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Interaction mode specific reorganization of gel phase monoglyceride bilayers by β-lactoglobulin

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Abstract

The interaction between β-lactoglobulin and sonicated aqueous dispersions of the gel phase forming monoglyceride monostearoylglycerol were studied using isothermal titration calorimetry, direct binding experiments, differential scanning calorimetry, leakage of a fluorescent dye and solid-state ^{31}P- and ^{2}H-NMR. In the absence of a charged amphiphile, monostearoylglycerol forms a precipitate. Under these conditions, no interaction with β-lactoglobulin was observed. In the presence of the negatively charged amphiphile dicetylphosphate, the gel phase monostearoylglycerol formed stable and closed, probably unilamellar, vesicles with an average diameter of 465 nm. β-Lactoglobulin interacts with these bilayer structures at pH 4, where the protein is positively charged, as well as at pH 7 where the protein is negatively charged. Under both conditions of pH, the binding affinity of β-lactoglobulin is in the micromolar range as observed with ITC and the direct binding assay. At pH 4, two binding modes were found, one of which is determined with ITC while the direct binding assay determines the net result of both. The first binding mode is observed with ITC and is characterized by a large binding enthalpy, a decreased enthalpy of the MSG L_β to L_α phase transition and leakage of a fluorescent dye. These characteristics are explained by a β-lactoglobulin induced partial L_β to coagel phase transition that results from a specific electrostatic interaction between the protein and the charged amphiphile. This explanation is confirmed by solid-state ^{2}H-NMR using 1-monostearoylglycerol with a fully deuterated acyl chain. Upon interaction with β-lactoglobulin, the isotropic signal in the ^{2}H-NMR spectrum of the monostearoylglycerol–dicetylphosphate mixture partially transforms into a broad anisotropic signal which could be assigned to coagel formation. The second binding mode probably results from an aspecific electrostatic attraction between the negatively charged bilayer and the positively charged protein and causes the precipitation of the dispersion. At pH 7, only the first binding mode is observed.

Keywords: Protein–lipid interaction; Isothermal titration calorimetry; Monoglyceride; β-Lactoglobulin

1. Introduction

Monoglycerides are becoming increasingly important for the food industry, in particular in low-fat products [1]. Besides, for this and other industrial applications, the potential of monoglycerides for biochemical research is also becoming more apparent. Their ability to form bilayers, like natural mem-

Abbreviations: β-LG, β-lactoglobulin; MSG, 1-monostearoyl-*rac*-glycerol; $^{2}H_{35}$-MSG, 1-mono-[$^{2}H_{35}$]-stearoyl-*rac*-glycerol; DCP, dicetylphosphate; TR, Texas red; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; IEP, isoelectric point

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branes, as well as non-bilayer structures may offer many interesting opportunities for studies on membrane structure and function [2–5]. For instance, the successful use of a monoglyceride cubic phase for the crystallization of membrane proteins underscores their large potential in this field [6].

Understanding the interaction with proteins is important for a technological application of monoglycerides as well as for biochemical studies. However, in contrast to the interaction of proteins with natural phospholipids, not much is known about their interaction with monoglycerides. We therefore set out to gain insight into the interactions between proteins and monoglycerides. To this end we used β-lactoglobulin (β-LG) and 1-monostearoylglycerol (MSG). Bovine β-LG was used because it is a well characterized globular protein and the main whey protein of 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 [7]. The gel phase forming MSG was used because the rigid bilayers of the gel phase are of particular interest since such systems allow the structuring of large amounts of water.

In a previous paper, we reported on the interaction between β-LG and a monomolecular layer of MSG spread at the air–water interface [8]. The presence of a surface charge, positive or negative, was found to be important for the binding of β-LG to the monolayer. In addition, we observed that β-LG is able to insert into the densely packed MSG monolayer in a charge-dependent manner. In particular, when interacting with a negative amphiphile containing monolayer the protein inserted even at very high surface pressures. However, it was found that the β-LG molecules inserting into a monolayer constitute only a small fraction of the total amount of β-LG associated with a monolayer.

In this paper, we study the interaction of β-LG with MSG bilayers. The experiments were performed under similar conditions of protein and surface charge as used in our monolayer experiments [8]. However, we now only used negative surface charge containing MSG bilayers because of the results of our monolayer experiments. We also made this limitation because negatively charged lipids are commonly present in membranes and because negatively charged amphiphiles are mainly used in technological applications. The interactions were studied using isothermal titration calorimetry (ITC). ITC is a versatile and sensitive technique that measures the heat released or absorbed by an interaction. It therefore not only allows the determination of binding parameters but also of thermodynamic parameters. We also used direct binding assays, differential scanning calorimetry (DSC) and leakage experiments. The latter approaches allowed us to determine whether the interaction with β-LG is affecting the lipid organization. Changes in lipid organization were also characterized by solid-state ^{31}P- and ^{2}H-NMR using 1-monostearoylglycerol with a fully deuterated acyl chain ($^{2}H_{35}$-MSG). Our results show that the presence of a surface charge is not only important for the formation of a homogeneous dispersion, but also for the interaction of β-LG with the MSG bilayers. Depending on the conditions, we observed two binding modes. A specific interaction that leads to a partial lipid L_β to coagel phase transition and a second more general electrostatic attraction between the protein and the bilayer.

2. Materials and methods

2.1. Materials

Bovine β-lactoglobulin (a mixture of genetic variants A and B), 1-monostearoyl-*rac*-glycerol and dicetylphosphate were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. Tris was obtained from Baker (Deventer, the Netherlands) and sodium acetate was from Merck (Darmstadt, Germany). Triton X-100 was obtained from Fluka (Buchs, Switzerland). Texas red covalently linked to a 3-kDa dextran was obtained from Molecular Probes Europe (Leiden, the Netherlands). All other materials were from Merck (Darmstadt, Germany). Fully deuterated stearic acid-d_{35} and deuterium depleted water were obtained from Cambridge Isotope Laboratories (Cambridge, USA). 1-[$^{2}H_{35}$]-Monostearoyl-*rac*-glycerol with a fully deuterated acyl chain was synthesized according to Buchnea [9].

2.2. General analytical procedures

β-LG stock concentrations were determined spec-

troscopically at 280 nm using an $E^{1\%}$ of 0.96 calculated according to Mach et al. [10].

Lipid concentrations were determined by measuring the phosphorus of DCP present in the sample using the Fiske–Subbarow assay [11]. The phosphorus was first liberated by destruction of DCP with perchloric acid.

2.3. Preparation and characterization of lipid dispersions

Lipid dispersions were prepared by mixing known amounts of MSG and dicetylphosphate (DCP) stock solutions in $CHCl_3$/MeOH (3:1). The solvent was evaporated under a stream of nitrogen. The lipids were subsequently hydrated by adding buffer and heating the samples at 65°C for at least 15 min and cooling to room temperature. After three cycles of heating and cooling the samples were dispersed using a Branson 1200 bath sonicator (Danbury, CN, USA) for 10 min at room temperature. The total lipid, i.e. MSG plus DCP, concentration of each dispersion was 5 mM. Buffers used were: 1 mM sodium acetate (pH 4) and 1 mM Tris (pH 7). Higher buffer concentrations resulted in a poor signal-to-noise ratio in our ITC experiments. A 1 mM buffer concentration proved to be sufficient for maintaining a constant pH throughout each experiment.

Particle sizes were determined by dynamic light scattering using a Zetasizer 3000 from Malvern (Malvern, UK).

^{31}P-NMR spectra were recorded on a Bruker MSL 300 spectrometer operating at a frequency of 121 MHz using a high resolution 10-mm broad-band probe. A CYCLOPS sequence with broad band gated proton decoupling was used. The recycling delay was 1.5 s and the $\pi/4$ pulse width 8 μs. Typically, 15 000 free induction decays were accumulated. An exponential multiplication with a line broadening factor of 200 Hz was used before performing the Fourier transformation. ^{2}H-NMR spectra of 46 MHz were obtained using a high power 7.5 mm selective probe. A quadrupolar echo technique [12] with a 3 μs $\pi/2$ pulse, a 40 μs τ delay, a 5 s recycling delay and a quadrature detection was used. Between 10 000 and 15 000 free induction decays were accumulated. An exponential multiplication with a line broadening factor of 300 Hz was used before performing the Fourier transformation. All ^{2}H-NMR spectra were symmetrized. All samples consisted of 10 mM lipid hydrated in buffer prepared with deuterium depleted water. All NMR experiments were performed at 20°C.

2.4. Isothermal titration calorimetry

Reaction heats were measured using a MCS from MicroCal (Northampton, MA, USA). The calorimeter was calibrated electrically. All experiments were performed at 20°C. All solutions were degassed under vacuum before usage. For each experiment, a sonicated 5 mM lipid dispersion was placed in the 1.345 ml reaction cell. A 150 μM β-LG solution in the same buffer as the lipid dispersion was put in a 250 μl syringe and added in 10 μl steps to the lipid dispersion. With each titration a 5 μl preinjection was used. Control experiments were performed by titrating a β-LG solution to a buffer solution without lipid and by titrating a buffer solution without β-LG to a lipid solution. All titrations were corrected for these background heat effects which amounted to no more than 10% of the total signal. The resulting binding curves were analyzed using the Origin software package included in the system. The binding model used is based on non-cooperative multiple binding steps [13]. The more elaborate model for the binding of charged peptides to charged lipid bilayers described by Seelig [14] was not used because we were unable to determine the exact charge of β-LG at pH 4 and 7.

2.5. Differential scanning calorimetry

DSC scans were measured using a Perkin–Elmer DSC-4 (Norwalk, CO, USA). MSG dispersions containing DCP at pH 4 were prepared as described above. After adding β-LG to a final concentration of 1.9 μM the dispersions were incubated at room temperature for 2 h. The lipids were pelleted by centrifugation at $100\,000 \times g$ for 1 h at 4°C using a Beckmann TL-100 ultracentrifuge (Palo Alto, CA, USA). The pellets were subsequently transferred to sample cups which were then sealed. Thermograms were recorded between 20 and 70°C at a scan rate of 5°C min^{-1}. Leakage of the sample cups did not allow scanning above 70°C. At the end of the experiment

the sample cups were opened and the total amount of lipid determined.

2.6. Determination of the entrapped volume and Texas red leakage

The entrapped volume of DCP containing MSG dispersions was determined by enclosing Texas red covalently linked to a 3 kDa dextran (TR). After preparing a 5-mM lipid dispersion in the presence of 0.25 mg ml^{-1} TR, the enclosed TR was separated from the non-enclosed TR by passing the sample over a Superose-6 column from Pharmacia (Uppsala, Sweden). The entrapped volume was then calculated from the amount of lipid and enclosed TR determined after the elution.

TR leakage was determined by following the appearance of TR in the supernatant after pelleting the vesicles by centrifugation. To this end, DCP containing MSG dispersions were prepared in the presence of 0.25 mg ml^{-1} TR. The enclosed TR was separated from the non-enclosed TR by centrifugation at $100\,000 \times g$ for 1 h using a TL-100 ultracentrifuge from Beckmann (Palo Alto, CA, USA). This resulted in an efficient pelleting of the lipid particles and allowed the preparation of sufficient material for these experiments. The pellets were subsequently washed with buffer after which the lipid particles were again pelleted. The TR containing lipid particles were then dispersed in the same volume of buffer as initially used. Samples of these lipid dispersions were incubated in the absence and presence of β-LG at room temperature. Aliquots were taken after 5 min or 24 h. These aliquots were centrifuged at $100\,000 \times g$ for 1 h after which the TR concentration in the supernatant was measured. The extent of TR leakage was determined by relating the TR concentration in the supernatant to the total TR concentration present in the sample. As a control, no TR was found to bind to DCP containing MSG dispersions under the conditions tested. Also, no TR was found to be pelleted in the absence of an MSG dispersion.

All TR concentrations were determined by measuring the fluorescence in the presence of Triton X-100 using an excitation and emission wavelength of 590 and 606 nm, respectively, and a bandwidth of 5 nm. To this end, a 50 μl aliquot of the sample to be measured was added to 950 μl of a 5% Triton X-100 solution. Under these conditions, the Texas red fluorescence is unquenched and the signal increases linearly with the Texas red concentration.

2.7. Direct binding assay

Lipid dispersions of 5 mM were prepared as described above. Increasing amounts of a 0.7 mM β-LG solution in the same buffer were subsequently added to the lipid dispersions. The lipid-to-protein ratio ranged from 1800 to 35. After incubating for 2 h at room temperature the unbound β-LG was separated from the bound protein and lipids by centrifugation at $100\,000 \times g$ for 1 h using a Beckmann TL-100 ultracentrifuge. This resulted in an efficient pelleting of the lipids also in the absence of protein. In the absence of lipids, no β-LG was found to be pelleted under these conditions. The amount of unbound β-LG present in the supernatant was determined using the enhanced BCA assay from Pierce (Rockford, IL, USA). Binding parameters were determined using the same binding model as used for the ITC data, but using an Excel 5.0 (Microsoft, Redmond, WA, USA) spreadsheet based on the mical program described by Hille et al. and Soares de Araujo et al. [15,16]. The difference between the programs used for ITC and the direct binding studies is related to the different manner of plotting the data. The ITC uses the signal of each individual titration and the corresponding lipid-to-protein ratio, while a cumulative signal and the free protein concentration are used for the direct binding assay.

3. Results

No homogeneous dispersion could be prepared with MSG in the absence of DCP. Large lumps of solid precipitated material formed which could not be dispersed by sonication. This may be due to the limited swelling of the monoglyceride bilayer [4,17]. With MSG containing 5 or 10 mol% DCP a homogenous and opalescent dispersion was obtained upon sonication, indicating that in both cases the amount of surface charge is sufficient to overcome the stacking tendency of the MSG bilayers. Without sonication the hydrated MSG-DCP mixtures formed extremely viscous dispersions excluding the use of

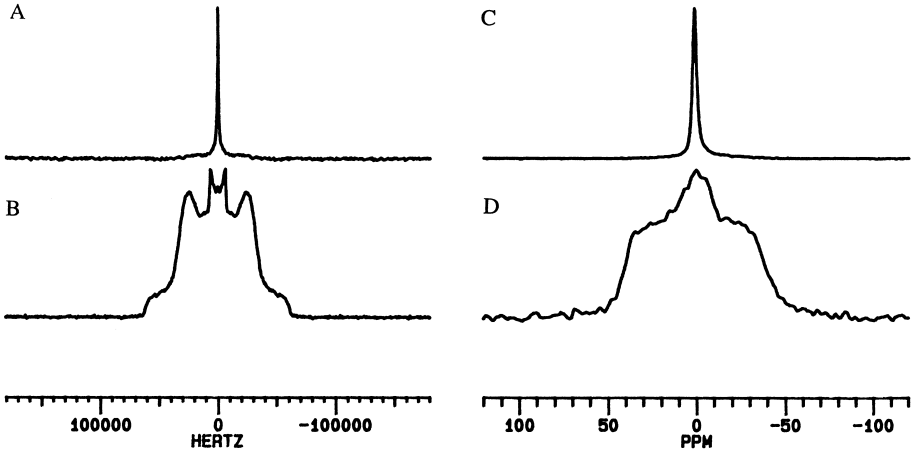

Fig. 1. ^{2}H- (A and B) and ^{31}P- (C and D) NMR spectra of non-sonicated (bottom) and sonicated (top) dispersions of $^{2}H_{35}$-MSG containing 10 mol% DCP at pH 7 and 20°C.

ITC. In addition, these dispersions probably consist of multilamellar liposomes meaning that the major part of the lipids would not be accessible to β-LG.

Fig. 1 shows ^{2}H- and ^{31}P-NMR spectra of non-sonicated and sonicated lipid dispersions composed of $^{2}H_{35}$-MSG containing 10 mol% DCP at pH 7 and 20°C. The ^{31}P-NMR spectrum of the non-sonicated dispersion (Fig. 1D) is close to the spectrum of a phosphate in the solid state and significantly differs from the spectra reported for phospholipids in the L_{β} phase [18]. This difference is due to the particular chemical structure of DCP. In the case of DCP, the phosphate group is anchored into the lipid bilayer by two long hydrocarbon chains. As a result, intramolecular rotations of the polar head group are restricted and the mobility of the phosphate group is strongly reduced. The ^{2}H-NMR spectrum of the non-sonicated dispersion (Fig. 1B) shows a signal with a smaller quadrupolar splitting (12 kHz) which corresponds to the methyl deuterons and a broader signal, with a quadrupolar splitting of 51 kHz, which corresponds to a superposition of the peaks due to the methylene deuterons. Both the magnitude of the splittings and the broadness are indicative for the L_{β} phase [19]. We found that at room temperature this phase is stable over a period of several weeks. Sonication results in isotropic signals in both the ^{2}H- and ^{31}P-NMR spectra (Fig. 1A,C). This reflects the averaging of the anisotropy of the quadrupolar interaction in the ^{2}H-NMR spectrum and the chemical shielding in the ^{31}P-NMR spectrum due to fast iso-

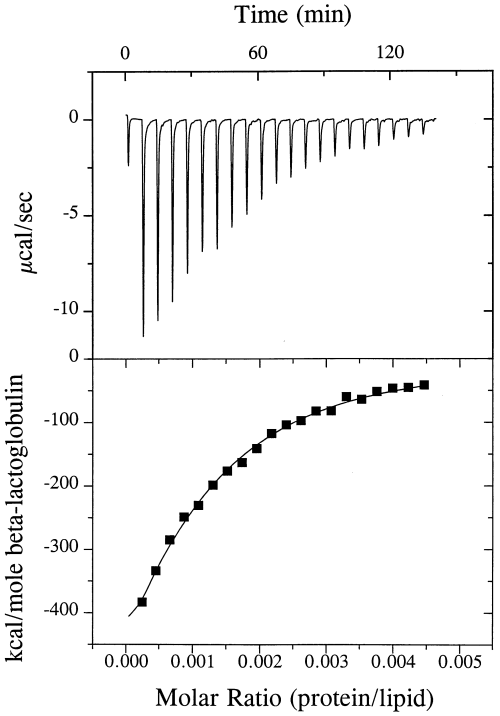

Fig. 2. Titration calorimetry curves of a 10 mol% DCP containing MSG dispersion with β-LG at pH 4 and 20°C. The initial lipid concentration (MSG+DCP) in the calorimetric cell was 5 mM. The β-LG concentration in the syringe was 150 μM. The top panel shows the calorimetric trace of the titration, while the bottom panel shows the derived binding curve. Each peak, except the first, in the calorimetric trace results from the addition of 10 μl of the β-LG solution to the lipid dispersion. The first peak results from the 5 μl preinjection.

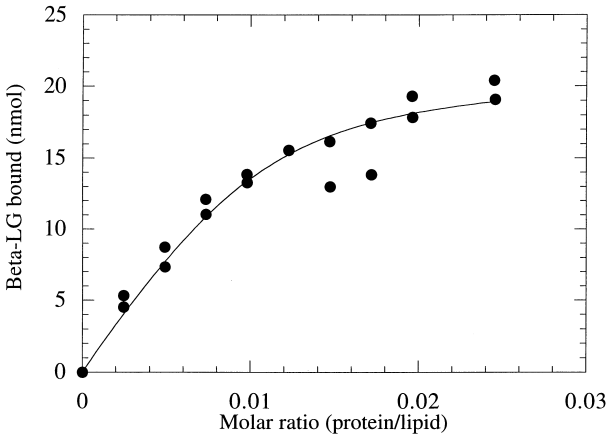

Fig. 3. The binding of β-LG to a 10 mol% DCP containing MSG dispersion at pH 4. The initial lipid (MSG+DCP) concentration was 5 mM. Increasing amounts of a 0.7 mM β-LG stock solutions were added to the lipid dispersion.

tropic motion which probably results from the tumbling of relatively small vesicles. The average size of these particles, as determined by dynamic light scattering, was found to be 465 nm. The particle size ranged from 330 to 635 nm for the different preparations. The trapped volume of the 10 mol% DCP containing MSG dispersions varied from 2.5 to 10 liter per mol of lipid with an average of 7.2 liter per mol of lipid. Assuming that the particles are spherical and unilamellar a particle diameter of 560 nm can be calculated from the average volume. The bilayers were stable as no significant TR release was observed after incubation for 24 h.

Using ITC we could not detect an interaction between β-LG and neutral MSG bilayers. As can be seen in Fig. 2, β-LG readily interacts with a 10 mol% DCP containing MSG dispersion at pH 4. Under these conditions, β-LG, with an isoelectric point of 5.2 [7], is positively charged. Both the calorimetric trace as well as the binding curve, derived by integration of the individual peaks of the calorimetric trace, are depicted. Under these conditions, it was also noted that at the end of each experiment a fluffy precipitate had formed in the sample. Similar titration curves were observed at pH 7 where β-LG carries a net negative charge (not shown). However, no visible precipitate was formed at pH 7. With the direct binding assay we could also observe the binding of β-LG to DCP containing MSG dispersions. In Fig. 3, the binding curve determined at pH 4 is depicted. The data are plotted as a function of the total protein-to-total lipid ratio in order to facilitate comparison with the ITC curve obtained under the same conditions. Comparing the two curves shows that at pH 4 the MSG dispersion is saturated with β-LG at a much lower protein-to-lipid ratio in the ITC experiment than in the direct binding assay. Apparently the two methods determine different binding processes. The difference could result from a difference in affinity of β-LG for the MSG bilayer, but can also result from a difference in stoichiometry. As can be seen in Table 1 the affinities determined with both methods are comparable while the stoichiometry shows a large difference. As a result of the more than 10-fold difference in stoichiometry the direct binding curve is mainly determined by β-LG molecules that are not observed with ITC. The resulting binding parameters can therefore be ascribed to these β-LG molecules. In Fig. 4, the amount of bound β-LG as function of the free protein concentration, as determined in the direct binding assay, at pH 4 and 7 is depicted. As can be seen β-LG binds to the bilayer at both pH values, but more β-LG is found to bind at pH 4 than at pH 7 using this assay.

From the ITC the binding parameters were calcu-

Table 1
Binding parameters and enthalpies of the interaction between β-LG and DCP containing MSG dispersions as determined with direct binding assays and ITC under various conditions at 20°C

	Direct binding assay		ITC			
	N (lipid/protein)	K_d (μM)	N (lipid/protein)	K_d (μM)	ΔH_o (kcal mol β-LG^{-1})	ΔH_o (kcal mol MSG^{-1})
10% DCP; pH 4	88	5.1	906	6.7	−756	−0.8
5% DCP; pH 4			2730	4.2	−600	−0.2
10% DCP; pH 7	1300	5.7	1530	3.1	−420	−0.3

The enthalpy is expressed per mol of β-LG as well as per mol of total lipid (MSG+DCP). The error in the numbers ranges from 5 to 10%.

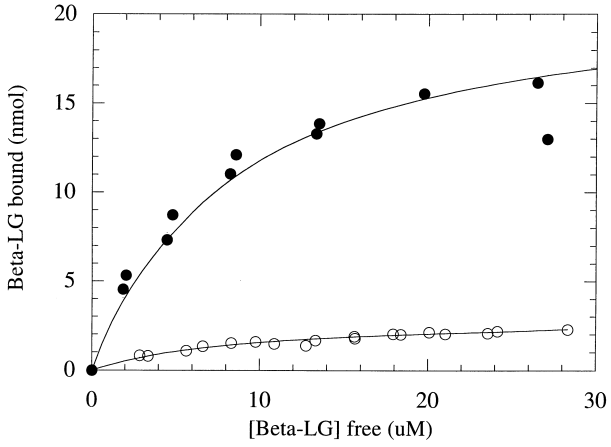

Fig. 4. Direct binding curves of a 10-mol% DCP containing MSG dispersion titrated with β-LG at pH 4 (closed circles) and pH 7 (open circles). The initial lipid (MSG+DCP) concentration was 5 mM. Increasing amounts of a 0.7 mM β-LG stock solutions were added to the lipid dispersion.

lated as depicted in Table 1. We like to stress here that the listed stoichiometries should not be seen as the exact amount of lipids per protein in a specific complex, but as the average ratio of lipids and proteins in a much larger structure. This structure can be visualized as a vesicle with a certain number of protein molecules bound to it. As can be seen in Table 1, the affinities of β-LG for DCP containing MSG bilayers are all in the micromolar range. At pH 4 a reduction of the amount of negative surface charge, from 10 to 5 mol%, results in only a small difference in affinity of the positively charged β-LG. Interestingly, β-LG also binds with a micromolar affinity to the bilayer at pH 7 where both the protein and the bilayer carry a net negative charge. This suggests that the binding characteristics are not simply determined by the overall charge difference between the lipid particle and the protein. The stoichiometries of the interaction determined under the various conditions also differ by less than an order of magnitude. Changing the net charge of β-LG in the presence of 10 mol% DCP results in only a difference of about 1.5-fold in the amount of protein bound to the bilayer. Reducing the amount of surface charge has a more profound effect. In the presence of 5 mol% DCP at pH 4 the lipid-to-protein ratio was found to be 2730 that is about 3-fold higher than 906 found for a MSG bilayer containing 10 mol% DCP at pH 4. This means that reducing the amount of DCP causes a reduction of the amount of binding sites for β-LG. At pH 7, the binding parameters observed by both methods are comparable (Table 1). In particular, the large difference in stoichiometry observed between ITC and the direct binding assay in the presence of 10 mol% DCP at pH 4 is not observed.

A common feature of all the studied conditions is the magnitude of the enthalpy of the binding process as observed with ITC, ranging from −420 kcal mol^{-1} to −765 kcal mol^{-1} when expressed in terms of β-LG (Table 1). These are very large values compared to the enthalpies normally observed for protein–lipid interactions that are in the order of several tens of kcal mol^{-1} [14]. It may be possible that the enthalpy of the protein–lipid interaction is masked by the enthalpy of protein induced changes in the lipid phase. The binding enthalpy per mol of lipid can be calculated by dividing the enthalpy per mol of β-LG by the stoichiometry (lipid/protein) of the interaction. This results in values ranging from −0.2 kcal mol^{-1} to −0.8 kcal mol^{-1} for the various conditions (Table 1). In order to determine the possible effects of the interaction on the lipid phase we analyzed the melting transition of the system using DSC. If β-LG indeed affects the lipids we may expect a change in enthalpy of the phase transition. For reasons of sensitivity, we limited the DSC experiments to the 10 mol% containing MSG dispersion at pH 4

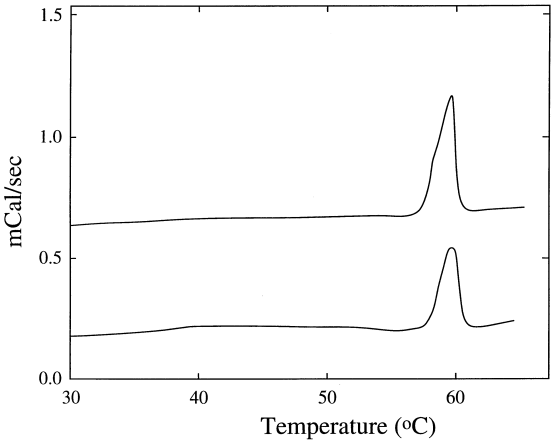

Fig. 5. DSC scans of pellets of a 10 mol% DCP containing MSG dispersion in the absence (lower curve) and presence (upper curve) of β-LG at pH 4. The total lipid concentration (DCP+MSG) in the incubations was 5 mM while the β-LG concentration was 20 μM. After an incubation time of 2 h, the material was pelleted by centrifugation at 100 000×*g* for 1 h.

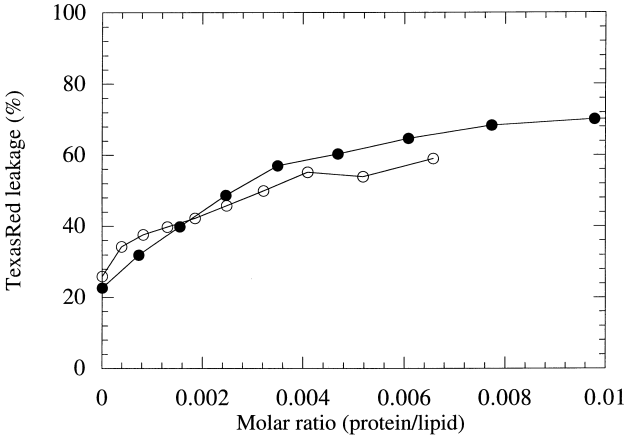

Fig. 6. TR leakage from a 10 mol% DCP containing MSG dispersion at pH 4 (closed circles) and pH 7 (open circles). TR leakage is expressed as a percentage of the total amount of enclosed TR. The initial lipid (MSG+DCP) concentration was 1.5 mM. Increasing amounts of a 120 μM β-LG stock solution were added to the lipid dispersion.

where the largest binding enthalpy is observed with ITC. The DSC scans obtained with this MSG dispersion in the absence and presence of β-LG are depicted in Fig. 5. The interaction of β-LG with the MSG bilayer does not have a large influence on the phase transition temperature of the lipid. In the absence of β-LG, the transition temperature was found to be 57.7 ± 0.2°C and in its presence 57.2 ± 0.2°C. However, the phase transition enthalpy is affected by β-LG. The enthalpy in the absence of β-LG was found to be 8.3 ± 0.4 kcal mol^{-1}, while in the presence of β-LG it was 7.1 ± 0.3 kcal mol^{-1}. The amount of β-LG used in this experiment is the same as that where the ITC signal is saturated. The enthalpy difference of -1.2 kcal mol^{-1} is similar to the binding enthalpy of -0.8 kcal mol^{-1} (Table 1) observed with ITC under the same conditions.

In order to establish whether the effects of β-LG on the lipid phase are reflected in changes in the barrier properties of the bilayer we performed leak experiments using vesicles containing enclosed reporter molecules. Initial experiments with carboxyfluorescein failed as a result of the poor solubility of carboxyfluorescein at pH 4. We therefore used TR covalently linked to a dextran because this is soluble at pH 4 and 7. At both pH values, the addition of β-LG to 10 mol% DCP containing MSG dispersions resulted in a partial release of the enclosed TR. This release is temporary rather than continuous. TR is released immediately after the addition of β-LG. No additional TR was found to be released from the dispersion, even after incubating for 24 h (not shown). On the other hand, addition of more protein resulted in additional TR leakage, both at pH 4 and 7 (Fig. 6). The initial levels of about 20% result from non-enclosed TR present after washing of the samples. As can be seen, the effect can be saturated since no more TR leakage is observed above a protein-to-lipid ratio of 0.01. Interestingly, comparing Fig. 2 with Fig. 6 shows that the calorimetric signals are observed at molar ratios comparable to those that give rise to TR leakage.

^{2}H-NMR was used to gain more structural insight into the lipid reorganization induced by β-LG. Fig. 7 shows that upon addition of β-LG to the dispersed monoglyceride the isotropic signal partially transforms into a broad spectrum in which there are two distinct anisotropic signals present (Fig. 7B). Such an anisotropic signal has been reported for fully deuterated fatty acids in the coagel phase [19] and corresponds to the ^{2}H-NMR spectrum of pure $^2H_{35}$-MSG in the coagel (Fig. 7C). The quadrupolar splitting of the methylene deuterons (118 kHz) is essentially the same at each position along the acyl

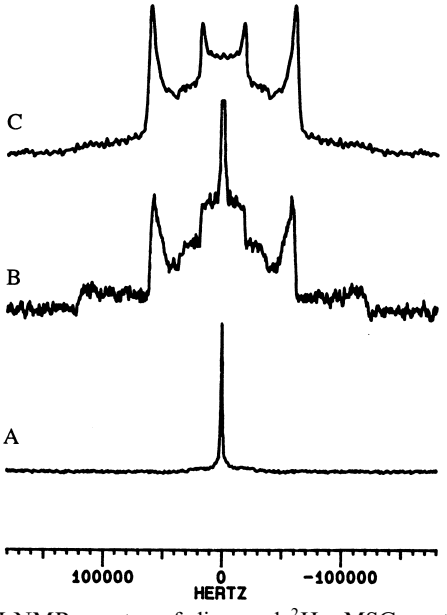

Fig. 7. ^{2}H-NMR spectra of dispersed $^2H_{35}$-MSG containing 10 mol% DCP at pH 7 and 20°C before (A) and after the addition of β-LG (B). The molar lipid-to-protein ratio was 100. C shows the ^{2}H-NMR spectrum of the coagel of pure $^2H_{35}$-MSG in water at 20°C.

chain. The quadrupolar splitting of the methyl deuterons (34 kHz) is less than that of the methylenes because this group rapidly reorientates around the terminal C–C bond. Strikingly, upon interaction with β-LG, the isotropic signal directly transforms in a coagel-like spectrum and no L_β phase could be detected (compare Fig. 1B and 7B).

4. Discussion

We set out to extend the study on the interaction between β-LG and aqueous dispersions of the gel phase forming monoglyceride MSG. In a previous study, using monomolecular layers at the air–water interface, we found that only small amounts of protein did bind to a neutral interface [8]. In this study, no interaction between β-LG and neutral MSG bilayers was observed. The neutral MSG dispersions formed precipitates that probably consist of large stacks of MSG bilayers with no, or hardly any, water between the layers, i.e. the coagel phase [1] as indicated by our NMR results. The absence of an interaction could therefore result from the inaccessibility of the stacked bilayers as well as from a low affinity of β-LG for neutral MSG systems, as observed with monolayers.

With the negatively charged DCP present in the bilayer, we were able to prepare stable dispersions of MSG. The surface charge is probably responsible for keeping the bilayers unstacked. The 10 mol% DCP containing MSG bilayers form structures with an average diameter of 465 nm. This is comparable to the diameter of 560 nm calculated from the entrapped volume which indicates that the dispersion mainly consists of unilamellar vesicle structures. The formation of relatively small vesicles is in agreement with our NMR data. These stable unilamellar vesicles of gel phase monoglycerides present us with an excellent model system for analyzing protein–monoglyceride interactions.

Both ITC and the direct binding experiments show that β-LG interacts with DCP containing MSG vesicles. The affinity of the protein for the vesicles at pH 4 and 7 was found to be similar with both ITC and the direct binding assay. Strikingly, at pH 4, a more than 10-fold difference in stoichiometry was observed between the two methods. This demonstrates the simultaneous occurrence of two binding modes under that condition. ITC measures a binding mode that saturates at about 900 lipids per protein while the direct binding assay, that saturates at about 90 lipids per protein, measures the net result of both. Apparently, 90% of β-LG binds to the vesicles without giving rise to a detectable calorimetric signal. Because β-LG carries a net positive charge at pH 4 we propose that this results from an aspecific electrostatic attraction between β-LG and the negatively charged bilayer. As described by Seelig [14] in his binding model for peptide–lipid interactions this attraction leads to an increase of the β-LG concentration close to the bilayer surface. These β-LG molecules, although very close to the bilayer, do not directly interact with the bilayer. This means that either there is no calorimetric signal or that the signal is not detected because it is much smaller than that of the remaining 10% of the β-LG molecules which apparently do interact directly with the bilayer. An aspecific electrostatic interaction also explains the precipitate observed at pH 4 which may result from the positively charged β-LG neutralizing the negative charge of the bilayer. Based on the model of Seelig [14], the binding detected with ITC at pH 4 and 7 could be the result of a hydrophobic partitioning of β-LG into the bilayer. A hydrophobic interaction might explain the binding observed at pH 7 where β-LG is also negatively charged and should therefore be repelled by the bilayer. However, our monolayer studies [8] indicated that only a very small fraction of β-LG is able to insert into a densely packed monoglyceride monolayer. Furthermore, this depends on the pH, surface charge and surface pressure such that no insertion is observed at pH 7 above a surface pressure of 32 mN m^{-1}. The binding observed with ITC under similar conditions indicates that the binding mode detected with ITC is not related to the insertion of β-LG into the lipid bilayer. It therefore probably involves contacts between the protein and the polar interface of the bilayer. Since lowering of the surface concentration of DCP resulted in a reduction of the amount of bound protein, we propose that this binding mode can be described as a specific interaction, in the form of hydrogen bonds or salt bridges, between the negatively charged amphiphile and residues exposed at the surface of β-LG. These are likely to be arginines or lysines which are posi-

tively charged at pH 4 and, despite the protein carrying a net negative charge, also at pH 7.

A striking result of this study is the magnitude of the exothermic binding enthalpy when expressed per mol of β-LG. The enthalpies are very large compared to those found for other protein–lipid interactions [14]. When proteins bind to a lipid bilayer contributions can be expected from protein–lipid interactions, protein (un)folding or changing lipid–lipid interactions. The lipid–protein interaction probably involves the positively charged side chains of arginines and lysines present in β-LG that interact with the negatively charged bilayer. Similar electrostatic interactions have been shown to be exothermic with enthalpies ranging from -5 to -7 kcal mol^{-1} for a single fatty acid binding to a protein [20,21]. If we assume that the contribution of each such interaction between β-LG and the bilayer yields -7 kcal mol^{-1} then this adds up to -126 kcal mol^{-1}, provided that all 3 arginines and 15 lysines [22] are able to interact with the negatively charged bilayer. This requires the unfolding of β-LG that would additionally contribute about 50 kcal mol^{-1} [23]. When comparing the total of these two effects, -76 kcal mol^{-1}, with the observed -756 kcal mol^{-1} it is clear that this cannot explain the large exothermic enthalpy. Hydrophobic interactions of β-LG with the MSG bilayer may also contribute. This interaction would require the insertion of hydrophobic parts of β-LG into the hydrophobic core of the bilayer. The insertion of β-LG was indeed demonstrated by our monolayer study [8], but only under certain conditions. Furthermore, the enthalpy associated with the transfer of a hydrophobic residue from an aqueous environment to a hydrophobic environment is in the order of a few kcal mol^{-1}. For instance, the enthalpy for the transfer of a methyl group from water to benzene is -3 kcal mol^{-1} [24]. Our monolayer data indicated that on average less than the area of one methyl group per β-LG inserts into the monolayer [8]. It is therefore unlikely that hydrophobic protein–lipid interactions explain the large enthalpies.

Taking all the above-discussed effects together, it becomes obvious that the major contribution to the observed binding enthalpies must come from changing lipid–lipid interactions. That lipid–lipid interactions are affected is supported by the effect of β-LG on the MSG bilayer as observed with DSC and TR leakage. The correlation between ITC and TR leakage shows that the binding enthalpy is indeed closely linked to changes in the bilayer. Probably packing defects that allow TR to leak out are transiently formed as the lipids reorganize. On the other hand, it could be that β-LG removes DCP from the bilayer. The latter possibility follows from the known binding of negatively charged amphiphiles to β-LG [7], but can be ruled out because no DCP was observed in the supernatants of the direct binding assay. Also, our monolayer studies [8] do not suggest that β-LG is able to remove lipids from the bilayer. Several calorimetric studies have shown that binding enthalpies can originate from a protein induced lipid reorganization [25–27]. The binding of Apolipoprotein–A1 to DPPC–triolein–cholesterol emulsions resulted in a very large enthalpy of no less than -940 kcal mol^{-1} protein [27]. Using DSC, it was shown that this enthalpy correlates with a change in enthalpy of one of the lipid phase transitions. Considering the phase behavior of MSG, three phases may be important: the L_α phase, the metastable L_β phase and the coagel phase [1]. Because the L_β to L_α transition of neutral MSG occurs at 55°C, we assign the transition observed with DSC at 57°C to this phase transition. As discussed above, after cooling to room temperature our dispersion consists mainly of unilamellar vesicles meaning that the lipids are in the L_β phase which is stabilized by the surface charge. The L_β phase can transfer exothermically into the coagel phase or endothermically into the L_α phase. The exothermic binding enthalpy points to a β-LG induced L_β to coagel transition. Indeed, our NMR data clearly shows the appearance of the coagel after the addition of the protein. The absence of an effect on the L_β to L_α phase transition temperature, as shown by DSC, is in agreement with this since the coagel transfers directly into the L_α phase at the same temperature as the L_β to L_α transition [1]. The binding enthalpy of -0.8 kcal mol^{-1} observed with ITC and the -1.2 kcal mol^{-1} difference in phase transition enthalpy observed with DSC are smaller than the -5 kcal mol^{-1} associated with the L_β to coagel transition [1] which suggests that the phase transition is only partial. Also our NMR data shows that the transition does not proceed to completion. The partial phase transition induced by β-LG results from the first binding mode observed at

pH 4 because this binding mode gives rise to the large calorimetric signal and TR leakage. The single binding mode observed at pH 7 also gives rise to these signals, albeit to a lesser extent. We therefore conclude that the stabilizing effect of the surface charge on the L_β phase is reduced as a result of the specific interaction between β-LG and DCP. This would allow the individual bilayers of the L_β phase to approach and eventually stack upon each other and thus form the coagel phase. Further research may shed light on the exact mechanism by which this specific interaction induces the lipid phase transition and why it is only partial.

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