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Biochimica et Biophysica Acta 1330 (1997) 61–70

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Charge-dependent insertion of β-lactoglobulin into monoglyceride monolayers

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Received 16 April 1997; revised 10 June 1997; accepted 12 June 1997

Abstract

The interactions between β-lactoglobulin and 1-monostearoyl-glycerol were studied in order to gain insight into protein–gel-phase monoglyceride interactions. Using a monomolecular layer at the air–water interface, we determined the insertion of β-lactoglobulin into the monoglycerides under different conditions of protein and surface charge by varying the pH and/or incorporating charged amphiphiles into the monolayer, respectively, and using subphases with either a low or high ionic strength. The interactions were quantified by determining the binding of ^{14}C-labeled β-lactoglobulin to the monolayer. Our results show the importance of electrostatics for binding of β-lactoglobulin to condensed monoglycerides. Moreover, electrostatic interactions were found to be important for specific insertion of β-lactoglobulin into the monolayer. A negatively charged surface in particular allowed positively charged β-lactoglobulin to insert in a surface charge density-dependent manner, even at surface pressures as high as 36 mN/m, whereas under other conditions, the limiting insertion pressure was 32 mN/m. The rheological properties of the monolayer were not affected by the interactions with β-lactoglobulin. © 1997 Elsevier Science B.V.

Keywords: Protein-lipid interaction; Condensed monolayer; Monoglyceride; β-Lactoglobulin

1. Introduction

Aqueous dispersions of commonly used long chain saturated monoglycerides are able to form densely packed gel-phase bilayer structures that are separated by aqueous compartments [1–3]. The size of these aqueous compartments can be increased by adding small amounts of charged amphiphiles to the monoglyceride, providing such systems with interesting properties for application in the food industry, in particular in low-fat products [4]. Proteins may be entrapped in the aqueous compartments and could interact with the lipid phase and thus influence the properties of the monoglyceride system. In contrast to the interactions between proteins and other lipid bilayers, i.e. biological membranes, virtually nothing is known about a possible interaction between proteins and gel-phase monoglycerides. Such interactions

Abbreviations: β-LG, β-lactoglobulin; MSG, 1-monostearoyl-*rac*-glycerol; MPG, 1-mono-palmitoyl-*rac*-glycerol; MOG, 1-monooleoyl-*rac*-glycerol; DCP, dicetylphosphate; SA, stearylamine; IEP, isoelectric point; π_i, initial surface pressure; $\Delta\pi$, surface pressure increase; π_l, limiting surface pressure; ϵ, surface elasticity dilatation modulus

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PII S0005-2736(97)00142-9

could involve binding and adsorption of a protein to the interface or even penetration of a protein into the bilayer. However, it is generally difficult for proteins to penetrate into gel-phase phospholipid bilayers. On the other hand, the nature and extent of any given lipid–protein interaction may not be entirely determined by the packing density of the lipids; the type of interface presented by the lipids is also an important parameter. Therefore, studying monoglyceride–protein interactions also offers many interesting opportunities from the point of view of understanding biomembrane structure and function, because a monoglyceride ''membrane'' presents a stable interface containing only free hydroxyls as polar groups to a protein. In this respect, it is intriguing that monoglyceride cubic phases allowed the crystallization of membrane proteins [5].

In the present study, we investigated, using the monolayer technique, the interaction between a globular protein and one layer of a gel-phase monoglyceride bilayer. For this purpose, the most abundant whey protein, β-lactoglobulin (β-LG), and a gel-phase-forming monoglyceride, 1-monostearoylglycerol (MSG), were used. The interactions between β-LG and MSG were characterized by determining the binding and insertion of β-LG into the monoglyceride monolayer and the effect of β-LG on the rheology of the monolayer. Bovine β-LG was used because it is a well-characterized globular protein of which two major, β-LG A and B, and several minor genetic variants are found in bovine milk. The protein consists of 162 amino acids and has a molecular weight of 18 kDa and an isoelectric point (IEP) of 5.2 [6]. The structure consists of an eight-stranded antiparallel β-barrel and one N-terminal α-helix, which is followed by a ninth β-strand. All of these structural elements are connected by large loops of random coil, which account for about 50% of the structure. The protein also contains one disulfide bridge and one free thiol group [7,8].

Since monoglycerides are uncharged, it can be envisioned that the addition of a surface charge will also affect the interaction between the monoglycerides and a protein. It has been shown that a surface charge can be an important factor for the interaction of peptides and proteins with membranes [9–11], including the interaction of β-LG with phospholipid monolayers [12]. Therefore, we included in our studies the effect of incorporating small amounts of dicetylphosphate (DCP) or stearylamine (SA) in the monolayer, to give a negatively or positively charged surface, respectively. The results show the importance of electrostatics for the interaction between β-LG and the monoglycerides, and, moreover, that the protein is, under specific conditions, able to penetrate into a densely packed MSG monolayer.

2. Materials and methods

2.1. Materials

Bovine β-lactoglobulin (a mixture of genetic variants A and B), 1-monostearoyl-*rac*-glycerol, 1-monopalmitoyl-*rac*-glycerol and 1-monooleoyl-*rac*-glycerol, DCP and SA were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. Ultra-pure bovine β-lactoglobulin (genetic variant A) was a generous gift from Dr. C.G. de Kruif (NIZO, the Netherlands). Tris was obtained from Baker (Deventer, the Netherlands), sodium acetate, KH_2PO_4 and NaCl were from Merck (Darmstadt, Germany), fast-flow Q-Sepharose was from Pharmacia (Uppsala, Sweden), [^{14}C]formaldehyde (58 μCi/μmol) was from Dupont NEN (Mechelen, Belgium) and $NaBH_3CN$ was obtained from Across (Geel, Belgium).

2.2. Modification of β-LG

A ^{14}C label was introduced into β-LG by reductive methylation with [^{14}C]formaldehyde and $NaBH_3CN$ [13]. β-LG was dissolved to a final concentration of 54 μM in a 0.2-M KH_2PO_4 buffer at pH 7.0. [^{14}C]Formaldehyde and $NaBH_3CN$ were added to give final concentrations of 0.16 and 1.6 mM, respectively. The mixture was then incubated overnight at room temperature. After the reaction, the incubation mixture was loaded on a 0.5-ml fast-flow Q-Sepharose column that had been equilibrated with 20 mM Tris, pH 7.0. After washing the column with 5.0 ml of 20 mM Tris, pH 7.0, β-LG was eluted from the column with 1.0 ml of 500 mM NaCl, 20 mM Tris, pH 7.0. The resulting β-LG had a specific radioactivity of 105 000 dpm/nmol, corresponding to a stoichiometry of the reaction of 0.8 mole label/mole protein.

β-LG was heat-denatured under conditions reported to induce the formation of large covalent aggregates [14,15]. To this end, 15 mg of β-LG dissolved in 1 ml of 20 mM Tris, 10 mM NaCl, pH 7.0, were incubated overnight at 65°C.

2.3. Monolayer experiments

Surface pressures were measured by the Wilhelmy plate method in Teflon troughs at 22°C, using a paper (π–A curves) or platinum plate [16]. Buffers used were: 20 mM sodium acetate (pH 4.0 and pH 5.2) and 20 mM Tris (pH 7.0), with or without 100 mM NaCl. Appropriate amounts of lipid stock solutions, dissolved in $CHCl_3$–MeOH (3:1, v/v), were spread on the subphase. Unless stated otherwise, a 9:1 monoglyceride-charged amphiphile molar ratio was used for experiments requiring a charged monolayer.

Surface pressure–area (π–A) curves were measured using a 0.8-l trough ($32.2 \times 17.3 \times 1.5$ cm) equipped with a moveable barrier. The films were compressed at a rate of 80 cm^2/min.

The insertion of β-LG into monoglyceride monolayers was studied using a 6-ml Teflon dish with a surface area of 8.8 cm^2. The whole experimental set-up was placed in a thermostated box. The subphase was continuously stirred with a magnetic bar. Initial surface pressures ranged from 25 to 36 mN/m. A 10-μl volume of a β-LG stock solution (0.82 mM) was injected under the monolayer through a separate hole in the Teflon dish, giving a final β-LG concentration of 1.4 μM. The addition of more β-LG did not result in larger increases in the surface pressure. The error in these experiments was 0.2 mN/m.

Binding experiments were performed using a 20-ml trough ($5.5 \times 6 \times 0.6$ cm) that was connected to two reservoirs via a circulation pump. Initial surface pressures were 26 or 32 mN/m. A 50-μl volume of a [^{14}C]β-LG stock solution (26.6 μM) was injected under the monolayer, giving a final β-LG concentration of 0.07 μM. This lower β-LG concentration gave similar surface pressure increases as those observed using 1.4 μM β-LG, as used for the insertion experiments. The surface radioactivity was determined with a Berthold LB 203E gas flow counter. After the surface tension and radioactivity of the monolayer became stable, the subphase was washed with 100 ml of the appropriate buffer by flushing at a flow-rate of 6 ml/min. The monolayer was subsequently collected in a scintillation vial by aspiration through a glass capillary while manually decreasing the surface area with a barrier. The radioactivity of the samples was determined with a Packard TRI-CARB 1500 scintillation counter (Downers Grove, IL, USA), and the result was corrected for the value measured in an equal volume of subphase.

The surface dilatation elasticity was measured as described by Paternotte et al. [17] using a 1.2-l trough ($45.0 \times 15.2 \times 1.5$ cm) equipped with a moveable barrier. A 1.0-ml volume of a β-LG stock solution (1.4 mM) was injected under the monolayer, giving a final β-LG concentration of 1.2 μM in the subphase. The initial surface pressure was 20 mN/m and final surface pressures ranged from 38 to 47 mN/m. Measurements were performed at several surface pressures by compressing the monolayer in a stepwise manner. After each compression step, the dilatation elasticity was determined by oscillating the barrier sinusoidally at a frequency of 0.15 Hz and at an amplitude of 4% of the mean surface area.

3. Results

In order to characterize the state of the monolayer, the surface pressure–area (π–A) curve of 1-monostearoyl-glycerol (MSG) was determined and compared to those of: 1-monopalmitoyl-glycerol (MPG) and 1-monooleoyl-glycerol (MOG) (Fig. 1). The π–A curve of MOG is typical for lipids in the expanded-

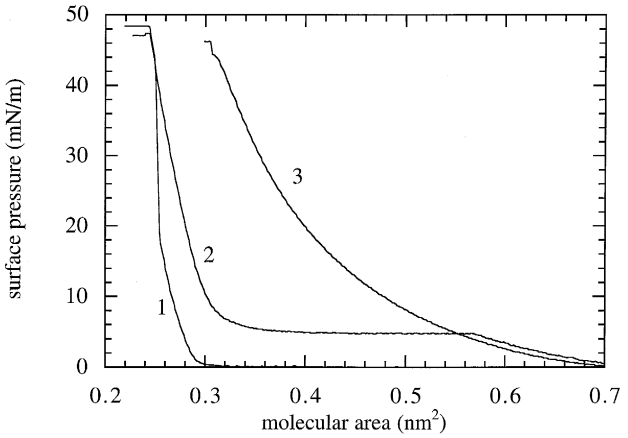

Fig. 1. Surface pressure–area curves for: 1-monostearoyl-glycerol (1), 1-monopalmitoyl-glycerol (2) and 1-monooleoyl-glycerol (3) at 22°C on water.

phase. The relatively large molecular area of 0.312 nm^2 at the collapse pressure can be attributed to the *cis*Δ9 double bond. MPG shows a phase transition from the expanded- to the condensed-phase at a surface pressure of 5 mN/m and a molecular area of 0.56 nm^2. In the expanded-phase, i.e. below 5 mN/m, the molecular area of MPG is comparable to that of MOG. The π–A curve of MSG is characteristic for a lipid in the condensed-phase with little compressibility, a phase similar to the gel-phase of lamellar systems, and its limiting molecular area of 0.254 nm^2 is significantly higher than those found for the single chain surfactants, stearic acid and stearyl alcohol [18]. This higher value of MSG could result from a tilted orientation of the acyl chain, as found in liquid crystals [1,19,20], or it could be attributed to the presence of the larger polar headgroup. The collapse pressure of 45 mN/m is remarkably high and comparable to that of phosphatidylcholines [16]. Because the aim of this study was to investigate the interaction between a protein and gel-phase, i.e. condensed monoglycerides, MSG was used for all further experiments. Furthermore, MSG forms very stable monolayers, whereas MPG shows a decline at higher surface pressures, as was also found by de la Fuente Feria and Rodriguez Patino [21].

In the absence of monoglycerides, the surface activity of β-LG results in a protein monolayer with a surface pressure of 22.8 mN/m, at pH 7 (Fig. 2) and pH 4 (not shown). The presence of 100 mM NaCl in the subphase increases the surface activity of β-LG at pH 7 to 27.4 mN/m (Fig. 2), indicating an increased hydrophobicity of the protein, while no significant effect was observed at pH 4 (not shown). Because of the surface activity of the protein, all insertion and binding experiments were performed using monolayers with initial surface pressures (π_i) of 25 mN/m or higher.

Injection of β-LG under a MSG monolayer leads to a protein-induced surface pressure increase ($\Delta\pi$) of the monolayer, the extent of which depends on the lipid composition of the monolayer, and on the pH and ionic strength of the subphase. The β-LG-induced $\Delta\pi$ as a function of the initial surface pressure of a neutral MSG monolayer (π_i–$\Delta\pi$) under various conditions is shown in Fig. 3. Extrapolation to high initial surface pressures provides the limiting inser-

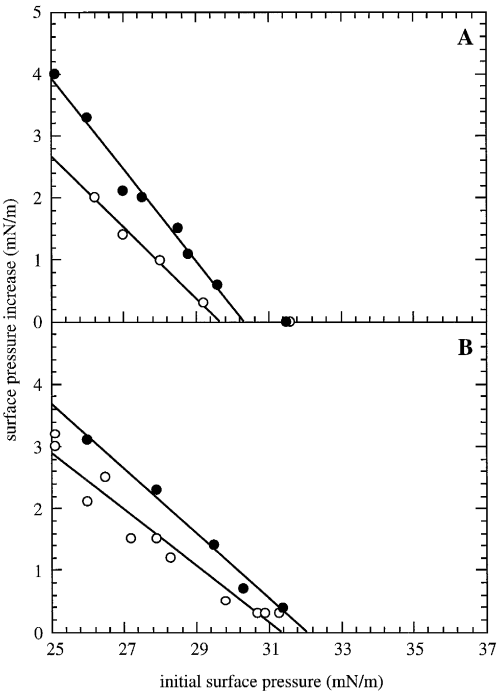

Fig. 3. β-LG-induced surface pressure increase as a function of the initial surface pressure of neutral MSG monolayers. The buffers used were 20 mM Tris, pH 7 (panel A) or 20 mM sodium acetate, pH 4 (panel B), either with (closed symbols) or without (open symbols) 100 mM NaCl. The β-LG concentration in the subphase was 1.4 μM.

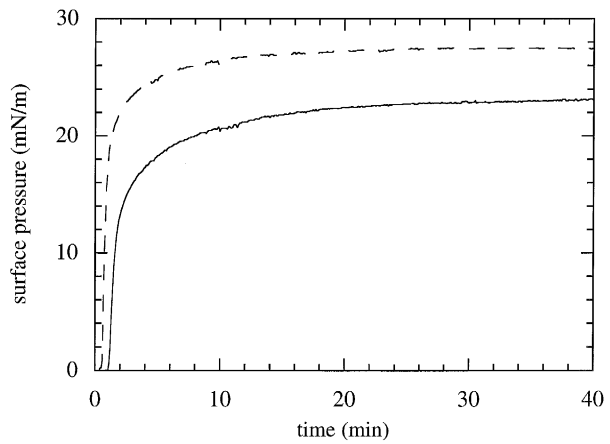

Fig. 2. Time dependence of the β-LG-induced surface pressure increase in the absence (solid line) or presence (dotted line) of 100 mM NaCl. The buffer used was 20 mM Tris, pH 7. At t = 0, β-LG was injected into the subphase to give a final concentration of 1.4 μM.

tion pressure (π_l) at which the protein is no longer able to insert into the monolayer. At pH 7, the π_l for β-LG insertion into a neutral MSG monolayer is 30 mN/m (Fig. 3A), while at pH 4, this was found to be 32 mN/m (Fig. 3B). Similar results were obtained at pH 5.2, the IEP of β-LG (not shown). Addition of 100 mM NaCl to the subphase has a stimulating effect on β-LG insertion, at pH values of 7 and 4.

The addition of charged amphiphiles to the MSG monolayer affects the interaction between the monolayer and β-LG. Fig. 4 shows the effect of incorporating small amounts of the anionic amphiphile, DCP, into a MSG monolayer with a π_i of 25 mN/m at pH 4, i.e. with β-LG and the monolayer having opposite charges. A non-linear relationship was observed. Initially, at DCP concentrations up to 5 mole%, no additional effect is observed. Above 5 mole% DCP, however, larger surface pressure increases were induced by β-LG, an effect which leveled off at 10 mole% DCP. No reliable data could be obtained above 10 mole% as a result of the monolayer becoming less stable. The increased insertion observed in the 5–10 mole% DCP range is specific for this set of conditions. Larger β-LG-induced $\Delta\pi$s were not observed at pH 7, where β-LG and the monolayer are both negatively charged. Moreover, larger $\Delta\pi$s were also not observed when the charges of β-LG and the monolayer were reversed by incorporating SA into the monolayer and performing the experiment at pH 7. Based on these observations, all further experiments with charged monolayers were performed using a charged amphiphile concentration of 10 mole%.

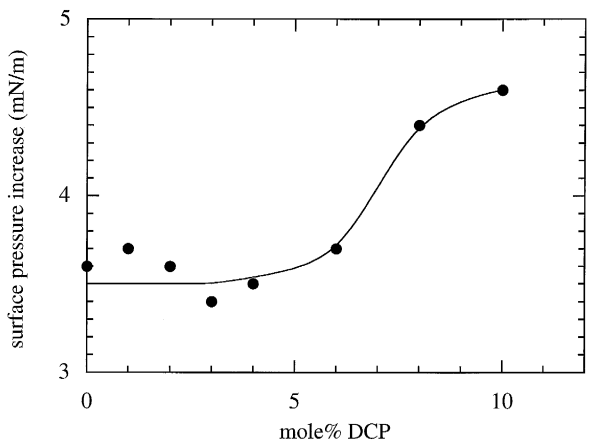

Fig. 4. β-LG-induced surface pressure increase as a function of the mole percentage of DCP in a MSG monolayer. The initial surface pressure was 25 mN/m. The buffer used was 20 mM sodium acetate, pH 4. The β-LG concentration in the subphase was 1.4 μM.

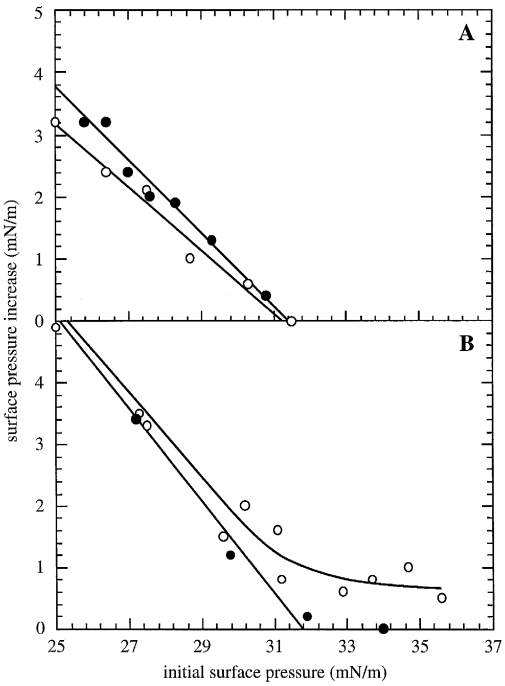

Fig. 5. β-LG-induced surface pressure increase as function of the initial surface pressure of negatively charged MSG/DCP monolayers. The concentration of DCP in the monolayer was 10 mole%. The buffers used were 20 mM Tris, pH 7 (panel A) or 20 mM sodium acetate, pH 4 (panel B), either with (closed symbols) or without (open symbols) 100 mM NaCl. The β-LG concentration in the subphase was 1.4 μM.

Incorporation of 10 mole% DCP into a MSG monolayer had little effect on the π_i–$\Delta\pi$ curve at pH 7 (Fig. 5A). Both in the presence and absence of 100 mM NaCl, the π–$\Delta\pi$ curves are similar to those observed using neutral MSG monolayers, and a comparable π_l of 32 mN/m was found in both cases. However, at pH 4, with β-LG and the surface having opposite charges, another interaction is observed and, as a result, β-LG insertion is increased (Fig. 5B). Consequently, the β-LG-induced $\Delta\pi$s are larger (cf. Fig. 4) and no clear π_l seems to be reached, distinct β-LG-induced $\Delta\pi$s are also observed above 32 mN/m, at surface pressures as high as 36 mN/m. In the presence of 100 mM NaCl, no β-LG-induced $\Delta\pi$s were observed above a π_i of 32 mN/m, but the $\Delta\pi$s observed at surface pressures below 32 mN/m are still larger than those observed using neutral

monolayers. Interestingly, heat denaturation did not abolish the ability of β-LG to insert at high initial surface pressures and a curve similar to that observed with the native protein was obtained (not shown). Furthermore, using an ultra-pure sample of β-LG A also resulted in a curve similar to that observed with the mixture of β-LG A and B obtained from Sigma, ruling out the possibility of contamination effects.

No large effects on the β-LG-induced $\Delta\pi$s were observed when 10 mole% of the positively charged amphiphile, SA, were incorporated into MSG monolayer (Fig. 6). Also, no stimulating effect of a higher ionic strength was observed at pH 7, while at pH 4, where both β-LG and the monolayer are positively charged, there is even a small negative effect on the insertion of β-LG into the MSG/SA monolayer, possibly as a result of charge shielding.

In order to quantify the binding of β-LG to the MSG monolayers, ^{14}C-labeled β-LG was used. In Fig. 7, two typical experimental curves are depicted.

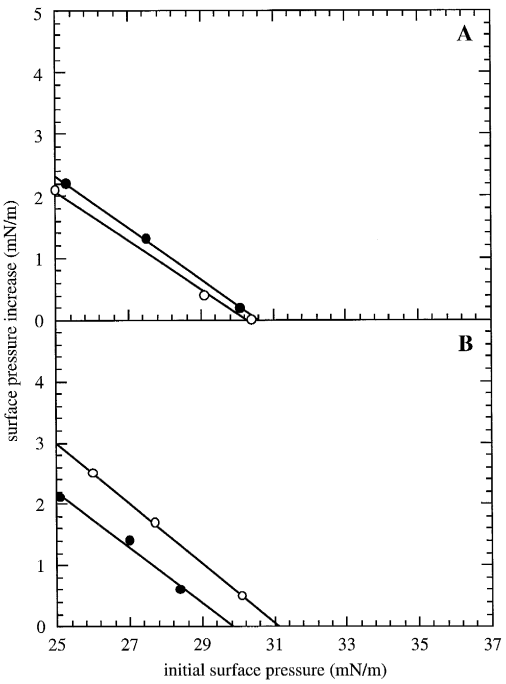

Fig. 6. β-LG-induced surface pressure increase as a function of the initial surface pressure of positively charged MSG/SA monolayers. The concentration of SA in the monolayer was 10 mole%. The buffers used were 20 mM Tris, pH 7 (panel A) or 20 mM sodium acetate, pH 4 (panel B), either with (closed symbols) or without (open symbols) 100 mM NaCl. The β-LG concentration in the subphase was 1.4 μM.

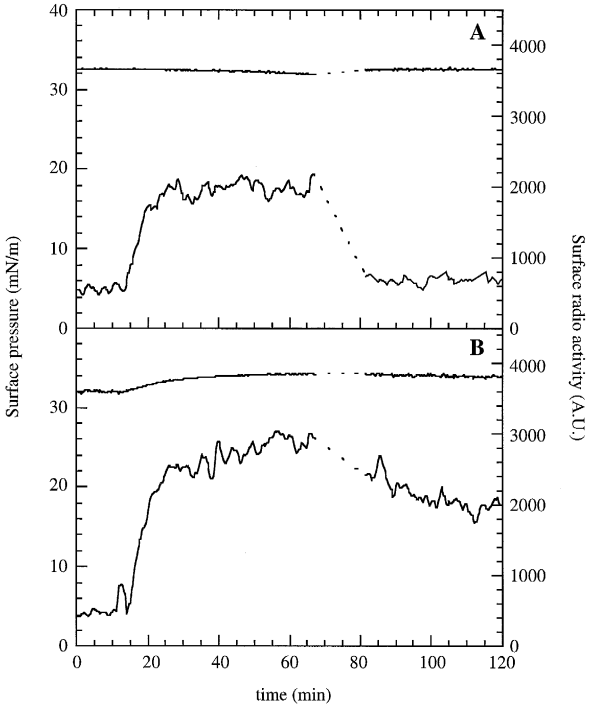

Fig. 7. Time dependence of β-LG binding (surface radioactivity) and insertion (surface pressure increase) into a MSG/SA monolayer at pH 7 (panel A) or a MSG/DCP monolayer at pH 4 (panel B). SA and DCP concentrations were 10 mole%. At t = 15 min, [^{14}C]β-LG was injected to give a final concentration of 0.07 μM in the subphase. Between t = 65 and t = 80 min, the subphase was washed with 100 ml of buffer (dotted line). The buffers used were 20 mM Tris, pH 7 or 20 mM sodium acetate, pH 4.

Although no insertion of β-LG into a MSG/SA monolayer with a π_i of 32 mN/m is observed at pH 7 (cf. Fig. 6), it is clear that β-LG binds to such a monolayer, as revealed by the increased surface radioactivity (Fig. 7A). However, most of the bound protein is removed from the monolayer upon washing, indicating a loose association. The interaction between β-LG and a MSG/DCP monolayer at 32 mN/m and pH 4 leads to insertion of the protein and a concomitant increase in the surface radioactivity (Fig. 7B). However, washing now results in only a partial loss of the β-LG bound to the surface. The role of electrostatics in the binding of β-LG to MSG monolayers is apparent from Fig. 8. When using monolayers containing a surface charge that is opposite to that of β-LG, optimal binding was observed with a low ionic strength in the subphase. A high

ionic strength in the subphase prevented the binding of β-LG to both positively and negatively charged MSG monolayers. Furthermore, a high ionic strength in the washing buffer resulted under all conditions in the removal of the β-LG bound to the monolayer. In contrast, a washing buffer with a low ionic strength did not completely remove the β-LG bound to MSG/DCP (cf. Fig. 7). Only small amounts of β-LG were found to interact with neutral MSG monolayers, even under conditions, i.e. at a π_i of 26 mN/m, where the protein does insert (cf. Fig. 3).

To gain further insight into the β-LG–MSG monolayer interaction, the effect of β-LG on the rheological properties of the monolayer were studied. In Fig. 9, the surface dilatation elasticity modulus (ϵ) of different MSG monolayers in the absence or presence of β-LG are shown as function of the surface pressure. The ϵ of the neutral MSG monolayer is comparable to that of a distearoylphosphatidylcholine monolayer [16]. Clear differences are indeed observed between the various MSG monolayers in the absence of β-LG, reflecting the sensitivity of this technique for small variations in the lipid composition of the monolayer. The presence of SA in the MSG monolayer causes a small decrease in ϵ at higher surface pressures, while ϵ is reduced at all surface pressures when DCP is added to the monolayer. However, the presence of β-LG had no detectable effect on ϵ under all of the conditions tested, indicating that the rheological properties are primarily determined by the lipid layer itself and less so by the adsorbed protein.

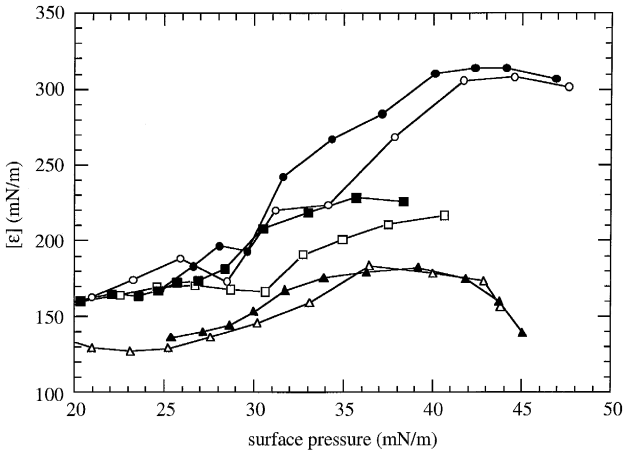

Fig. 9. Surface dilatation elasticity modulus (ϵ) of neutral MSG (circles), positively charged MSG/SA (squares) and negatively charged MSG/DCP (triangles) monolayers as a function of the surface pressure, either in the absence (open symbols) or presence (closed symbols) of β-LG. DCP and SA concentrations were 10 mole%. The buffers used were 20 mM Tris, pH 7 (positively charged monolayer) or 20 mM sodium acetate, pH 4 (neutral and negatively charged monolayers). The β-LG concentration in the subphase was 1.2 μM.

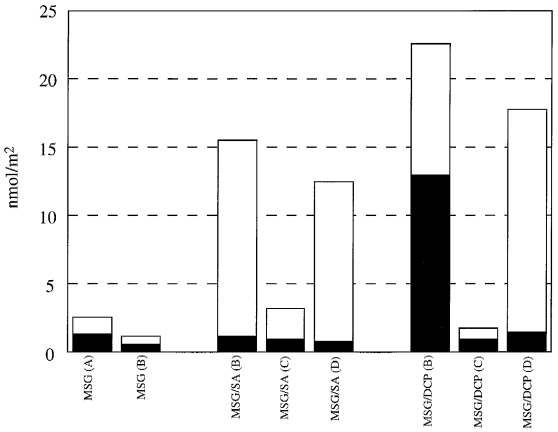

Fig. 8. Surface binding of [^{14}C]β-LG, before (open + closed bar) and after (closed bar) washing of the subphase, to MSG, MSG/SA and MSG/DCP monolayers at pH values of 5.2, 7 and 4, respectively. Conditions: (A) $\pi_i = 26$ mN/m, (B) $\pi_i = 32$ mN/m, (C) $\pi_i = 32$ mN/m with 100 mM NaCl added to the subphase and (D) $\pi_i = 32$ mN/m with 100 mM NaCl added to the washing buffer. The buffers used were 20 mM sodium acetate (pH 4), 20 mM sodium acetate (pH 5.2) and 20 mM Tris (pH 7). SA and DCP concentrations were 10 mole%. The [^{14}C]β-LG concentration in the subphase was 0.07 μM. β-LG binding was determined by measuring the surface radioactivity before and after washing and by measuring the radioactivity of the monolayers collected after washing of the subphase.

4. Discussion

It is clear from our results that β-LG is able to interact with condensed monoglyceride monolayers under various conditions. When using neutral MSG monolayers, the protein is able to insert into the monolayer at initial surface pressures of up to 32 mN/m. The hydrophobic nature of this interaction follows from the stimulating effect of the increased ionic strength of the subphase, in particular, at pH 4, where the hydrophobicity of the protein itself is unaffected. The interaction therefore probably in-

volves some of the hydrophobic patches on the surface of β-LG, one of which could be the putative binding site for retinol and other hydrophobic compounds [8]. The pH of the subphase, on the other hand, had no effect on the insertion of β-LG into a neutral MSG monolayer, indicating that, in contrast to a protein monolayer, the conformational change of β-LG, which occurs between pH values of 4 and 6 [22,23], does not affect its interaction with a neutral MSG monolayer.

No reduction of the β-LG-induced surface pressure increase was observed when a surface charge of the same sign as that of β-LG was introduced into the monolayer, meaning that an electrostatic repulsion by the monolayer does not affect the insertion of β-LG, further pointing to the importance of hydrophobic contacts for its insertion. The importance and effects of electrostatics were revealed by the interaction of β-LG with a monolayer that was oppositely charged. When injected under a positively charged MSG/SA monolayer, the binding of β-LG to the monolayer was found to be increased at pH 7. However, the insertion of β-LG into the monolayer was comparable to that observed with neutral monolayers, π_1 not exceeding 31 mN/m, and the β-LG bound to a MSG/SA monolayer could be largely removed by washing with a low ionic strength buffer. Both of these observations indicate that the electrostatic interaction between β-LG and a positively charged monolayer is relatively weak. Interestingly, the reduced insertion of β-LG observed below 31 mN/m at pH 4 in the presence of a high ionic strength, i.e. as a result of charge shielding, indicates that this electrostatic interaction can still stimulate the insertion of β-LG into a MSG/SA monolayer, albeit weakly. In the reversed situation, with β-LG being positively- and the monolayer negatively charged, a stronger interaction was observed. Firstly, because, at pH 4, β-LG is able to insert into negatively charged MSG/DCP monolayers at initial surface pressures far above 32 mN/m. Secondly, only a fraction of β-LG was removed from the monolayer after washing. The importance of electrostatics for this stronger interaction between β-LG and a MSG/DCP monolayer follows from the results obtained in the presence of 100 mM NaCl. A high ionic strength first of all effectively prevented, or abolished, the binding of β-LG to the monolayer and, furthermore, abolished the ability of β-LG to insert at initial surface pressures higher than 32 mN/m.

From the amounts of protein associated with a monolayer, it is possible to estimate the average area occupied by a protein in the monolayer, assuming that the molecular areas of the components of a monolayer are additive and knowing the area occupied by the lipids as a function of surface pressure [24,25]. The observed amounts of β-LG bound to a MSG/DCP monolayer at pH 4 mean that, when taking into account the dimensions of β-LG (molecular area ≈ 21 nm^2 [7,8]), the protein covers 30 or 18% of the total available area before and after washing of the subphase, respectively. These amounts of β-LG induce a surface pressure increase of about 1 mN/m in a monolayer with an initial surface pressure of 32 mN/m. Inspection of the π–A curve of the monolayer shows that, under similar conditions, the area occupied by the lipids decreases by 0.06% and that only 0.07 nm^2, i.e. less then the area of a methyl group, is available per bound β-LG molecule for insertion. From this, we conclude that only a fraction of the β-LG bound to the MSG/DCP monolayer inserts and that, consequently, two populations of β-LG are present at the MSG/DCP surface, one inserted and one adsorbed. It is as yet unclear what the exact nature of the two different β-LG populations at the MSG/DCP monolayer is. It is, however, highly unlikely that this behavior of β-LG is due to the presence of minor contaminations in the protein sample, because similar results were obtained using an ultra pure sample prepared at the NIZO. The conclusion that only a fraction of the bound β-LG inserts into the monolayer is supported by the finding that only very small amounts of β-LG were bound to a neutral MSG monolayer under conditions, i.e. at a π_i of 26 mN/m, where a $\Delta\pi$ was observed that is higher than that observed with a MSG/DCP monolayer, of 32 mN/m at pH 4. Furthermore, no effects were observed on the surface dilatation elasticity, which can be expected when large amounts of protein insert into a lipid monolayer [17].

The difference in insertion of β-LG bound to either a MSG/SA monolayer at pH 7 or a MSG/DCP monolayer at pH 4 probably does not find its origin in simple electrostatics because the relationship between surface charge and β-LG-induced $\Delta\pi$s was either not observed (pH 7) or not linear (pH 4). Also,

the increased insertion of β-LG into a MSG/DCP monolayer below 32 mN/m at pH 4 was unaffected by a high ionic strength; a high ionic strength only affected the insertion above 32 mN/m. The observed difference could result from structural differences of β-LG bound to the surface. These structural differences are, however, not likely to be those found in solution, because no differences between pH 4 and pH 7 were observed with neutral monolayers and heat-denaturation of the protein also had no effect. We therefore propose that the difference results from an anionic amphiphile-induced structural change in β-LG. This structural change may not be very dramatic but is probably large enough to allow additional hydrophobic parts of the protein to insert into the densely packed monolayer. That anionic amphiphiles are able to affect the structure of a protein is not unlikely — SDS being a well known example, also for β-LG [26,27] — while cationic amphiphiles are normally less destructive, thus explaining the absence of an increased insertion of β-LG into a positively charged monolayer.

The different types of interactions observed at high surface pressures are schematically summarized in Fig. 10. Only weak interactions are found in the absence of charge differences between β-LG and the monolayer and the protein will thus be mainly present in solution (Fig. 10A). Above its IEP, i.e. when negatively charged, β-LG will bind to a positively charged surface. Binding in this case does not, however, result in an increased insertion of β-LG into the monolayer (Fig. 10B). Below its IEP, i.e. when positively charged, β-LG will bind strongly to a negatively charged monolayer and will probably undergo a structural change. As a result, parts of the protein will also insert into the monolayer (Fig. 10C).

In conclusion, the results presented in this study show the importance of a negatively charged surface for the insertion of β-LG into condensed-phase monoglyceride monolayers, which compares well with the reported importance of a negative charge for the interaction of β-LG with phospholipid monolayers [12]. This increase in understanding protein–monoglyceride interactions will not only prove valuable for industrial use, but, moreover, also for the application of monoglycerides in studies on biological systems, as so elegantly demonstrated by Landau and Rosenbusch [5] who recently used monoglyceride cubic phases for the crystallization of membrane proteins.

Acknowledgements

This work was supported by the IOP-industrial proteins project IIE95001a. The authors would like to thank I. Heertje, F.A.M. Kleinherenbrink, A. Sein, G. Wijngaards, L.G.J. Verhoeven and B. Klarenbeek for fruitful discussions. The authors also would like to thank M. Kooijman for help with the surface dilatation elasticity measurements.

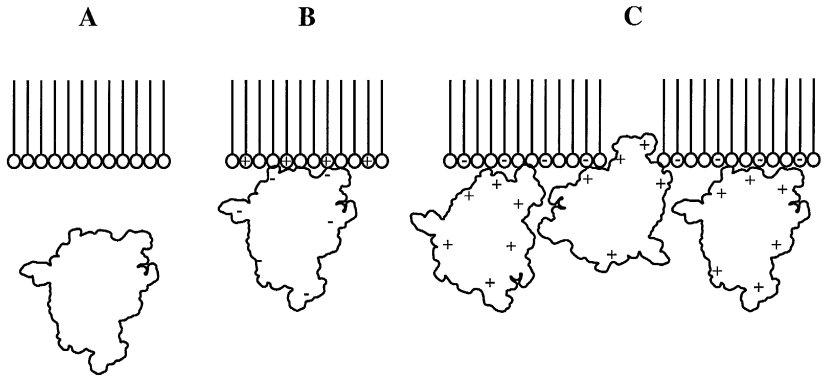

Fig. 10. Schematic representation of the observed interactions between β-LG and different MSG monolayers. β-LG does not bind to neutral monolayers (A). When negatively charged, β-LG is able to bind to positively charged monolayers. Binding does not, however, result in insertion (B). When positively charged, β-LG not only binds strongly to negatively charged monolayers but a fraction also inserts into the monolayer (C).

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