the transition temperature.

Fluorimetric measurements

Fluorescence spectra obtained for α-lactalbumin are typical of those generally observed for tryptophan-containing proteins. The wavelength distribution of fluorescence depends upon the polarity of the immediate surroundings of the tryptophan groups. Tryptophans exposed to a polar medium have an emission maximum at 350 nm. Buried tryptophans in nonpolar regions are expected to have an emission maximum at 300 nm. However, the close proximity of relatively polar groups (such as the peptide linkage) shifts the emission wavelength to longer wavelength. Of the four tryptophans in native bovine α-lactalbumin, it is suggested that tryptophan 118 is exposed to the solvent and that tryptophan 26 is buried near the center of the molecule, while the tryptophans 60 and 104 should be in a labile region which is relatively close to the molecular surface. This structure of the native protein results in a net exposure of 1.7 tryptophan residues [28].

Fluorescence of pure α-lactalbumin. From Table I it is seen that in the pH region 4—7, the emission maxima shift towards the red when increasing the temperature from 17 to 50°C. At these pH values, between 17 and 34°C, the fluorescence intensity (at λ_{max}) decreases with increasing temperature, owing to an increase in nonspecific quenching of tryptophan fluorescence at higher

TABLE I

EMISSION MAXIMA AND INTENSITIES AT THE EMISSION MAXIMUM FOR α-LACTALBUMIN AT pH 4, 5 AND 7, AND DIFFERENT TEMPERATURES

Concentration of α-lactalbumin 0.40 mg/ml.

Temperature (°C)	pH 4 (0.1 M NaCl; 0.01 M acetate buffer)		pH 5 (0.1 M NaCl; 0.01 M acetate buffer)		pH 7 (0.1 M NaCl; (0.01 M phosphate buffer)	
	λ_{max} (nm)	I_{max} (arbitrary units)	λ_{max} (nm)	I_{max} (arbitrary units)	λ_{max} (nm)	I_{max} (arbitrary units)
17	330	165	330	165	329	170
22	332	156	331	155	330	159
27	332	152			330	154
32	333	148	331	144	331	150
34	333	151			332	148
37	339	155	334	152	334	157
43	341	167			340	171
50	344	167			343	175

temperature. The observed increase in fluorescence above 34°C is the net result of two effects: a further nonspecific quenching and a more important increase in fluorescence which is due to conformational change in α-lactalbumin. It is suggested that in bovine α-lactalbumin (and other α-lactalbumins with tryptophan residues at positions 60 and 104) at lower temperatures, transfer of the excited-state energy takes place from tryptophan 104 to tryptophan 60 with subsequent quenching by vicinal disulfide bridges [29]. Conformational changes should disrupt this aromatic pair at higher temperatures [30].

From Table I it is also seen that between pH 7 and 5, the fluorescence maximum does not vary in wavelength while a small decrease in maximum intensity (and quantum yield) is observed. Between pH 5 and 4 this maximum shifts to a higher wavelength and a small increase in maximum intensity (and quantum yield) is observed.

White [31] has observed that the fluorescence quantum yield of tryptophan is decreased when the carboxyl groups becomes protonated. And studies with proteins [32] have shown a pH-dependent decrease in fluorescence intensity in the acidic region which is attributed to excited-state interactions between carboxyl and tryptophan groups. In the same way, it is believed that the observed decrease in fluorescence quantum yield between pH 7 and 5 is related to protonation of a vicinal side chain of tryptophan [30]. Below pH 5, the small increase in λ_{max} and the increase in the quantum yield of the emission spectra of α-lactalbumin indicate different changes in the environment of the tryptophans. Between pH 4 and 3, this trend is more pronounced than between pH 5 and 4. At 25°C, the quantum yield increases by a factor 1.4 from pH 4 to 3 and λ_{max} has a red shift of 5—6 nm [33]. It is demonstrated that α-lactalbumin does not really denaturate in this pH region, but rather expands by about 30% [13]. In this state, α-lactalbumin is partially unfolded but keeps the helical content of the native structure. This expansion at 25°C does not lead to an increase in exposure of the tryptophans as is demonstrated by the perturbation of the absorption spectra [34].

TABLE II

RELATIVE FLUORESCENCE AND EMISSION MAXIMUM OF α-LACTALBUMIN IN THE PRESENCE OF DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES AT A MOLAR RATIO OF 100 (1.8 mg/ml DIMYRISTOYL PHOSPHATIDYLCHOLINE IN THE PRESENCE OF 0.38 mg/ml α-LACTALBUMIN)

a, pure α-lactalbumin; b, α-lactalbumin/vesicle, first scan (2—6 min after mixing); b′, same as b, but after 15 min incubation; c, same as b, but after 1 h incubation. F/F_0 = area under the emission curve of α-lactalbumin in the presence of vesicles, corrected for the light scattered by control samples of free vesicles/area under the emission curve of pure α-lactalbumin at the same concentration, pH, temperature and NaCl concentration.

Temperature (°C)	pH4		pH5		pH7	
	λ_{max}	F/F_0	λ_{max}	F/F_0	λ_{max}	F/F_0
17 a	330	1	330	1	329	1
b	334	1.25	331	0.87	331	0.94
c	334	1.38	331	0.97	332	1.08
23 a	332	1	331	1	330	1
b	336	1.60	331	0.96	330	0.74
c	336	1.85	334	1.20	330	0.89
37 a	339	1 *	334	1	334	1
b	336	1.50 *	333	0.82	335	0.83
b′	336	1.32 *				
c	precipitate		precipitate		335	0.90

* Values for a more dilute solution (0.64 mg/ml dimyristoyl phosphatidylcholine in the presence of 0.15 mg/ml α-lactalbumin).

Fluorescence of α-lactalbumin/vesicle mixtures. For α-lactalbumin/vesicle mixtures, the pH and temperature dependence of the emission maxima and the relative quantum yield are summarized in Table II. It is observed that α-lactalbumin in contact with vesicles behaves differently at acidic and neutral pH values.

At pH 4

Fluorescence spectra. At pH 4 and at 23°C, a red shift (4—5 nm) of the emission maximum accompanied with a marked increase in the relative fluorescence ($F/F_0 \cong 2$) is noticed (Fig. 7). The same effects, but smaller, are found below the transition temperature (17°C). Above the transition temperature (37°C), the fluorescence intensity vs. time plot evolves to a maximum, then decreases while the mixture becomes turbid. This evolution was very rapid at lipid and α-lactalbumin concentrations 1.8 and 0.38 mg/ml, respectively, but could easily be followed at more dilute concentrations of 0.64 mg/ml lipid and 0.15 mg/ml α-lactalbumin. It is noteworthy that at pH 4, α-lactalbumin complexed to dimyristoyl phosphatidylcholine has a λ_{max} which is nearly independent of the temperature, this is in contrast to λ_{max} of pure α-lactalbumin which is temperature dependent. As a consequence of this, the complexation of α-lactalbumin at 37°C is accompanied by a blue shift with regard to pure α-lactalbumin at pH 4 and 37°C.

Evolution of the fluorescence spectra versus time. As observed by Kronman [33], the changes in fluorescence accompanying the conformational changes of α-lactalbumin, induced by acid, temperature or urea, occur quite rapidly and are completed by the time of preparing the measurements (3 min). From

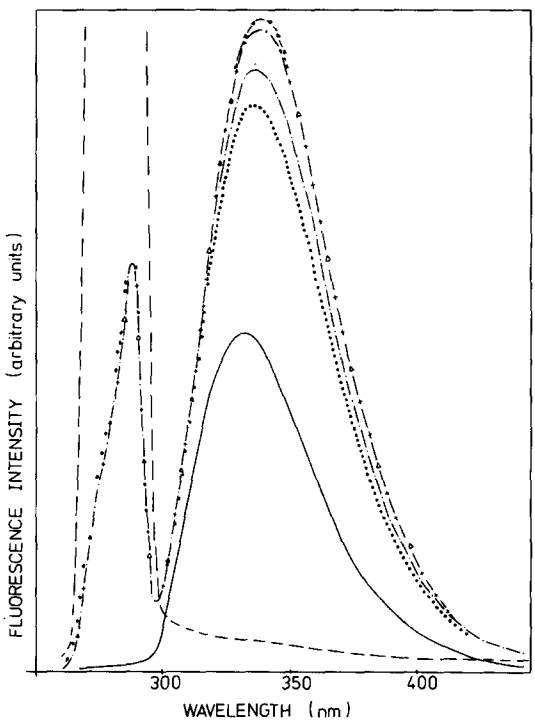

Fig. 7. Fluorescence spectra of the α-lactalbumin/dimyristoyl phosphatidylcholine system at pH 4, 23°C and 0.1 M NaCl. Excitation wavelength 280 nm. ———, α-lactalbumin (0.4 mg/ml); - - - - - -, dimyristoyl phosphatidylcholine vesicles (1.8 mg/ml); ○, dimyristoyl phosphatidylcholine vesicles (1.8 mg/ml) and α-lactalbumin (0.4 mg/ml) 2—6 min after mixing; · — · — ·, same but after 14 min; +, same but after 1 h; △, same but after 2 h.

measurements of the extinction at 293 nm with a rapid reaction analyser, Sugai and co-workers [35] determined that the denaturation of bovine α-lactalbumin at pH 4, 25°C and 2.4 M guanidine hydrochloride follows a first-order kinetic reaction with a halftime of 0.35 s. In contrast (Fig. 7), the fluorescence of the α-lactalbumin/vesicle mixture at pH 4 and 23°C continues to increase up to a period of 2 h. Identical data are obtained at pH 4 and 17°C and at pH 5 and 23°C. (It is difficult to follow the shift of λ_{max} with time as difference of 1–2 nm may result from the scan procedure). These observations could lead to the supposition that the complex formation between α-lactalbumin and the vesicles proceeds for up to 2 h. However, at pH 4 and 23°C, the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the lipid phase increases to its final value, within 5 min after the addition of α-lactalbumin, indicating that the penetration of the protein in the bilayer is completed in that time (Herreman, W., van Tornout, P., Hanssens, I. and van Cauwelaert, F., unpublished results). The results of the light-scattering measurements, described above (see Fig. 5) also indicate that at 23°C and pH 4, a reconstruction of the vesicles takes place within 5 min after mixing. Therefore, the continuation of the fluorescence increase must be an effect that follows the complex formation and does not precede it. Since conformational changes occurring in free α-lactalbumin are very rapid, the fluorescence intensity changes as

function of time must be declared as a function of the complex formed, e.g., a (partial) rearrangement of α-lactalbumin in the lipid phase that can be hindered by the limited space.

Accessibility of complexed α-lactalbumin to changes in the water phase. It was attempted to obtain some information about the accessibility of α-lactalbumin in a reconstituted vesicle for changes in the water phase. For that purpose, a 2 ml sample of α-lactalbumin bound to dimyristoyl phosphatidylcholine vesicles at pH 4 and 23°C (obtained by a 2 h incubation in a buffer containing 0.1 M NaCl and 0.01 M sodium acetate/acetic acid) was added to 2 ml phosphate buffer at pH 7 and with greater capacity (0.1 M K_2HPO_4/NaH_2PO_4). The pH of the mixture was maintained at 7. The emission maximum of α-lactalbumin in this solution remained at 336 nm and was unchanged after 1 h. Also, the fluorescence intensities did not differ from a reference solution which was diluted with NaCl/acetate buffer. These fluorescence characteristics differ from what is obtained by direct mixing of α-lactalbumin and phospholipid vesicles at pH 7 (see Table II). This result indicates that, at pH 4, α-lactalbumin in contact with lipid becomes insensitive to changes in the water phase. A more drastic method to control the accessibility of the complexed α-lactalbumin is obtained by adding aqueous solutions of protein denaturants to a stabilized suspension of lipid/α-lactalbumin. Urea and guanidine hydrochloride are widely used denaturants of proteins; concentrated solutions of these agents denaturate globular proteins to linear random coils. α-Lactalbumin is completely denaturated in a 4 mM guanidine hydrochloride solution [35] or in a 8 M urea solution [28]. Concentrated aqueous solutions of guanidine hydrochloride have also been used to extract membrane proteins. The solubilized proteins appear to be random coils free of intermolecular associations. However, concentrated (6 M) guanidine hydrochloride solubilizes only some membrane proteins and leaves certain others associated with the lipid [36]. Urea also has been found to be useful as an adjunct to the extraction of membrane proteins. We observed that at pH 4 and 23°C, free α-lactalbumin denatured in 5 M urea and had an emission maximum at 350 nm. However, after α-lactalbumin has interacted with the vesicles, a 5 M urea solution only shifts the emission maximum of the preformed complex from 336 to 340 nm. Free α-lactalbumin in the presence of 4 M guanidine hydrochloride has an emission maximum at 351 nm, while α-lactalbumin complexed to the phospholipid has an emission maximum of 344 nm at the same guanidine hydrochloride concentration. These data demonstrate the reduced exposure of tryptophan residues to the aqueous denaturants and consequently prove that under the experimental conditions α-lactalbumin is not completely released from the apolar lipid moieties.

Lipid-to-protein molar ratio in the complexes. Fig. 8 shows the relative fluorescence when, at pH 4 and 23°C, to an increasing amount of phospholipid the same amount of α-lactalbumin is added (final concentration of α-lactalbumin 0.38 mg/ml). A doubling of the total fluorescence is found when the lipid-to-protein ratio increases from zero to 80. Further addition of lipid has practically no effect on the fluorescence. This observation indicates that up to a ratio of 80, all α-lactalbumin molecules interacted with the vesicles, and

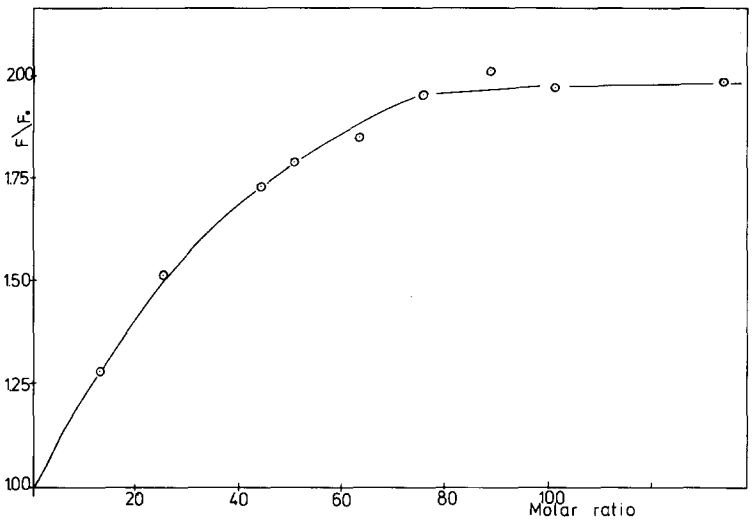

Fig. 8. Relative fluorescence, F/F_0, of α-lactalbumin (0.38 mg/ml) with increasing amounts of dimyristoyl phosphatidylcholine. pH 4, 23°C and 0.1 M NaCl.

that at lower lipid concentrations, a part of the α-lactalbumin does not interact with dimyristoyl phosphatidylcholine.

At pH 7

Fluorescence spectra and evolution in time. At pH 7 and different temperatures, no significant shift of the emission maximum was detected. As can be seen in Table II, a limited change in the fluorescence intensity was observed. At all temperatures, the relative fluorescence (F/F_0) changed with time: in the first spectrum after mixing, a relatively important quenching of the tryptophan fluorescence of α-lactalbumin was always measured, while after a longer incubation period the quenching was reduced or even evolved into a fluorescence increase as is the case at 17°C.

Accessibility of α-lactalbumin to protein denaturants. For free α-lactalbumin in 5 M urea at pH 7, a λ_{max} at 348 nm is measured. After α-lactalbumin has interacted with the vesicles at pH 7, a 5 M urea concentration shifts the emission maximum from 330 to 345 nm.

The λ_{max} of free α-lactalbumin in the presence of 4 M guanidine hydrochloride is not influenced by the pH and remains at 351 nm. α-Lactalbumin that interacted with dimyristoyl phosphatidylcholine at pH 7 produces an emission maximum of 349 nm in 4 M guanidine hydrochloride.

Electron microscopy

Electron micrographs were taken from dimyristoyl phosphatidylcholine/α-lactalbumin mixtures at 23°C at pH 4 and 7 compared with the micrographs of pure vesicles at the same pH. At pH 7, as depicted in Fig. 9a, no difference is observed between micrographs of pure vesicles and vesicles/α-lactalbumin. Single lamellar vesicles are shown with a diameter of ±300 Å. Fig. 9b shows the micrograph of a vesicle/α-lactalbumin mixture incubated at 23°C at a molar

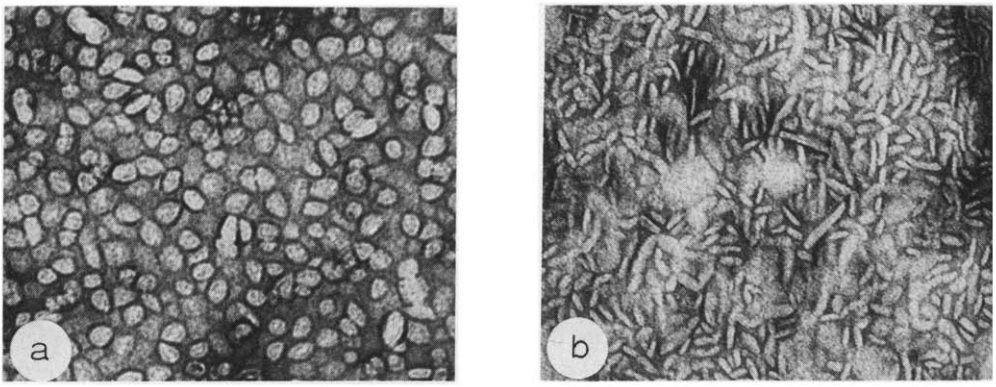

Fig. 9. Electron micrographs: (a) pH 7; (b) pH 4. 0.1 M NaCl, molar ratio 20. Magnification × 120 000.

ratio of 20. Bar-shaped structures, different from the original vesicles, appear in the micrograph.

Discussion

α-Lactalbumin is used in this work as a prototype to study the influence of the conformation of a protein on its behaviour towards phospholipid vesicles. Two possible interaction mechanisms can occur between the vesicles and the protein: adsorption at the vesicle outer surface or adsorption-penetration in the vesicle bilayer. Our experimental data will be discussed in view of these two possibilities.

pH 7

Since α-lactalbumin is known to be a peripheral protein at physiological pH, it is most probable that its interactions with dimyristoyl phosphatidylcholine will be weak. This is confirmed by the microcalorimetric data: low ΔH values which do not show a maximum in the ΔH vs. T graph but are dependent on the ionic strength (an operational criterion that indicates that the interaction is electrostatic in nature [37]). Furthermore, the chromatographic data indicate that only a small amount of protein is bound to the vesicles. Although it is possible that a small fraction of the adsorbing protein molecules penetrates the vesicles, no species of a new size or shape are detected by gel chromatography or by light scattering (Figs. 5 and 6). Electrostatic interactions with a protein do not necessarily change the phospholipid bilayer thickness as shown in X-ray studies by Rand and Sen Gupta [38] in a study with insulin as protein and by Shechter et al. [37].

The increase in fluorescence intensity with time after the initial decrease upon the interaction of α-lactalbumin with the vesicles suggests two distinct steps for the interaction. The first step is dominated by the electrostatic interaction, while the second step may be a limited conformational change and/or even a limited penetration into the hydrophobic phase. The data in which urea and guanidine hydrochloride are added to the vesicle/protein mixture, are strongly in favour of a penetration, since it is found that the

protein is partially protected (but much less than at pH 4) from aqueous denaturants. Summarized, all data at pH 7 (and pH 6 as well) are in agreement with an adsorption of α-lactalbumin to the outer surface of the vesicle through electrostatic interaction, followed by a slow penetration of a small fraction of the molecules. At physiological pH, α-lactalbumin behaves, therefore, like a peripheral protein.

pH 4 and 5

In the acidic pH region, the results obtained for the interaction between dimyristoyl phosphatidylcholine and α-lactalbumin are totally different: the ΔH values are much higher than at pH 7, the dependence of ΔH on the ionic strength of the solution disappears and the curve of ΔH as a function of temperature shows a maximum around the transition temperature of the lipid. Furthermore, in the gel-filtration chromatographs, a peak due to a lipid-α-lactalbumin complex appears. These data can be explained by assuming that the interacting α-lactalbumin molecules break down the original vesicles and form a lipid-protein complex with a molar ratio of $\simeq 80$. The drastic decrease in light scattering around the transition temperature is in agreement with the formation of such a complex. In parallel to the monolayer behaviour [20], α-lactalbumin in the acidic pH region adsorbs to and penetrates the vesicles, so that interaction becomes possible between the fatty acid chain of the phospholipid molecules and the protein. The shape and the maximum in the ΔH vs. (T) curve at pH 4 and at pH 4.6 signifies that this interaction is strongly facilitated around the transition temperature but less so at higher and lower temperatures. A similar maximum in the microcalorimetric data was also found by Rosseneu [25] for the interaction of apolipoproteins A-I and A-II with dimyristoyl phosphatidylcholine vesicles. The electron-microscopic data show that at 23°C, protein-lipid complexes are formed of a size and shape different from the complexes at pH 7. The fact is also that the high ΔH values (2000 kJ/mol protein) observed at about 23°C at pH 4 and 5 are probably not due to simple adsorption. When adsorption and penetration become possible, supplementary interactions can contribute to ΔH, i.e., hydrophobic interaction between the fatty acid chains of the vesicles and hydrophobic parts of the protein, conformational changes in the proteins resulting in the possibility of protonation of more carboxylic groups in α-lactalbumin [9] and binding of water. If hydrophobic interactions are present, fluorescence measurements on the protein must present indications. Morrisett et al. [39], studying fluorescence spectra of the interaction between the apolipoprotein-containing C-terminal alanine (apo-LP-Ala) interacting with phosphatidylcholine, observed a λ_{max} of 350.4 for the free protein. In 4 M guanidine hydrochloride, the λ_{max} shifted to 351 nm, suggesting that the three tryptophans are highly exposed to the polar solvent. Addition of the phosphatidylcholine shifts λ_{max} to 345.2 nm at a molar ratio of phosphatidylcholine to apo-LP-Ala of 90 and decreases the intensity. This is interpreted as a shift of one or more of the three tryptophan residues from a more polar to a more hydrophobic environment. Dufourcq et al. [40] studied the interaction between cytochrome b_5 and egg lecithin liposomes: the formation of the lipid-protein complex with a molar ratio of 50 is accompanied by a drastic increase in the

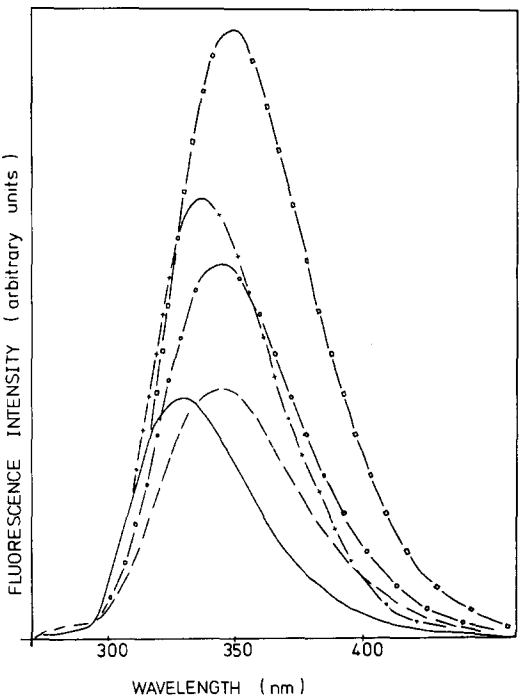

Fig. 10. Fluorescence spectra of α-lactalbumin under different denaturating conditions: [α-lactalbumin] = 0.6 mg/ml, [NaCl] = 0.1 M in all spectra. ———, α-lactalbumin in acetate buffer, pH 4, 23°C, λ_{max} = 330 nm, - - - - - -, α-lactalbumin in acetate buffer, pH 4, 51.2°C, λ_{max} = 354 nm, ○———○, α-lactalbumin at pH 2, λ_{max} = 345 nm; +———+, α-lactalbumin + dimyristoyl phosphatidylcholine vesicles (1.8 mg/ml) in buffer at pH 4, 23°C, λ_{max} = 336 nm; □———□, α-lactalbumin in 6 M urea at pH 4, λ_{max} = 350 nm.

intensity and a slight blue shift of λ_{max}. By eliminating the hydrophilic moiety of cytochrome b_5, they were able to show that only the hydrophobic part of the molecule is concerned in this interaction.

In our experiments at 23°C, a shift of λ_{max} is observed from 331 to 334 nm at pH 5 and to 336 nm at pH 4, while the fluorescence intensity strongly increases up to a molar ratio of dimyristoyl phosphatidylcholine to α-lactalbumin of 80. Also, the red shift and the increase of fluorescence yield are effects commonly found in α-lactalbumin denaturation and conformational changes. In Fig. 10, the fluorescence spectra of α-lactalbumin are recorded in different circumstances which cause typical conformational changes. In this figure, the spectrum of α-lactalbumin in contact with phospholipid vesicles at pH 4 and 23°C is corrected for the scattering background of the vesicles. From the comparison of the spectra, it is obvious that although the area of the emission curve of α-lactalbumin in contact with the vesicles at pH 4 has strongly increased, the shift of the emission maximum is comparatively low and is less than the red shift caused by temperature- or acid-induced conformational changes. As the red shift of α-lactalbumin at pH 2 is not due to the exposure of tryptophan groups to an external polar solvent [34], it is not probable that the red shift of the α-lactalbumin-vesicle complex at pH 4 is due to a contact of the tryptophan groups with water or other strongly polar groups. In the same way as is suggested for temperature-induced confor-

mational changes [30], it is believed that the increase of fluorescence yield in the complex is due to the reduction of fluorescence quenching by the separation of the tryptophans 60 and 140. In the α-lactalbumin-vesicle complex, the protein expands in an apolar lipid phase and the separated tryptophans have less opportunity to enter into contact with relatively polar groups (such as peptide linkages), than at the temperature and in the acid formation of α-lactalbumin. This representation also declares that λ_{max} of the complex is less temperature dependent than λ_{max} of α-lactalbumin itself: it results in a hypsochromic shift of α-lactalbumin combined to the vesicles at 37°C, from 340 nm in free α-lactalbumin to 336 nm. The complex with a molar ratio of $\simeq 80$ is very stable, since denaturation of the protein in the presence of 5 M urea or 4 M guanidine hydrochloride does not occur and since it can be separated by gel chromatography.

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