*Biochimica et Biophysica Acta* 817 (1985) 154–164
Elsevier

BBA 72662

# Influence of the protein conformation on the interaction between α-lactalbumin and dimyristoylphosphatidylcholine vesicles

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(Received February 19th, 1985)

Key words: Protein-lipid interaction; α-Lactalbumin; Lipid solubilization; Dimyristoylphosphatidylcholine; Circular dichroism; Fluorescence polarization

**α-Lactalbumin is a globular protein containing helical regions with highly amphiphathic character. In this work, the interaction between bovine α-lactalbumin and sonicated dimyristoylphosphatidylcholine vesicles has been compared in different circumstances which influence the protein conformation i.e., pH, ionic strength, decalcification, guanidine hydrochloride denaturation. Above the isoelectric point the interaction is mainly electrostatic; improved electrostatic interaction results in better contact with the apolar lipid phase. Below the isoelectric point, hydrophobic forces dominate the interaction and the vesicles are solubilized. The mode of interaction is not determined to a great extent by the demetallization of the protein. However, by a more explicit unfolding of the globular structure with guanidine hydrochloride, micellar complexes can be formed with the lipid, even at neutral pH. From this study it is obvious that the presence or capability for formation of helices with high amphipathic character is not a sufficient condition for lipid solubilization by a globular protein. Also, the capability of a globular protein to unfold its tertiary structure seems to be a prerequisite for its capability to lipid solubilization.**

## Introduction

A good knowledge of protein-lipid interaction is a key to the understanding of many biological processes. Intrinsic membrane proteins penetrate into the hydrocarbon region, while extrinsic proteins are located adjacent to the outer or inner surface of the membrane. The extrinsic proteins are arranged in such a manner that their polar groups are either exposed to the aqueous environment or associated electrostatically with the hydrophilic groups of the bilayer, while apolar parts of the protein (may) maintain contact with the inner hydrophobic region. One way to segregate the apolar groups of the protein is the formation of α-helices in which the hydrophobic residues of the protein are located on one face of the helix. As for serum lipoprotein particles, the creation of amphiphatic helices is often accompanied by the formation of micellar complexes [1]. Synthetic lipid-binding peptide [2], as well as fragments corresponding to lipid-binding regions of apolipoproteins [3] and low-molecular-weight model proteins [4,5] have been used to study this last-mentioned aspect of the protein-lipid interaction. In these studies it has been found that peptides of at least 20 amino-acid residues which are not very hydrophilic and have the potential to form an amphipathic helix can form comparable protein-lipid micellar particles.

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We have attempted to supply further evidence on the association of proteins with lipids by using a protein which is able to interact in different ways, dependent upon the external environment. The choice of α-lactalbumin was inspired by the abundance of data that were available on different aspects of the conformational behavior of this protein as a function of pH, temperature and, since the recent discovery by Hiraoka [6], that it is a metalloprotein, also as a function of the $Ca^{2+}$-content.

Our first studies [7–9] with the native protein (i.e., the calcium-conformer) were mainly as a function of pH and temperature. In summary, at physiological pH and in the presence of 0.1 M NaCl no clear interaction with dimyristoylphosphatidylcholine vesicles is observed. In absence of NaCl, a pronounced release of heat indicates lipid-protein interaction, although this interaction did not result in a destruction of the vesicles. At pH 4, the protein has an apolipoprotein-like behavior and forms micellar complexes. These complex particles are formed with small unilamellar vesicles at temperatures between 18 and 33°C (with a composition of the complex which depends on temperature). With large vesicles the complex formation is more restricted to a small temperature range above the transition temperature [10].

Hitherto, we thought that this capability of α-lactalbumin to form complexes at pH 4 was due to a more expanded and hydrophobic conformation. However, since the discovery that α-lactalbumin is a calcium protein, fluorescence studies [11] have indicated that the decalcified protein (i.e., the apo-protein) has its most compact conformation at pH 4–5, and a more expanded structure at pH 2 and pH 8. Warme et al. [12] suggested that in the expanded conformers a highly apolar part of the molecule will be exposed to the solvent. Addition of $Ca^{2+}$ to the decalcified expanded conformer at pH 8 leads to a more compact conformer [11].

In this paper we have investigated, therefore, whether the decalcified, expanded conformer penetrates better into the apolar lipid phase and if its ability for micellar complex formation is enhanced.

The results demonstrate that the removal of $Ca^{2+}$ enhances somewhat the protein penetration at pH 6–8, but does not increase its capability to destroy the vesicle structures. We propose an explanation for the capability of α-lactalbumin to form micellar complexes at more acid pH.

## Materials and Methods

*Materials.* Bovine α-lactalbumin and L-α-dimyristoylphosphatidylcholine were from Sigma. The phospholipid was used without further purification. α-Lactalbumin is purified as described in Ref. 11. The purified α-lactalbumin is decalcified by ion-exchange chromatography on a Chelex 100 column (1.5 cm diameter, 30 cm high) at pH 10–11. The solutions of $Ca^{2+}$ free protein are brought to neutral pH and are lyophilized. After this procedure, a $Ca^{2+}$ content of about $3 \cdot 10^{-6}$ M is found by atomic absorption spectrophotometry (Varian Techtron AA 6) in a solution of $1.6 \cdot 10^{-4}$ M α-lactalbumin.

1,6-Diphenyl-1,3,5-hexatriene was purchased from Eastman Kodak Co. NaCl is a suprapure product from Merck, except in the studies of guanidine hydrochloride denaturation, where no comparison is made between $Ca^{2+}$- and $Ca^{2+}$-free α-lactalbumin.

*Vesicle preparation.* Single-shelled vesicles of dimyristoylphosphatidylcholine were prepared as described in Ref. 10. The lipid concentrations are controlled by phosphate analysis [13]. Atomic absorption spectrometry indicates that the lipid vesicles do not contain any appreciable amount of $Ca^{2+}$ (the limit of $Ca^{2+}$ detection is $1 \cdot 10^{-6}$ M).

*Lipid-protein mixtures.* For further measurements, demetallized α-lactalbumin powder has been dissolved in $Ca^{2+}$-free buffer containing 5 mM Tris (pH 8), 5 mM Mes (pH 7 and 6), 5 mM acetate (pH 5 and 4) or diluted HCl (pH 3 and 2). The protein concentrations are determined by the excitation at 280 nm using a value of $E_{1\%} = 20.1$. To 1.25 ml decalcified α-lactalbumin, 0.25 ml of the appropriate buffer, 1 M NaCl or $Ca^{2+}$ solution is added along with 1.0 ml vesicle suspension or buffer. The concentrations of lipid and protein are adapted to obtain a molar ratio of 70, which corresponds to the ratio found in the lipid-protein complexes after incubation at pH 4 with 0.1 M NaCl at 23°C [9,10]. The lipid/protein mixtures are incubated at 23°C for at least 2 h before any

measurement. Polypropylene test-tubes and polypropylene pipettes are used to contain and to transfer $Ca^{2+}$-free α-lactalbumin solutions.

*Fluorescence measurements.* The fluorescence measurements are performed with an Aminco SPF-500 spectrofluorimeter, connected with a Hewlett-Packard 7225 plotter and a Hewlett-Packard 9815 A desk-top computer. The latter calculates corrected spectra, the maximum wavelength and the area under the corrected emission spectrum. Excitation was made at 280 nm (bandwidth, 4 nm). The emission bandwidth is 1 nm. Fluorescence quantum yields of lipid-free α-lactalbumin solutions are evaluated by comparing the area of the corrected spectra with those of tryptophan solutions (quantum yield 0.13 at 23°C) [14] with the same absorbance at 280 nm. The fluorescence yield of α-lactalbumin in vesicle suspensions is obtained by a direct comparison of the area of the corrected spectra with that of a lipid-free α-lactalbumin solution containing the same amount of protein. In earlier studies [7] another fluorimeter was used which did not allow such precise measurements. The fluorescence data of α-lactalbumin in the absence of lipid vesicles are described elsewhere [11].

*Fluorescence polarization.* In order to label the vesicle suspension, 4 μl of 0.5% (w/v) diphenylhexatriene in tetrahydrofuran is added per mg phospholipid. The final samples for polarization measurements are prepared in a similar way as for the fluorescence measurements, but the concentrations of protein, lipid and $Ca^{2+}$ are halved. The fluorescence polarization is measured with an Elscint MV-1A microviscosimeter.

*Circular dichroism measurements.* The CD spectra of the peptide region were obtained by using a Cary model 61 spectropolarimeter. The instrument was calibrated with (+)-camphor-10-sulfonic acid. Cuvettes of 1.0 mm light-path were used. Protein concentrations were about 0.4 mg/ml. The bandwith of the incident light was 4 nm and the scan rate 0.1 nm/s. The amino acid residue weight of α-lactalbumin was calculated to be 117. The fraction of helical structure, $f_H$, is derived from the mean residue ellipticity, $[\theta_\lambda]$, at different wavelengths, λ, between 210 and 234 nm, by means of the equation proposed by Siegel et al. [16]:

$$f_H = \frac{|\theta_\lambda| - C_\lambda}{\theta_{H,\lambda}}$$

In this equation, the mean residue ellipticity for 100% helix ($\theta_{H,\lambda}$) and the transferable constant ($C_\lambda$) are derived from the ellipticity of 16 reference proteins of which the secondary structure was studied by X-ray diffraction.

Light scattering at 400 nm has been measured as a function of the lipid:protein molar ratio, by means of the spectrofluorimeter.

## Results

*Fluorescence changes occurring in α-lactalbumin after interaction with phosphatidylcholine vesicles under different environmental conditions*

The influence of the environmental conditions (pH, ionic strength and presence of $Ca^{2+}$ ions) on the interaction between α-lactalbumin and dimyristoylphosphatidylcholine vesicles was followed primarily by the fluorescence characteristics of the protein. The results are summarized in Table I. The following comments can be given on these results.

(a) The interaction with vesicles increases the fluorescence quantum yield of the protein at all pH values for the apo- as well as for the $Ca^{2+}$-protein. The size of the increase is hardly influenced by the presence of $Ca^{2+}$ or by the ionic strength, but it is primarily determined by the pH value.

(b) In earlier work [11] it was shown that the $Ca^{2+}$-α-lactalbumin at pH 8 and the apo-α-lactalbumin at pH 4–5 have a compact conformation with similar quantum yield and wavelength maximum. Since the quantum yields and wavelength of these both conformers change in a different way in the presence of the vesicles, it is clear that their mode of interaction with the vesicles must be different.

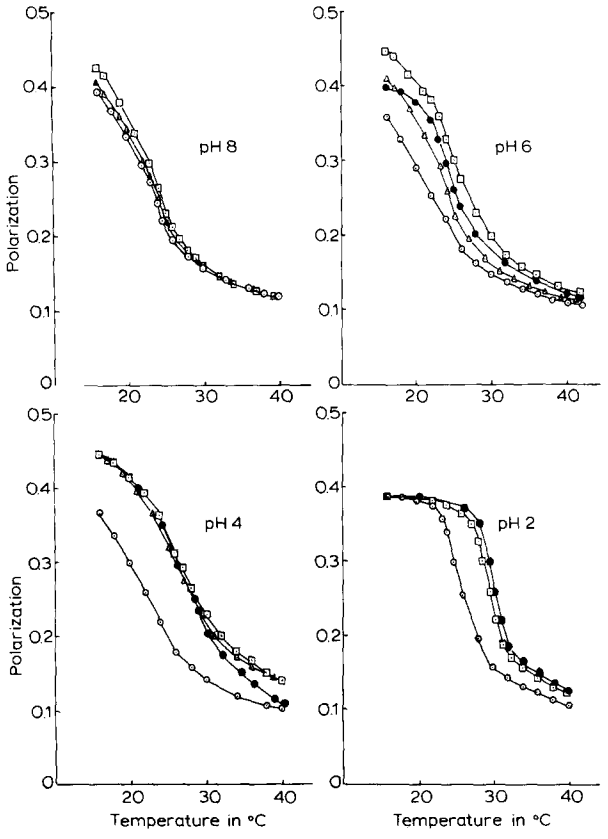
(c) A similarity in conformation between the apo-protein at pH 8 and pH 2 has been proposed from their similar quantum yields and wavelengths [17]. These conformers are rather expanded [11]. After interaction with dimyristoylphosphatidylcholine vesicles, the quantum yield of both conformers increases and the emission maximum shifts to a lower wavelength. Such a blue shift accompanied with an increase in quantum yield is a strong indication that the tryptophan groups enter into a more apolar medium. The result can be interpreted by a penetration of tryptophan

TABLE I

TRYPTOPHAN FLUORESCENCE PARAMETERS OF α-LACTALBUMIN

Comparison of the tryptophan fluorescence parameters of α-lactalbumin ($1.5 \cdot 10^{-5}$ M). (a) In the absence of lipid (see also Ref. 11). (b) In the presence of dimyristoylphosphatidylcholine vesicles (0.75 mg/ml) (lipid: protein molar ratio = 70). Different environmental conditions are compared: absence of divalent ions, presence of $3.8 \cdot 10^{-5}$ M $Ca^{2+}$, presence of 0.1 M NaCl, different pH values. Temperature, 23°C.

| | pH | Quantum yield | | | $\lambda_{max}$ | | |
|---|---|---|---|---|---|---|---|
| | | apo-protein | 2.5 $Ca^{2+}$ protein | 0.1 M NaCl | apo-protein | 2.5 $Ca^{2+}$ protein | 0.1 M NaCl |
| (a) | 8 | 0.051 | 0.032 | 0.036 | 337 | 325 | 325 |
| | 7 | 0.047 | 0.032 | 0.034 | 334 | 325 | 325 |
| | 6 | 0.043 | 0.030 | 0.033 | 331 | 325 | 325 |
| | 5 | 0.033 | 0.028 | 0.030 | 327 | 325 | 325 |
| | 4 | 0.032 | 0.027 | 0.036 | 329 | 325 | 331 |
| | 3 | 0.042 | 0.040 | 0.042 | 337 | 334 | 337 |
| | 2 | 0.046 | 0.046 | 0.045 | 339 | 338 | 341 |
| (b) | 8 | 0.058 | 0.038 | 0.043 | 333 | 325 | 327 |
| | 7 | 0.059 | 0.038 | 0.043 | 334 | 325 | 327 |
| | 6 | 0.065 | 0.041 | 0.050 | 333 | 327 | 331 |
| | 5 | 0.066 | 0.048 | 0.061 | 331 | 329 | 331 |
| | 4 | 0.077 | 0.072 | 0.076 | 331 | 331 | 331 |
| | 2 | 0.056 | – | 0.059 | 331 | – | 331 |




groups into the apolar lipid phase. This effect is more pronounced at pH 2 than at pH 8.

(d) While for the expanded protein (pH 2 and apoprotein at pH 8) a blue shift is observed, for the more compact conformations (at pH 4–5 and higher pH in presence of $Ca^{2+}$ or NaCl) the fluorescence shifts to red in presence of phosphatidylcholine. A simultaneous increase in quantum yield indicates that the red shift is not due to a simple displacement of tryptophan groups to a more polar phase. A more detailed inspection of the data reveals that the emission maximum of α-lactalbumin in contact with vesicles tends to 331 nm, independent of pH and ionic strength and independent of whether the lipid free protein has a higher or a lower emission maximum. As a consequence, the compact conformers of α-lactalbumin tend to an arrangement with the lipid similar to that achieved by the expanded forms. The low

Fig. 1. Fluorescence polarization of diphenylhexatriene in dimyristoylphosphatidylcholine vesicles ($5.3 \cdot 10^{-4}$ M) as a function of temperature at different pH values, in the absence of α-lactalbumin (○), after incubation with α-lactalbumin ($6.9 \cdot 10^{-6}$ M) in the absence of ions (□), with $Ca^{2+}$ ($18 \cdot 10^{-6}$ M) (△) and with NaCl 0.1 M (●). Incubation temperature: 23°C at pH 8, 6 and 4; 28°C at pH 2.

fluorescence yield of the protein in a compact state is explained by energy transfer between tryptophans 28, 109 and 63, and a further quenching by disulfide bridges neighboring the last tryptophan group [18]. The increased fluorescence intensities therefore indicate that the mentioned tryptophan groups move away from each other upon interaction with phospholipid. From the fluorescence red shift it can be deduced that the hydrophobic environment of the tryptophans in the compact protein is partially lost by that expansion. However, the red shift remains limited by the contact of these groups with the apolar lipid phase.

*Study of the changes occurring in the bilayer of the phosphatidylcholine vesicles on interaction with α-lactalbumin under different environmental conditions*

*Temperature scan of the fluorescence polarization.* Scans of the fluorescence polarization of diphenylhextatriene in pure vesicles are compared with those in vesicles incubated with apo-α-lactalbumin and with the $Ca^{2+}$-protein. The scans shift to higher values when α-lactalbumin is added to the vesicles. This is somewhat more pronounced in the presence of the apo-protein than with $Ca^{2+}$-protein. The increase in the polarization is primarily determined by the pH values of the medium. The results of the fluorescence polarization confirm the conclusions from the tryptophan fluorescence: at pH 8 the interaction between the vesicles and α-lactalbumin remains restricted, even for the apoprotein. At pH 6 and 4 a much stronger interaction is observed.

In earlier work without $Ca^{2+}$-control, it was observed that after interaction with α-lactalbumin at pH 4, the vesicles were transformed to typical complex particles, while at neutral pH the vesicles remained intact. From the fluorescence and polarization data as a function of the pH and calcium content, one cannot decide, however, from which pH on the micellar complexes are found. This problem is solved by light scattering.

*Light scattering as a function of the molar ratio.* The break-up of vesicles in small complex particles can be measured by a decrease of the light scattering. The light scattering of similar vesicle suspensions (containing 0.1 mg lipid/ml) at which different amounts of α-lactalbumin are added has been measured. The measurements were carried out after an incubation period of 20 h at 25°C to be certain that the interaction had reached equilibrium.

At pH 8–6 the light scattering does not change over the whole range of the lipid/protein ratios, neither with the $Ca^{2+}$-protein (not shown) nor with the $Ca^{2+}$-free protein (Fig. 2), indicating that the vesicle structure is not influenced by the presence of α-lactalbumin.

At pH 4 and pH 2 the light scattering decreases upon addition of α-lactalbumin to the vesicles (molar ratios 200 to 50): the vesicles are broken up to form miceller complexes with the protein. When enough α-lactalbumin is present to break up all the vesicles to complex particles, further addition of protein no longer influences the light scattering. As a consequence, a plateau is found at low lipid-to-protein molar ratios. Also in that pH range, the curve is identical for the $Ca^{2+}$-protein (not shown) and the $Ca^{2+}$-free protein (Fig. 2).

At pH 5, the light scattering shows a limited vesicle breakdown.

*Influence of the vesicle charge on the complex formation between lipid and α-lactalbumin*

At pH 8, the vesicles were made positively charged by the cosonication of 10% stearylamine with dimyristoylphosphatidylcholine. As a consequence, a good electrostatic interaction can be expected between the positively charged vesicles and the negatively charged α-lactalbumin. The interaction was measured in 5 mM Tris buffer without other ions, in the presence of $Ca^{2+}$, and in presence of 0.1 M NaCl. The incubation occurred at 30°C, the transition temperature of the stearylamine/myristoylphosphatidylcholine mixture. The fluorescence characteristics of the α-lactalbumin were measured at 23°C before and after the interaction, and the results are found in Table II.

A second experiment to control the influence of the electrostatic interaction was performed at pH 6 (5 mM Mes buffer). The vesicles are negatively charged by 10% dimyristoylphosphatidylglycerol to cause an electrostatic repulsion between the lipid and the protein. The fuorescence characteristics are measured after 2 h incubation at 23°C (Table II).

At pH 8 and 10% stearylamine incorporated, the fluorescence yield as well as the emission maxi-

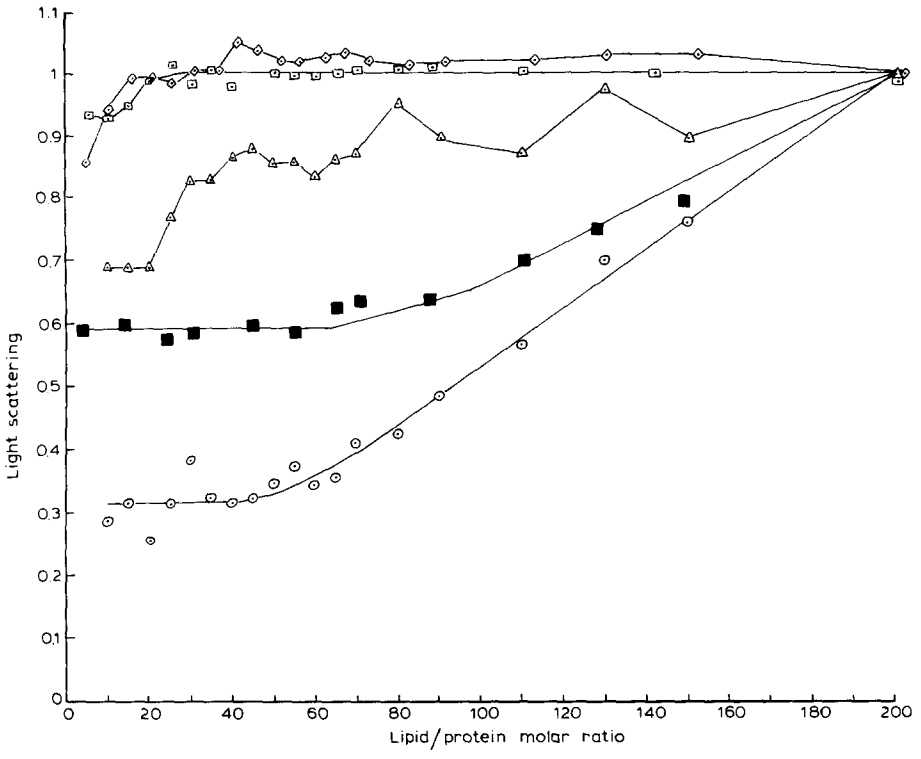



Fig. 2. Light scattering at 23°C of dimyristoylphosphatidylcholine vesicle suspensions after the addition of different amounts of α-lactalbumin, in the absence of $Ca^{2+}$ and NaCl, and at different pH: (◇) pH 8, (□) pH 6, (△) pH 5, (○) pH 4 and (■) pH 2. The final lipid concentration is always 0.1 mg/ml. Before measuring, the mixtures are incubated for 20 h at 25°C. The light scattering of freshly prepared vesicles is taken as unity.

mum of α-lactalbumin tryptophans indicate good contact of the partially unfolded protein with the apolar lipid bilayer. In spite of the good interaction, no lipoprotein-like complexes are formed at pH 8. On the contrary, the vesicle suspension containing stearylamine becomes turbid very quickly after the addition of α-lactalbumin: the vesicles coagulate by α-lactalbumin links. Table II shows that the interaction firmly diminishes in the presence of NaCl. At pH 6 and 10% phosphatidylglycerol (Table IIB) the protein-lipid interaction is weakened compared to the situation described in Table I. These data demonstrate, therefore, that above the isoelectric pH 4.9 the quantum yield increases to a greater extent when the electrostatic interaction is improved and increases to a lesser extent when an electrostatic repulsion between vesicles and protein is expected. Below the isoelectric pH, at pH 4 and at pH 2 the fluores-

TABLE II

COMPARISON OF THE QUANTUM YIELD AND THE EMISSION MAXIMUM OF α-LACTALBUMIN ($1.5 \cdot 10^{-5}$ M)

(a) At pH 8 in the absence and presence of cosonicated vesicles of stearylamine (0.07 mg/ml) and dimyristoylphosphatidylcholine (0.7 mg/ml). [Tris] = 5 mM. (b) At pH 6 in the absence and presence of cosonicated vesicles of dimyristoylphosphatidylglycerol (0.07 mg/ml) and dimyristoylphosphatidylcholine (0.7 mg/ml). [Mes] = 5 mM.

| | | Without vesicles | | With charged vesicles | |
|---|---|---|---|---|---|
| | | $q$ | $\lambda_{max}$ | $q$ | $\lambda_{max}$ |
| (a) | Apo-protein | 0.051 | 337 | 0.070 | 331 |
| | $3.8 \cdot 10^{-5}$ M $Ca^{2+}$ | 0.032 | 325 | 0.065 | 329 |
| | 0.1 M NaCl | 0.036 | 325 | 0.050 | 325 |
| (b) | Apo-protein | 0.043 | 331 | 0.054 | 331 |
| | $3.8 \cdot 10^{-5}$ M $Ca^{2+}$ | 0.030 | 325 | 0.038 | 327 |
| | 0.1 M NaCl | 0.033 | 325 | 0.038 | 329 |

cence characteristics obtained by interaction with the phosphatidylcholine vesicles are quite similar in presence or absence of NaCl (Table I). Also the light scattering of the vesicle suspension decreases upon the formation of protein-lipid complexes and nearly equal light scattering is obtained in the presence or absence of NaCl. Furthermore, at pH 2 the fluorescence characteristics and the fluorescence polarization indicate a good protein-phospholipid interaction, although both are positively charged at that pH.

The study in this paragraph demonstrates that by passing the isoelectric point, the interaction between α-lactalbumin and dimyristoylphosphatidylcholine changes from mainly electrostatic to mainly hydrophobic. The elimination of $Ca^{2+}$ did not change this behavior.

*Circular dichroism measurements*

Below the isoelectric point, α-lactalbumin solubilizes phospholipids by forming micellar complexes. Similar complexes are formed with apolipoproteins. It is believed that the formation of such complexes is enabled by segregration of the protein hydrophobic residues as a result of the formation of an amphipathic helix [1]. Circular dichroic spectra have been taken to look for this property in α-lactalbumin. In Fig. 3, these spectra of α-lactalbumin in different circumstances are given. From the mean residue ellipticity at different wavelengths between 210 nm and 234 nm the helical content is calculated. The pH and $Ca^{2+}$ content cause some differences in the circular dichroic spectra of α-lactalbumin. In the absence of phospholipid, these differences are rather small. Statistically mean helical contents of 25–33% are calculated. In contact with the vesicles, the pH effect on the circular dichroic spectra is large. Especially at pH 4, the fraction of helix was increased considerably. At pH 8 the helicity does not vary by contact with the vesicles; this is true for the $Ca^{2+}$-protein as well as for the $Ca^{2+}$-free protein. A comparison of Table III and Table I demonstrates that at different pH values the presence of vesicles has qualitatively similar effects on the increase in fluorescence yield and on the increase

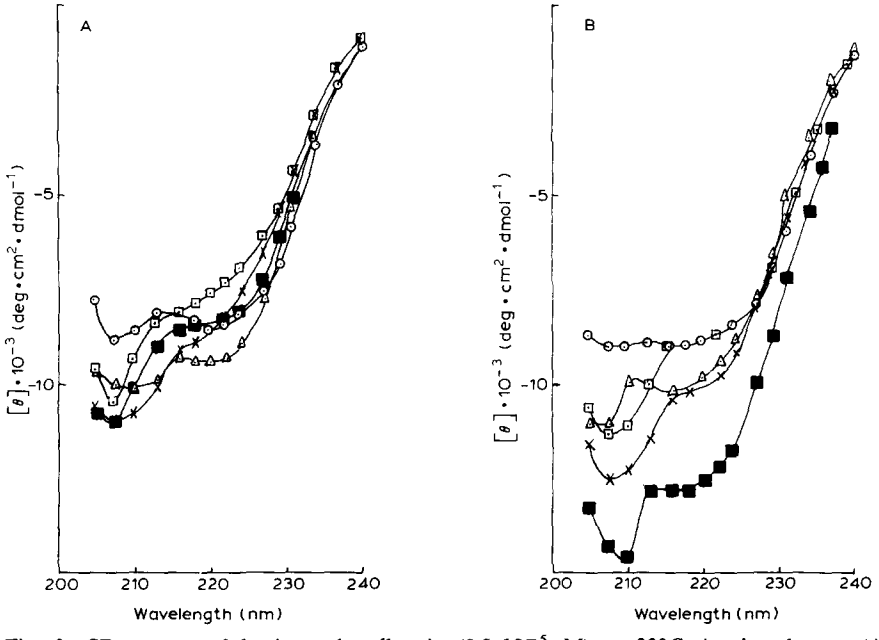



Fig. 3. CD spectra of bovine α-lactalbumin ($2.8 \cdot 10^{-5}$ M) at 23°C, in the absence (A) and in the presence (B) of dimyristoylphosphatidylcholine vesicles $2.1 \cdot 10^{-3}$ M, (○) at pH 8 (5 mM $Na_2HPO_4$-$NaH_2PO_4$) and $5 \cdot 10^{-5}$ M $Ca^{2+}$, (△) at pH 8 and in the absence of $Ca^{2+}$, (□) at pH 6, (■) at pH 4 and (X) pH 2. The solutions at pH 6, pH 4 and pH 2 are not buffered and no $Ca^{2+}$ is present.

TABLE III

HELICAL CONTENT OF BOVINE α-LACTALBUMIN (0.4 mg/ml) IN THE ABSENCE AND PRESENCE OF DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES (1.4 mg/ml)

The helical content is calculated by means of mean residue ellipticity at wavelengths between 210 nm and 234 nm, by the method proposed by Siegel et al. [16].

| | | $f_H$ in absence of vesicles | $f_H$ in contact with vesicles |
|---|---|---|---|
| $Ca^{2+}$-protein | pH 8 | 0.29±0.05 | 0.32±0.05 |
| Apo-protein | pH 8 | 0.33±0.03 | 0.33±0.03 |
| | pH 6 | 0.25±0.03 | 0.33±0.03 |
| | pH 4 | 0.30±0.03 | 0.50±0.03 |
| | pH 2 | 0.28±0.05 | 0.36±0.06 |

in helicity. Therefore a cause and effect relationship between both seems to exist, i.e., because α-lactalbumin forms (amphipathic) helices when it comes in contact with lipid vesicles, some tryptophan groups in the protein move away from each other.

*Influence of a protein denaturants on the lipid-protein interaction*

The influence of a gradual guanidine hydrochloride denaturation on the lipid-protein interaction has been studied at neutral pH and at pH 4. The fluorescence parameters as well as the light scattering (Table IV) indicate guanidine hydrochloride-dependence at both pH values. At pH 7 and 2 M guanidine hydrochloride, the data of Table IV suggest vesicle solubilization by α-lactalbumin. This result has been verified by gel chromatography on a Sepharose 6B column with GdnHCl-NaCl as eluents (not shown). A common lipid-protein peak eluting between the pure vesicles and pure α-lactalbumin confirms that small lipid-protein complex particles are formed.

TABLE IV

TRYPTOPHAN FLUORESCENCE PARAMETERS OF α-LACTALBUMIN IN ABSENCE AND PRESENCE OF DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES AS A FUNCTION OF GUANIDINE HYDROCHLORIDE CONCENTRATION

(a) At pH 7: [protein] = 0.20 mg/ml, phospholipid = 0.70 mg/ml, total salt concentration: [Gdn·HCl]+[NaCl] = 5 M. (b) At pH 4: [protein] = 0.16 mg/ml, phospholipid = 0.52 mg/ml, total salt concentration: [Gdn·Hcl]+[NaCl] = 4 M. $q$ = quantum yield, $L$ = light scattering of lipid suspension in presence of α-lactalbumin/light scattering of vesicles in absence of protein. prec., precipitate.

| | [Gdn·HCl] (M) | Absence of vesicles | | Presence of vesicles | | $L$ |
|---|---|---|---|---|---|---|
| | | $q$ | $\lambda_{max}$ | $q$ | $\lambda_{max}$ | |
| (a) | 0 | 0.038 | 325 | – | – | – |
| | 1 | 0.034 | 325 | 0.060 | 328 | 0.74 |
| | 1.5 | 0.035 | 325 | 0.063 | 331 | 0.73 |
| | 2.00 | 0.040 | 327 | 0.070 | 331 | 0.41 |
| | 2.25 | 0.057 | 333 | 0.071 | 334 | 0.45 |
| | 2.50 | 0.051 | 340 | 0.067 | 334 | 0.66 |
| | 2.75 | 0.056 | 340 | 0.065 | 337 | 1.02 |
| | 3.00 | 0.059 | 340 | 0.064 | 340 | 1.01 |
| | 3.5 | 0.064 | 341 | 0.065 | 341 | 1.03 |
| | 4.0 | 0.063 | 341 | 0.068 | 341 | 1.12 |
| (b) | 0 | prec. | prec. | – | – | – |
| | 1.0 | prec. | prec. | 0.082 | 331 | 0.50 |
| | 1.5 | 0.043 | 336 | 0.082 | 328 | 0.33 |
| | 2.0 | 0.043 | 339 | 0.084 | 331 | 0.27 |
| | 2.25 | 0.050 | 340 | 0.081 | 331 | 0.30 |
| | 2.50 | 0.052 | 340 | 0.082 | 331 | 0.35 |
| | 2.75 | 0.054 | 341 | 0.083 | 332 | 0.35 |
| | 3.0 | 0.054 | 340 | 0.079 | 331 | 0.41 |
| | 3.5 | 0.053 | 3.41 | 0.077 | 334 | 0.54 |
| | 4.0 | 0.057 | 343 | 0.074 | 337,5 | 0.74 |

## Discussion

Above the isoelectric point, the interaction of α-lactalbumin with phosphatidylcholine is mainly electrostatic and the vesicle entity is conserved. Some tryptophan groups move away from each other (decrease of internal energy transfer quenching) and a pH-dependent penetration in the hydrophobic region of the lipid is observed (increased fluorescence polarization of diphenylhextriene, blue-shifted spectrum relative to that of the expanded protein). These effects are strongly enhanced by the incorporation of postively charged stearylamine in the vesicles, since even the $Ca^{2+}$-conformer displays a fluorescence emission which nearly equals that of the expanded apoprotein. This effect of the positive vesicles on the protein resembles the binding effect of $Zn^{2+}$, which also changes the fluorescence spectral parameters of the $Ca^{2+}$-protein to those of the apoprotein [19]. The wavelength of the fluorescence maximum suggests that tryptophan groups of the expanded protein enter in the apolar phase of the lipid bilayer. Thus the protein-lipid interaction illustrates the possibility of α-lactalbumin to involve a hydrophobic region in its binding with other molecules. The interaction with galactosyltransferase has been suggested to be (partly) hydrophobic [20,21] and involves a hydrophobic region on α-lactalbumin containing Trp-118 and His-32 [22,23]. The hydrophobic region with Trp-118 and His-32 can be exposed by a distortion of the C-tail [12]. Just as a strong electrostatic interaction between lipid and protein induces a conformational change by which an apolar site of the protein interacts hydrophobically with the lipid bilayer, so may galactosyltransferase induce a same conformational change on this co-enzyme to improve the hydrophobic interaction. This can explain why $Zn^{2+}$ or $Ca^{2+}$ has no influence on the binding between α-lactalbumin and galactosyltransferase [19].

Below the isoelectric point, the lipid-protein interaction results in the formation of discrete complexes which can be isolated by chromatography on Sepharose. From circular discroism measurements it is obvious that the helical content of α-lactalbumin strongly increases when complex particles are formed. The following regions of bovine α-lactalbumin have been designated as potentially helical regions [24,25]: 5–16, 37–43, 77–89, 91–99, 105–110 and 111–123.

These regions represent 48% of the length of the chain, which is 15–20% more than the helicity estimated from the CD spectra in absence of phospholipid. The formation of an helix possessing a sharply defined topography with one side polar and the other side apolar has been proposed as a basic structural element for lipid-complexing apolipoproteins [1]. This requirement is satisfied for most proteins which solubilize phospholipids [2,4,5,26,27]. The helical wheel projections of the α-lactalbumin segments designated for high helical probability indicate that they are nearly completely amphipathic. In order to quantify the degree of amphipathic character of a helix, the mean helical hydrophobic moment has been defined [28]. Another characteristic of lipid-complexing peptides is that they are rather poorly hydrophilic [2,5,27]. Therefore, the mean hydrophobicity of the amino acids in a helix is also calculated. The hydrophobicity values proposed Janin [29] are used: with these values, a poor hydrophilic character correspond to small negative values.

In Table V the mean helical hydrophobic moments, $\langle \mu_H \rangle$, and mean hydrophobicity, $\langle H \rangle$, of α-lactalbumin segments at neutral pH are tabulated. Between brackets, the values of $\langle \mu_H \rangle$ and $\langle H \rangle$ are calculated at pH 4 assuming that by picking up a proton $H$ of histidine decreases from $-0.1$ to $-1.2$ (the value of the positively charged arginine is $-1.4$) while $H$ of aspartic and glutamic acid increases from $-0.7$ and $-0.6$ to $-0.2$ (the value of threonine). A comparison with the values of potentially amphipathic helices of lipid-complexing protein (Table V) indicates that for a comparable hydrophobicity the potential helical domains of α-lactalbumin are even more amphipathic. As a consequence, at neutral and at acid pH the helices, if formed, will have an amphipathic character favorable to the formation of micellar complexes with the vesicle. The question remains then why, at neutral pH, the interaction of α-lactalbumin with the vesiles does not lead to micellar complexes, but only to an adsorption which is mainly electrostatic in nature, while at acid pH the complexes do form.

In contrast to most enzymatic and synthetic peptides used in lipid-solubilization studies, α-

TABLE V

MEAN HYDROPHOBICITY $\langle H \rangle$ AND MEAN HELICAL HYDROPHOBIC MOMENT $\langle \mu_H \rangle$ OF α-LACTALBUMIN SEGMENTS WITH HELICAL PROPENSITY AND OF POTENTIALLY AMPHIPATHIC HELICES OF LIPID-COMPLEXING PROTEINS

$\langle H \rangle$ and $\langle \mu_H \rangle$ are calculated with hydrophobicity values of Janin [32]. The values between brackets are estimated for acid pH.

| Protein | Segment | $\langle H \rangle$ | $\langle \mu_H \rangle$ |
|---|---|---|---|
| α-Lactalbumin | 7–15 | −0.35(−0.19) | 0.51(0.46) |
| | 77–87 | −0.21(−0.14) | 0.35(0.38) |
| | 91–99 | −0.31(−0.27) | 0.57(0.54) |
| | 105–110 | −0.05(−0.23) | 0.41(0.45) |
| | 111–119 | −0.19(−0.09) | 0.38(0.34) |
| | 38–43 | 0.00(+0.16) | 0.28(0.14) |
| Apolipoprotein A II | 18–30 | −0.42 | 0.41 |
| | 39–47 | −0.92 | 0.46 |
| Melittin | 5–22 | −0.02 | 0.40 |
| Glucagon | 18–29 | −0.13 | 0.28 |
| Salmon calcitonin | 8–22 | −0.24 | 0.35 |
| Apolipoprotein CI | 18–29 | −0.33 | 0.36 |
| | 7–14 | −0.35 | 0.39 |
| | 33–53 | −0.42 | 0.29 |

lactalbumin is a globular protein of which the hydrophobic core is protected by a polar shell. We believe that the different behavior as a function of pH is related to that globular structure of α-lactalbumin which must unfold to some degree in order to form micellar complexes. Above the isoelectric point, the increased interaction with the apolar lipid phase demonstrated that some apolar part of the protein is more accessible in the apoprotein then in the calcified protein. However, the influence of the demetallization on the lipid interaction is small, which indicates that the protein remains mainly globular, even in contact with lipid vesicles. Since it is possible to change the protein conformation by a gradual guanidine hydrocholoride denaturation [30], we could study more thoroughly the influence of the tertiary structure on the lipid interaction. At neutral pH and moderate denaturation by guanidine hydrochloride ([Gdn · HCl] < 2.5 M) the yield and emission maximum of the tryptophan fluorescence indicate a good hydrophobic interaction of the protein and the lipid. Also lipid solubilization appears from the decrease in light scattering of the suspension and from the appearance of the micellar complex in the gel chromatography (result not shown). These results support the hypothesis that a removal of the globular character of the protein is a prerequisite for the formation of lipid-protein complex particles. In analogy, complexes of β-lactoglubulin and phosphatidylcholines are formed by dissolving both in $H_2O/CHCl_3/CH_3OH$, followed by removal of the organic solvent. These complexess, however, are not formed by cosonication of β-lactoglobulin with the lipid [27]. At guanidine hydrochloride concentrations higher than 2.5 M, the secondary protein structure unfolds at pH 7 [30] and the formation of lipid-protein complex micelles is prevented by a lack of helical structures.

At pH 4 the light scattering and fluorescence data indicate good lipid solubilization by protein interaction. Even at 4 M guanidine hydrochloride, the scattering of the vesicle suspension decreases upon addition of α-lactalbumin. This corresponds to the observation that the unfolding of the secondary structure of α-lactalbumin needs a higher concentration of guanidine hydrocholoride in an acid medium (pH < 4.9) than at neutral pH [30,31]: due to local interactions, the parameters for helix

formation of glumatic acid (and aspartic acid) residues are significantly increased at acid pH when the glutamyl (and aspartyl) side-chains are uncharged [32]. At pH 4 and in absence of denaturants, even $Ca^{2+}$-free $\alpha$-lactalbumin keeps a compact form due to electrostatic interactions [11], the helicity of the protein is not influenced markedly (Table III). However, the increased capability for helix formation of uncharged glutamic (and aspartic) acid residues helps to explain the increased capability of the protein for lipid solubilization. It has been demonstrated that the conformational change of $\alpha$-lactalbumin at acid pH can be described in terms of four abnormal carboxyl groups and one abnormal histidine residue. The carboxyl groups change their $pK_a$ value from 3.3 and 3.8 in the compact form to 4.4 in the partially unfolded form, while the $pK_a$ value of the histidine changes from 5.8 to 6.3 [30]. As a consequence, when at pH 4 some electrostatic interactions are loosened in the presence of phospholipid vesicles, the carboxyl groups will neutralize. Upon neutralization, the tendency to create new helical regions helps to destroy the globular conformation of the protein by which interaction of the separated amphipathic helices with the bilayer interface can occur.

## Acknowledgements

This study was supported by a grant of the National Fonds voor Wetenschappelijk Onderzoek. We thank Dr. C. Gielens (Laboratory of Biochemistry at the University of Louvain) for his aid with the CD measurements. The technical assistance of Mrs. G. Dewitte, N. Holvoet and Mr. W. Noppe is gratefully acknowledged.

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