ELSEVIER

Biochimica et Biophysica Acta 1510 (2001) 401–413

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

The specificity of monoglyceride–protein interactions and mechanism of the protein induced L_β to coagel phase transition

J.-W.P. Boots *, V. Chupin, J.A. Killian, R.A. Demel, B. de Kruijff

Department of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received 14 July 2000; received in revised form 24 October 2000; accepted 8 November 2000

Abstract

This study aims at gaining insight into the specificity and molecular mechanism of monoglyceride–protein interactions. We used β-lactoglobulin (β-LG) and lysozyme as model proteins and both monostearoylglycerol and monopalmitoylglycerol as defined gel phase monoglycerides. The monoglycerides were used in different combinations with the two negatively charged amphiphiles dicetylphosphate and distearylphosphate. The interactions were characterized using the monolayer technique, isothermal titration calorimetry, ^{2}H-nuclear magnetic resonance (NMR) using deuterium labelled monoglycerides and freeze fracture electron microscopy (EM). Our results show that lysozyme inserts efficiently into all monolayers tested, including pure monoglyceride layers. The insertion of β-LG depends on the lipid composition of the monolayer and is promoted when the acylchains of the negatively charged amphiphile are shorter than that of the monoglyceride. The binding parameters found for the interaction of β-LG and lysozyme with monoglyceride bilayers were generally similar. Moreover, in all cases a large exothermic binding enthalpy was observed which was found to depend on the nature of the monoglycerides but not of the proteins. ^{2}H-NMR and freeze fracture EM showed that this large enthalpy results from a protein mediated catalysis of the monoglyceride L_β to coagel phase transition. The mechanism of this phase transition consists of two steps, an initial protein mediated vesicle aggregation step which is followed by stacking and probably fusion of the bilayers.

Keywords: Monoglyceride; Monoglyceride–protein interaction; Monolayer insertion; ITC; NMR; Freeze fracture EM

1. Introduction

The ability of monoglycerides to form bilayers, like natural phospholipids, as well as non-bilayer structures offers many interesting opportunities for studies on membrane structure and function [1–4]. The successful use of a monoglyceride cubic phase for the crystallization of membrane proteins as well as soluble proteins [5–8] underscores their large potential in this field. Because of their emulsifying properties monoglycerides are also becoming increasingly important for the food industry [9]; gel phase monoglycerides are for example used in low-fat products. Proteins are present in many of these products and therefore a good understanding of the interac-

Abbreviations: β-LG, β-lactoglobulin; MSG, 1-monostearoyl-*rac*-glycerol; $^2H_{35}$-MSG, 1-mono-[$^2H_{35}$]-stearoyl-*rac*-glycerol; MPG, 1-mono-*rac*-palmitoylglycerol; $^2H_{35}$-MPG, 1-mono-[$^2H_{35}$]-palmitoyl-*rac*-glycerol; DCP, dicetylphosphate; DSP, distearylphosphate; π_i, initial surface pressure; $\Delta\pi$, surface pressure increase; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; EM, electron microscopy

* Corresponding author. Fax: +31-30-2522478;
E-mail: j.w.p.boots@chem.uu.nl

0005-2736/01/$ – see front matter
PII: S0005-2736(00)00372-2

tion between proteins and monoglycerides is needed. However, in contrast to the interaction of proteins with phospholipids not much is known about their interaction with monoglycerides.

In two previous papers [10,11] we reported on the interaction of the soluble protein β-lactoglobulin (β-LG) with the gel phase forming 1-monostearoyl-*rac*-glycerol (MSG), either in the absence or presence of the negatively charged amphiphile dicetylphosphate (DCP). Monolayer experiments showed that β-LG is able to insert between the densely packed monoglycerides in a pH, surface charge and pressure depending manner. Studies using dispersed MSG bilayers stabilized with DCP showed that β-LG is able to induce a phase transition of the lipids [11] from the L_β phase, which consists of single gel phase bilayers interspaced by water, to the coagel phase which consists of stacks of gel phase bilayers [12,13].

In this study we aim to answer the following questions: how general is the observed interaction and what is the underlying molecular interpretation? Since in the previous studies only one gel phase forming monoglyceride, one negatively charged amphiphile and one protein was used, it is possible that the observed effects are related to some specific property of those model compounds. To investigate the lipid specificity of these effects we therefore extended our studies to include a different chain length monoglyceride and negatively charged amphiphile (Fig. 1). 1-Mono-*rac*-palmitoylglycerol (MPG) was chosen because it is able to form the L_β and coagel phase, forms relatively stable monolayers at the air–water interface and has the same acylchain length as DCP. The negatively charged amphiphile distearylphosphate (DSP) was included to match the acylchain length of MSG. To investigate the protein specificity we used β-LG and lysozyme which has a similar size and number of positively charged amino acids as β-LG but has no apparent sequence, structural or functional homology with β-LG. Understanding the interaction of lysozyme with monoglycerides is of particular interest because it has been crystallized using a monoglyceride cubic phase [8].

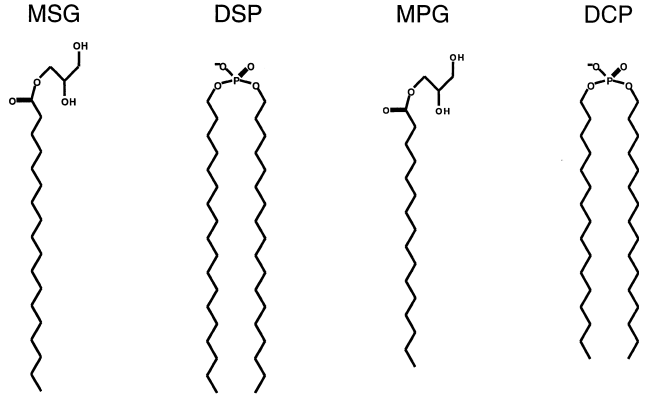


Fig. 1. Structure of (left to right): MSG, DSP, MPG and DCP.

In order to investigate whether β-LG and lysozyme can insert between the monoglycerides we studied the interaction of these proteins with various monoglyceride monolayers. The interaction of both proteins with dispersed bilayers was characterized with isothermal titration calorimetry (ITC). This technique not only gives the binding parameters but also the enthalpy of the interaction which gives further insight into the consequences of the interaction [11]. Freeze fracture electron microscopy (EM) was used to study the morphology of the monoglyceride dispersions in the absence and presence of protein. Finally, solid-state ^{2}H-nuclear magnetic resonance (NMR) using monoglycerides with perdeuterated acylchains was used to obtain structural information on the lipids. The experiments with both proteins were performed at neutral pH and with β-LG also at pH 4 where, like lysozyme at pH 7, it is positively charged. Our monolayer results show that the type of interaction of β-LG and lysozyme with monoglycerides is mostly similar. However, the results also reveal a difference. Lysozyme inserts efficiently into all tested monolayers, whereas insertion of β-LG is dependent on the lipid composition of the monolayer. The binding parameters of both proteins to the various monoglyceride bilayers were found to be rather similar. Furthermore, a remarkably large exothermic binding enthalpy is observed in all cases. ^{2}H-NMR and freeze fracture EM showed that this large enthalpy results from a protein induced L_β to coagel lipid phase transition, the mechanism of which is shown to consist of two steps.

2. Materials and methods

2.1. Materials

Bovine β-LG (a mixture of genetic variants A and

B), MSG, MPG and DCP were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. Lysozyme was obtained from Boehringer (Mannheim, Germany). Tris was obtained from Baker (Deventer, The Netherlands). All other materials were from Merck (Darmstadt, Germany). Fully deuterated stearic and palmitic acid and deuterium depleted water were obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). 1-Mono-[$^2H_{35}$]-stearoyl-*rac*-glycerol ($^2H_{35}$-MSG) and 1-mono-[$^2H_{35}$]-palmitoyl-*rac*-glycerol ($^2H_{35}$-MPG) with a fully deuterated acyl-chain were synthesized according to Buchnea [14]. DSP was synthesized as described by Tsiourvas et al. [15] but using carbontetrachloride as a solvent.

2.2. General analytical procedures

β-LG and lysozyme stock concentrations were determined spectroscopically at 280 nm using an $E^{1\%}$ of 0.96 or 2.69, respectively, calculated according to Mach et al. [16].

2.3. Monolayer experiments

Surface pressures were measured by the Wilhelmy method in Teflon troughs with a volume of 5 ml and a surface area of 8.8 cm^2 at 20°C [17]. The buffers used were: 1 mM sodium acetate (pH 4) or 1 mM Tris (pH 7), with or without 100 mM NaCl. A 1 mM buffer concentration proved to be sufficient for maintaining a constant pH throughout each experiment. 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 in all experiments. The whole experimental setup was placed in a thermostated box. The subphase was continuously stirred with a magnetic bar. Initial surface pressures (π_i) ranged from 25 to 36 mN/m. Ten μl of a protein stock solution, 0.8 mM for β-LG and 1.2 mM for lysozyme, was injected under the monolayer through a separate hole in the Teflon dish. The final protein concentrations in the subphase were 1.4 μM for β-LG and 2 μM for lysozyme. The addition of more protein did not result in larger increases of the surface pressure. The error in these experiments was 0.2 mN/m.

2.4. Preparation of the lipid dispersions

Lipid dispersions were prepared by mixing known amounts of monoglyceride and amphiphile stock solutions in $CHCl_3$:MeOH (3:1). A 9:1 monoglyceride:amphiphile molar ratio was used in all experiments. The solvent was removed with a stream of nitrogen after which the sample was placed under vacuum for at least 2 h. 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) filled with hot water (70°C) for 1 min. This protocol is slightly different from that used in our previous study [11] in which the dispersions were sonicated at room temperature. However, this did not result in a stable MSG/DSP dispersion. For a proper comparison of the results we also used the higher sonication temperature for MSG/DCP and MPG/DCP bilayers. MSG and MPG did not form stable dispersions in the absence of either DCP or DSP and only large lumps of precipitated lipid were found. The total lipid, i.e. monoglyceride plus amphiphile, concentration of each dispersion was 5 mM for ITC, 15 mM for EM and 15 mM for NMR experiments. The buffers used were: 1 mM sodium acetate (pH 4) or 1 mM Tris (pH 7). Higher buffer concentrations resulted in a poor signal to noise ratio in the ITC experiments whereas higher monoglyceride concentrations resulted in solutions which were too viscous to be used in the ITC.

2.5. Freeze fracture EM

Samples for freeze fracture EM were sandwiched between two hat-shaped copper holders interspaced by a copper spacer. The samples were prepared at room temperature and no cryoprotectants were added. The samples were fast-frozen by plunging them into liquid propane, cooled to its melting point with liquid nitrogen, using a KF80 plunge-freezing device (Reichert Jung, Vienna, Austria). The samples were fractured and subsequently replicated with platinum according to standard procedures using a BAF400 freeze fracture device (Bal-tec AG, Baltzers, Liechtenstein). The replicas were stripped from the copper holders and cleaned with chromic acid

followed by distilled water according to Costello et al. [18]. A CM10 electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV was used for examining the replicas.

2.6. ^{2}H-NMR

NMR spectra were recorded on a Bruker MSL 300 spectrometer. 46 MHz ^{2}H NMR spectra were obtained using a high power 7.5 mm selective probe. A quadrupolar echo technique [19] with a 3 μs π/2 pulse, a 40 μs τ delay, a 5 s recycling delay and a quadrature detection was used. Between 3000 and 8000 free induction decays were accumulated in the case of gel phase monoglycerides and between 20 000 and 30 000 in the case of monoglycerides in the coagel. 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 15 mM lipid hydrated in buffer prepared with deuterium depleted water. All NMR experiments were performed at 20°C.

2.7. ITC

Reaction heats were measured using an 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 protein 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 pre-injection was used. Control experiments were performed by titrating a protein solution to a buffer solution without lipid and by titrating a buffer solution without protein 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 [20]. The more elaborate model for the binding of charged peptides to charged lipid bilayers described by Seelig [21] was not used because it was not possible to determine the exact charge of both proteins at pH 4 and pH 7.

3. Results

3.1. Monolayer insertion

Before looking at the binding of β-LG and lysozyme to monoglyceride bilayer systems, we first investigated the insertion of both proteins using monolayers of monoglycerides. Our previous monolayer study [10] showed that β-LG is able to insert into MSG monolayers under various conditions of surface charge, pH and ionic strength at π_i below 32 mN/m. Intriguingly, insertion above 32 mN/m was only observed in an MSG monolayer containing 10 mol% DCP under conditions of low ionic strength and at pH 4 where the protein carries a net positive charge. We therefore used these same conditions to study the lipid specificity of the insertion of β-LG. As can be seen in Fig. 2 β-LG is able to insert into all mixtures of monoglyceride and negatively charged amphiphile used at a π_i lower than 32 mN/m. However, the observed surface pressure increase ($\Delta\pi$) is much lower with MPG/DCP, MSG/DSP and MPG/DSP monolayers than with MSG/DCP monolayers. Moreover, β-LG does not insert into either the

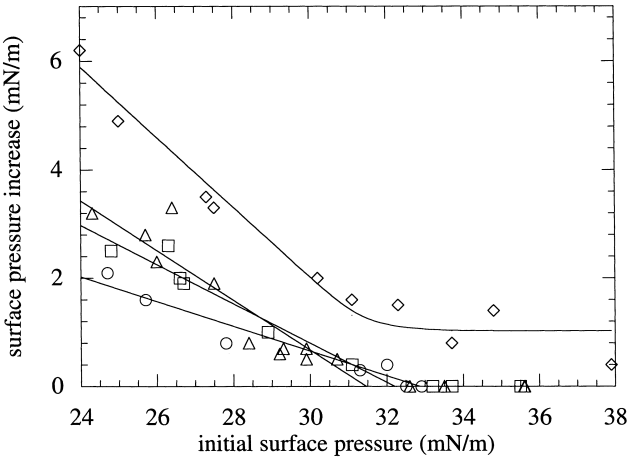


Fig. 2. β-LG induced $\Delta\pi$ as function of the π_i of MSG/DCP (diamonds, taken from [10]), MPG/DCP (squares), MSG/DSP (circles) monolayers and MPG/DSP (triangles) monolayers. The buffer used was 1 mM acetate, pH 4. The β-LG concentration in the subphase was 1.4 μM.

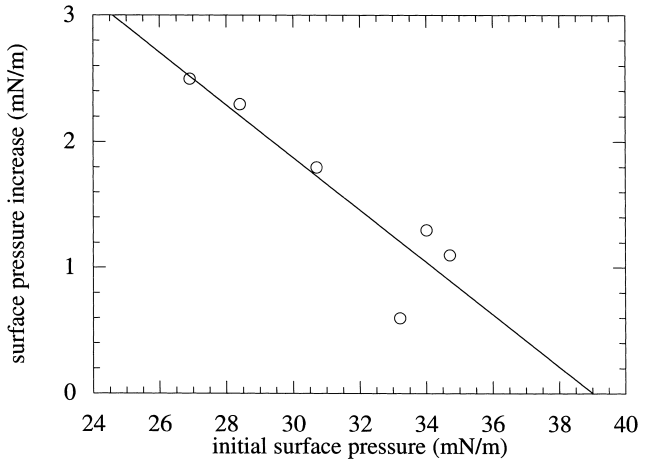


Fig. 3. Lysozyme induced $\Delta\pi$ as function of the π_i of MSG monolayers. The buffer used was 1 mM Tris, pH 7. The lysozyme concentration in the subphase was 2 μM.

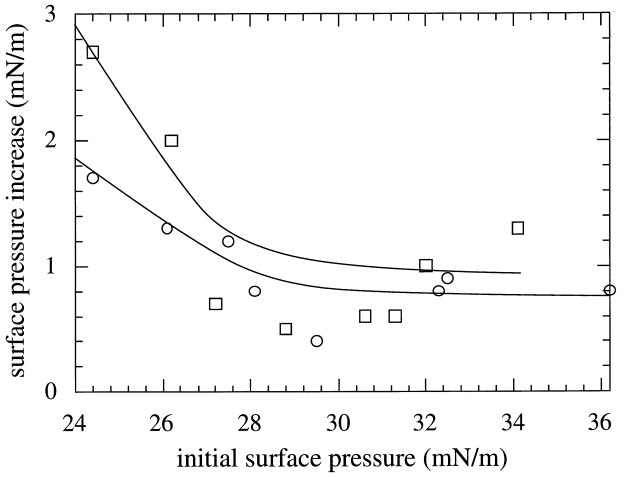


Fig. 5. Lysozyme induced $\Delta\pi$ as function of the π_i of MPG/DCP (squares) or MSG/DSP (circles) monolayers. The buffer used was 1 mM Tris, pH 7. The lysozyme concentration in the subphase was 2 μM.

MPG/DCP, MSG/DSP or MPG/DSP monolayers at a π_i higher than 32 mN/m. Apparently the shorter acylchain length of DCP promotes the insertion of β-LG into an MSG monolayer.

Insertion experiments with lysozyme were performed at pH 7 where, similar to β-LG at pH 4, the protein is positively charged. As can be seen in Fig. 3 lysozyme is able to insert into a neutral MSG monolayer up to very high π_i. Extrapolation of the data reveals a cut off pressure for the insertion of 39 mN/m which is much higher than reported for β-LG in pure MSG [10]. This demonstrates the profound interaction between lysozyme and monoglycerides. The efficient penetration of lysozyme into monoglyceride monolayers with a very high surface pressure can also be observed with monoglyceride monolayers containing negatively charged amphiphiles. Fig. 4 shows that lysozyme can insert into MSG/DCP monolayers above a π_i of 32 mN/m but only under conditions of low ionic strength. Adding 100 mM NaCl to the subphase abolishes the ability of lysozyme to insert at a π_i above 32 mN/m pointing to the importance of the surface charge for this interaction. This is similar to the behavior of β-LG interacting with the same monolayer (Fig. 2, [10]). However, in contrast to β-LG the insertion of lysozyme did not appear to have any lipid specificity as shown in Fig. 5. Lysozyme is also able to insert into MSG/DSP and MPG/DCP monolayers at a π_i higher than 32 mN/m, but again not under conditions of high ionic strength (not shown). This shows that the negative surface charge is important for the interaction of both proteins with monoglyceride monolayers.

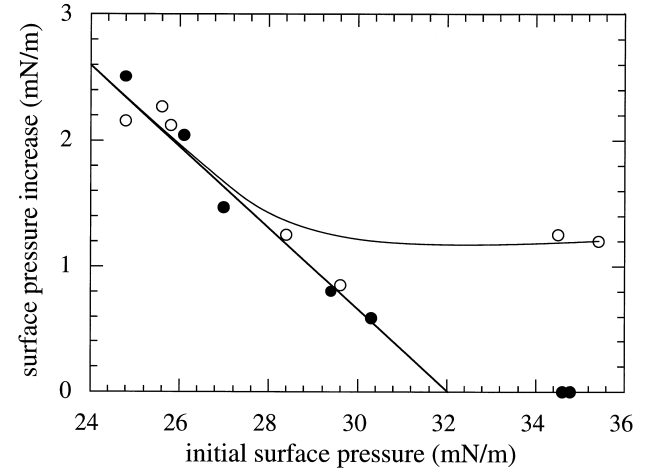


Fig. 4. Lysozyme induced $\Delta\pi$ as function of the π_i of MSG/DCP monolayers. The buffer used was 1 mM Tris, pH 7, either with (closed circles) or without (open circles) 100 mM NaCl. The lysozyme concentration in the subphase was 2 μM.

3.2. *Interaction of β-LG and lysozyme with monoglyceride bilayers*

We next investigated the interaction of both proteins with monoglyceride bilayers using ITC. Fig. 6 shows the ITC curves obtained for the binding of

β-LG to MSG/DSP bilayers at pH 7 where the protein carries a net negative charge. The curve could be fitted to a single binding site model [20], which is depicted by the solid line in the lower panel, allowing the determination of the stoichiometry and affinity of the interaction. The exact enthalpy of the interaction can, however, not be determined accurately from this curve because saturation effects already occur after the first addition of β-LG. Nevertheless, the signal of the first titration step does give a lower limit for the actual ΔH value. Integration of this signal (Fig. 6, lower panel) gives a ΔH of -436 kcal mol^{-1} for this particular titration step. This is very large compared to the values determined for other lipid–protein interactions [21] which are usually much smaller than -100 kcal mol^{-1}. Because of the saturation effects, an accurate determination of the ΔH requires another setup of the ITC experiment. The common approach for this is to either increase the lipid concentration in the cell or to reverse the titration setup by filling the cell with the protein solution and the syringe with the lipid dispersion. Both approaches proved to be impractical as a result of the viscosity of the dispersions. Therefore, and because the ΔH is apparently very high, we decided to lower the protein concentration in the syringe 50-fold. We were indeed able to obtain a measurable signal with such a low protein concentration (Fig. 7). Importantly, no saturation is observed meaning that every added protein molecule binds to the lipids and that the enthalpy of the interaction can be obtained by integration of the signal.

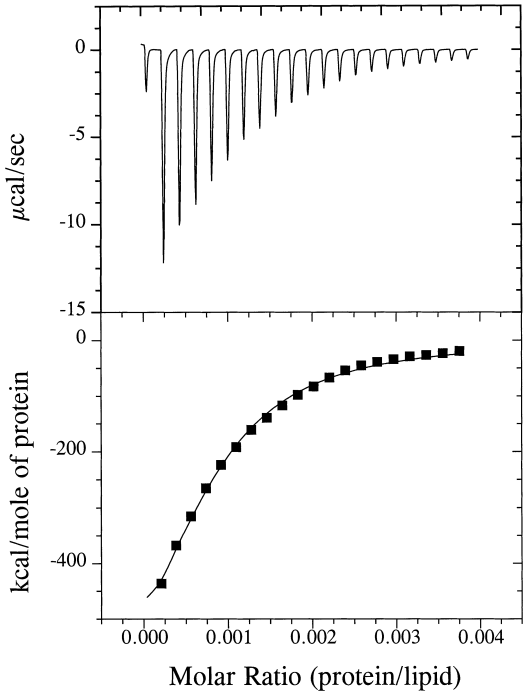


Fig. 6. Binding of β-LG to a 10 mol% DSP containing MSG dispersion at pH 7 and 20°C as determined with ITC. The top panel shows the calorimetric trace while the bottom panel shows the heat of reaction as evaluated from the peak areas. The solid line corresponds to the theoretical binding isotherm. The buffer used was 1 mM Tris, pH 7. The total lipid concentration (MSG+DSP) in the cell was 5 mM. The β-LG concentration in the syringe was 116 μM.

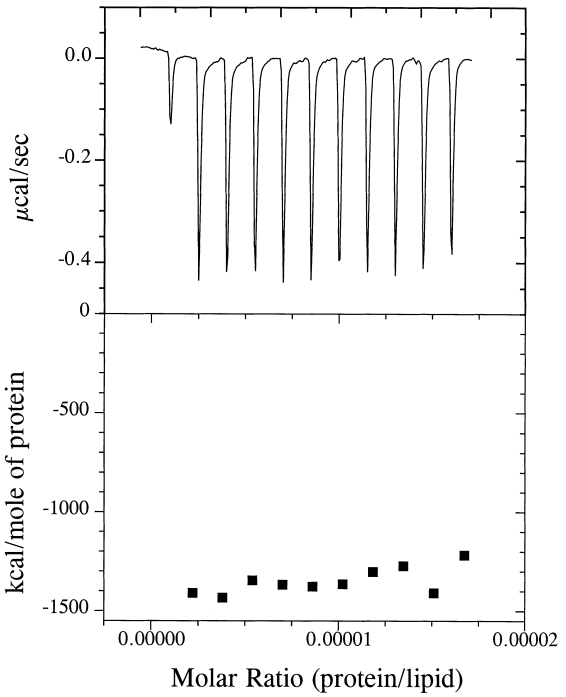


Fig. 7. Determination of the enthalpy of the binding of β-LG to a 10 mol% DSP containing MSG dispersion at pH 7 and 20°C. The top panel shows the calorimetric trace while the bottom panel shows the heat of reaction as evaluated from the peak areas. The buffer used was 1 mM Tris, pH 7. The total lipid concentration (MSG+DSP) in the cell was 5 mM. The β-LG concentration in the syringe was 2 μM.

The parameters for the interaction of β-LG with all tested gel phase monoglyceride bilayers are listed in Table 1 which also include measurements at pH 4. The parameters obtained with MSG/DCP are comparable to the results of a previous study [11] showing that the modification in the sonication protocol (see Section 2) does not affect the interaction with

Table 1
Binding parameters and enthalpies of the interaction of β-LG with 10 mol% negatively charged amphiphile containing monoglyceride dispersions at pH 4 and pH 7 and 20°C

	β-Lactoglobulin at pH 4				β-Lactoglobulin at pH 7			
	N (lipid/protein)	K_d (μM)	Δ*H* (kcal/mol β-LG)	Δ*H* (kcal/mol lipid)	*N* (lipid/protein)	K_d (μM)	Δ*H* (kcal/mol β-LG)	Δ*H* (kcal/mol lipid)
MSG/DCP	1592	3.9	−697	−0.43	2512	5.1	−1120	−0.44
MSG/DSP	1919	2.2	−630	−0.33	2341	3.4	−1270	−0.54
MPG/DCP	649	3.3	−459	−0.70	1083	3.6	−1090	−1.01

The binding enthalpies are expressed per mole of β-LG as well as per mole of total lipid (amphiphile+monoglyceride) which is derived by dividing the binding enthalpy per mole of β-LG by the stoichiometry (lipid/protein) of the interaction. The listed values are the average of three independent experiments. The standard errors were not more than 10%.

β-LG. The overall conclusion to be drawn from Table 1 is that β-LG binds to the tested monoglyceride bilayers at pH 4 and pH 7 with comparable affinities, stoichiometries and thermodynamic parameters. However, small but systematic differences are observed. The stoichiometries observed at pH 4 are all smaller than those observed at pH 7. This means that more protein binds to the bilayer at pH 4 which points to the importance of the electrostatic attraction between the positively charged protein and the negatively charged bilayer. Also more β-LG binds to MPG bilayers than to MSG bilayers which is most likely related to the expected slightly looser lipid packing of MPG compared to MSG [10]. The Δ*H* values when expressed per mol of β-LG are all very large and exothermic. Since they can originate from a structural transition of the lipids (see [11] and further results) it is more informative to express the Δ*H* per mole of lipid. This value is derived by dividing the Δ*H* value per mole of protein by the stoichiometry in lipids per protein. The data now show a pronounced difference between the 18 (MSG) and 16 (MPG) carbon acylchain length monoglyceride systems. Much more energy is released upon the interaction of β-LG with the shorter chain systems which is mainly due to the binding of larger amounts of protein to the MPG bilayers. No systematic difference is observed when comparing the Δ*H* values per mole of lipid at pH 4 and pH 7. The data therefore seem to indicate that at both pH values the same structural transition takes place within a given lipid system.

Next, the interaction of lysozyme with dispersed monoglyceride bilayers was studied with ITC at pH 7. The resulting binding parameters are listed in Table 2. Comparing these results with the binding parameters listed in Table 1 shows that they are rather similar to those observed for β-LG at pH 4. The same differences in behavior between MPG and MSG are observed, further indicating that they result from differences in properties of the lipids and not of the proteins.

3.3. Structural consequences

The structural consequences of the interaction of both proteins with the monoglyceride bilayer systems

Table 2
Binding parameters and enthalpies of the interaction of lysozyme with 10 mol% negatively charged amphiphile containing monoglyceride dispersions at pH 7 and 20°C

	Lysozyme at pH 7			
	N (lipid/protein)	K_d (μM)	Δ*H* (kcal/mol lysozyme)	Δ*H* (kcal/mol lipid)
MSG/DCP	1259	4.1	−578	−0.46
MSG/DSP	1972	4.1	−789	−0.40
MPG/DCP	649	13.2	−686	−1.06

The binding enthalpies are expressed per mole of lysozyme as well as per mole of total lipid (amphiphile+monoglyceride) which is derived by dividing the binding enthalpy per mole of lysozyme by the stoichiometry (lipid/protein) of the interaction. The listed values are the average of three independent experiments. The standard errors were not more than 10%.

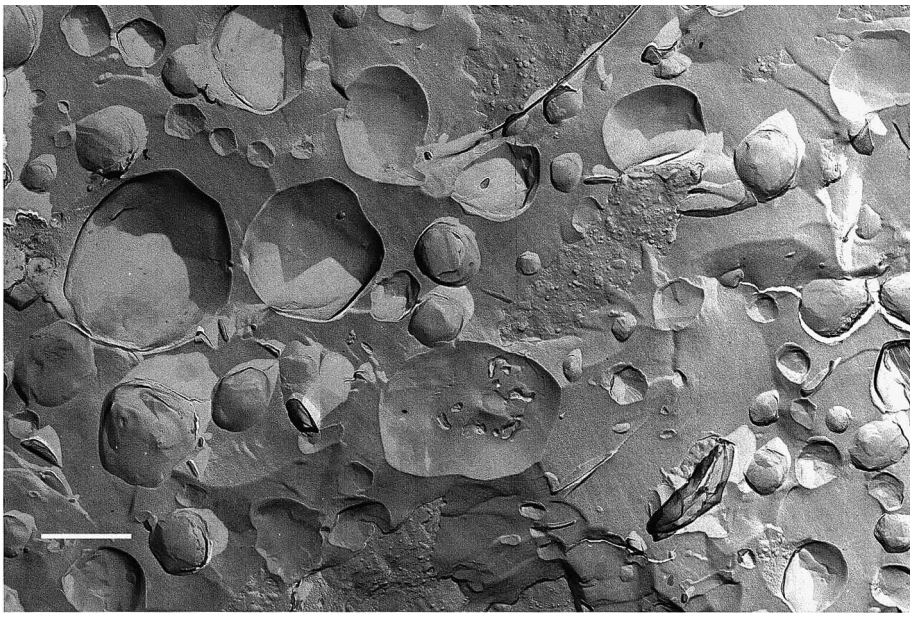
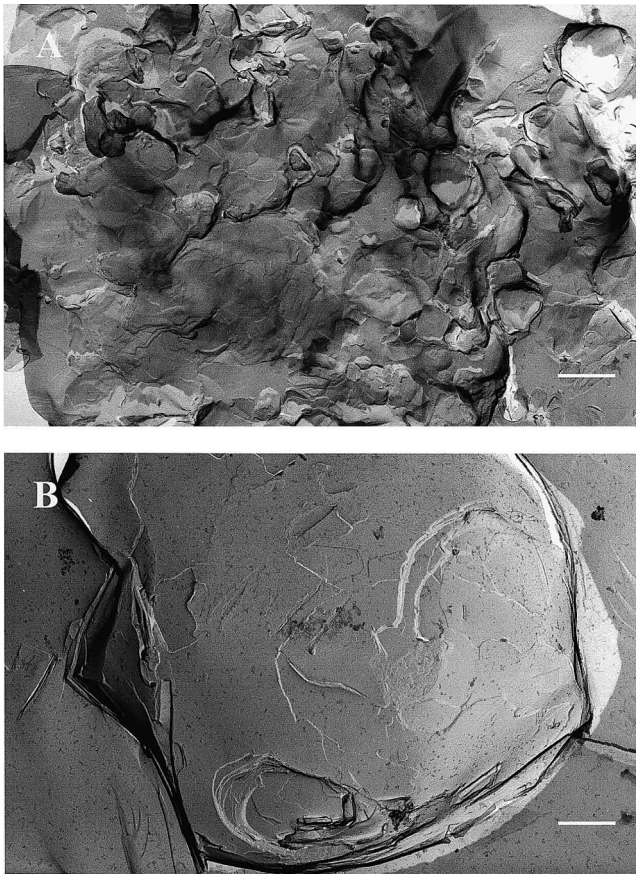


Fig. 8. MSG/DCP (9:1) vesicles at pH 7 as visualized by freeze fracture EM. Bar = 500 nm.

used was investigated with freeze fracture EM and ^{2}H-NMR. Fig. 8 shows a freeze fracture EM image of an MSG/DCP dispersion quenched from room temperature. The dispersion consists of vesicles with a size ranging from 90 to 1100 nm. The absence of intravesicular fracture faces indicates that the vesicles are unilamellar. Interestingly, the vesicles have a facetted structure. Possibly this can be attributed to the lipids being in the gel phase because such a tightly packed bilayer may not be easily bent. Dispersed MPG/DCP and MSG/DSP bilayers were found to consist of unilamellar vesicles of similar morphology and size (not shown). The addition of either lysozyme (Fig. 9A) or β-LG (not shown) changes the appearance of the dispersions dramatically. Instead of single unilamellar vesicles, large lumps of aggregated lipid structures are now observed. Vesicle-like structures are still present at the rim of the aggregate but other parts have a smoother appearance and seem to consist of tightly stacked



→

Fig. 9. (A) MSG/DCP (9:1) vesicles at pH 7 in the presence of lysozyme as visualized by freeze fracture EM. The lipid particle consists of a rim of aggregate vesicles and a coagel core. (B) Freeze fracture image of pure MSG in the coagel consisting of stacked bilayers. Bars are 500 nm.

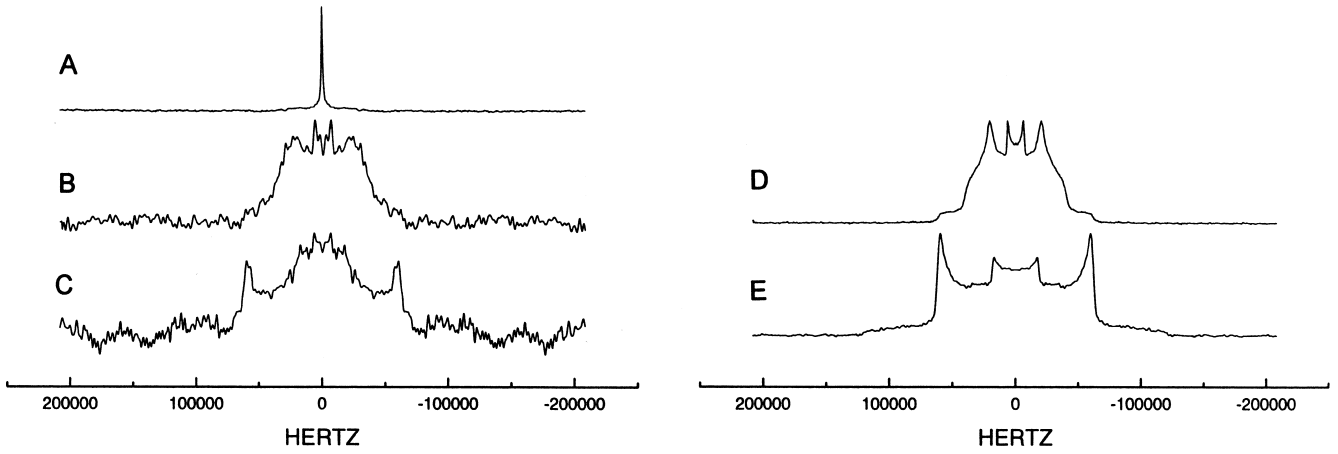


Fig. 10. ^{2}H-NMR spectra of dispersed $^2H_{35}$-MSG containing no or 10 mol% DCP at pH 4 and 20°C. The left panel shows the spectra of MSG/DCP vesicles in the absence of protein (A), just after the addition of β-LG (B) and after 5 h after the addition of lysozyme (C). The molar lipid-to-protein ratio was 100:1. The right panel shows the spectra of pure $^2H_{35}$-MSG in the gel phase (D) and coagel phase (E).

sheets. For comparison Fig. 9B shows a freeze fracture image of MSG in the coagel phase, which also consists of large sheets of tightly stacked bilayers. This suggests that the core of the lipid aggregate shown in Fig. 10A consists of the monoglycerides in the coagel and that the vesicle-like structures at the rim of the aggregate are intermediates in the L_β to coagel phase transition.

In order to obtain further insight into the influence of both proteins on the organization of the lipids, ^{2}H-NMR was applied to dispersions made of chain deuterated monoglycerides. ^{2}H-NMR can give information on the molecular ordering and phase structure of lipid systems such as monoglycerides [22,23]. In the absence of protein an isotropic signal is obtained (Fig. 10A), even though the monoglycerides are in the L_β phase. This indicates that the tumbling of the vesicles is apparently fast enough to average the anisotropy of the quadrupolar interaction. Upon addition of β-LG the isotropic signal is lost and transiently transforms into the broad spectrum depicted in Fig. 10B, suggesting that the tumbling of the vesicles is now arrested. The spectrum is characterized by a doublet with a quadrupolar splitting of 11 kHz and a doublet with a larger splitting of 50 kHz. These spectral features are characteristic for MSG in the gel phase as shown in Fig. 10D. The component with the larger quadrupolar splitting results from the methylene deuterons. The narrower splitting originates from the three deuterons of the chain terminal methyl group which have additional rotational freedom. Eventually the spectrum shown in Fig. 10C appears, which is even broader than the spectrum shown in Fig. 10B, indicating an increase in the molecular ordering of the monoglycerides. This spectrum is also characterized by two doublets which originate from the methylene and methyl deuterons of the acylchain but the quadrupolar splittings are larger, 35 and 120 kHz, respectively. These spectral features are characteristic for the coagel phase (Fig. 10E) in which the motional freedom of the monoglycerides is reduced due to interbilayer hydrogen bonds [12]. Similar results were obtained with lysozyme as well as with the other monoglyceride systems (not shown) which indicates that both MSG and MPG respond similarly to the binding of protein.

4. Discussion

This study was aimed to answer the questions: what is the specificity of monoglyceride–protein interactions and what is the underlying molecular mechanism? By using two different gel phase forming monoglycerides and two different water soluble proteins we were able to show the generality of the interaction and its consequences as well as some interesting differences. We will first discuss the lipid and protein specific effects and next the more general structural consequences that allow us to pro-

pose a mechanism for the protein induced phase transition.

4.1. Lipid specificity

Our current results show that the composition of the monolayer has an effect on the insertion of β-LG but not of lysozyme. Compared to MSG/DCP monolayers [10] the insertion of β-LG into both MPG/DCP, MSG/DSP and MPG/DSP monolayers is clearly reduced, in particular above 32 mN/m where β-LG does not insert at all. Given these results an explanation must lie in the unique packing properties of an MSG/DCP monolayer which are the result of the shorter acylchains of DCP. Two scenarios seem to be possible. Firstly, if the acylchains would all be lined up at the methyl ends then the shorter acylchains of DCP could cause small undulations in the interface of an MSG monolayer which could allow an electrostatic interaction to pull the protein into the monolayer. Alternatively, if the position of the polar headgroup is in line with that of the monoglyceride, the shorter acylchain of DCP could allow it to be pushed more easily into the monolayer. Both possibilities would provide a way for β-LG to penetrate into an MSG/DCP monolayer which is absent in the other monolayers. Because lysozyme is intrinsically more able to insert, its insertion is independent on the packing properties of the monolayer.

Lipid specificity is also apparent for the interaction of both β-LG and lysozyme with monoglyceride bilayers. A significant difference in ΔH per mole of lipid and a difference in stoichiometry can be observed when comparing the MPG containing bilayers with both MSG containing bilayers with more protein molecules binding to MPG. This is probably related to an intrinsic difference between MPG and MSG which allows more protein binding sites to be exposed. For instance, a likely possibility would be that the shorter acylchain of MPG results in a slightly looser packing of the MPG bilayers compared to MSG bilayers as was suggested previously [10].

4.2. Protein specificity

The differences in behavior between β-LG and lysozyme are most apparent in our monolayer results. A striking observation is the ability of lysozyme to insert into a neutral MSG monolayer at very high surface pressures. This observation suggests that lysozyme will also interact and insert into more loosely packed monoglycerides such as in the liquid crystalline phase. It is therefore very likely that lysozyme interacts with monoglycerides in the cubic phase as used for its crystallization [8]. Since no surface charge is present the interaction of proteins with neutral MSG monolayers is likely to be hydrophobic in nature. Because lysozyme inserts more efficiently into neutral monolayers than β-LG [10], lysozyme has apparently more hydrophobic side chains exposed. However, inspection of the structures of lysozyme [24] and β-LG [25] does not reveal a clear difference in hydrophobicity of the surfaces of the two proteins. The exposure of more hydrophobic side chains therefore probably involves some structural changes of lysozyme upon the interaction with the monoglycerides. Possibly this is related to the overall lower stability of lysozyme compared to β-LG as shown by the reversible thermal unfolding of the two proteins [26]. Also, in mixtures of monoglycerides with negatively charged amphiphiles, lysozyme appeared to have a higher penetrating power than β-LG. The effect of a high ionic strength clearly showed the importance of electrostatics also for this interaction. Therefore, lysozyme, like β-LG [10], probably has a preference for the negatively charged amphiphiles when these are present in a monoglyceride monolayer.

Electrostatic interactions also appear to be important for the interaction of lysozyme and β-LG with monoglyceride bilayers. Firstly, this follows from the comparable binding parameters found with ITC for lysozyme and β-LG, because the one feature that β-LG and lysozyme have in common is their comparable number of positively charged residues. These residues will most likely interact with the negatively charged amphiphiles which fits with our monolayer results. Secondly, at pH 4 more β-LG binds to the bilayer than at pH 7 which is probably caused by the overall electrostatic attraction of the positively charged protein to the negatively charged bilayer. The larger amount of bound β-LG molecules at low pH probably relates to the increased protein concentration near the charged surface of the bilayer as described by Seelig [21] and the additional bound

β-LG molecules found with a direct binding assay as described in our previous paper [11].

4.3. Structural consequences of the interaction

The observed binding enthalpies indicate that structural changes are taking place upon the interaction of β-LG or lysozyme with the monoglyceride bilayers. Since the ΔH values per mol of protein are too large to be explained by binding or insertion of the proteins or by unfolding of the proteins [11] these structural changes must involve the lipids. Indeed, our NMR and freeze fracture EM studies show that in all cases the proteins induce an L_β to coagel monoglyceride phase transition. This suggests that the L_β to coagel transition is a general response of negatively charged amphiphile containing gel state monoglycerides to soluble proteins and is not restricted to the β-LG, MSG and DCP system which we used in our previous study [11]. The transition is strongly exothermic and since the L_β phase is metastable the effect of the proteins can be understood as a catalysis of the L_β to coagel transition. When comparing the ΔH values a larger enthalpy per mol of lipid for the MPG containing bilayer compared to both MSG containing bilayers can be observed. In principle this could indicate a difference in ΔH value of the L_β to coagel phase transition of MPG and MSG. However, this is unlikely since the ΔH values of phase transitions in related systems suggest that the ΔH value of MPG should be lower than that of MSG [27]. Therefore, the difference in ΔH indicates that the extent of the protein induced L_β to coagel phase transition is larger in MPG than in MSG containing bilayers. Nevertheless, also with MPG the transition is only partial because the ΔH values per

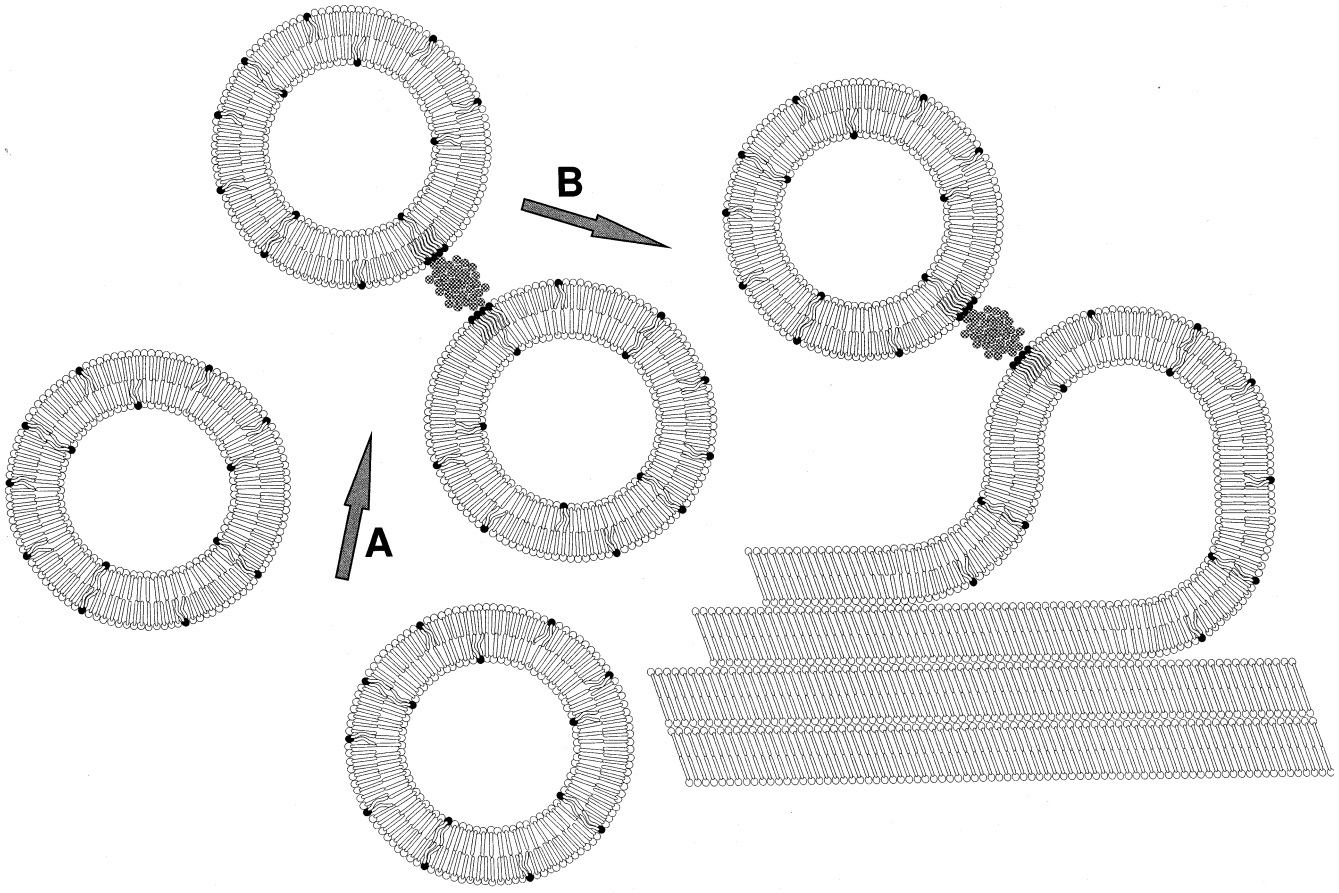


Fig. 11. Proposed mechanism of the protein induced monoglyceride L_β to coagel phase transition. Depicted are the initial protein mediated vesicle aggregation (A) and subsequent stacking of the bilayers (B). The putative fusion of the bilayers and segregation of the negatively charged amphiphiles are also depicted.

mole of lipid are all much smaller than the −5 kcal mol^{-1} associated with a complete L_β to coagel phase transition of MPG [9]. The reason for the larger amount of coagel formation in the case of MPG is probably the exposure of more protein binding sites since the ΔH values per mole of protein are similar.

4.4. Mechanism of the protein induced phase transition

Our ^{2}H-NMR results clearly show that after the addition of either β-LG or lysozyme to the vesicles the isotropic signal does not transform directly into a coagel spectrum but proceeds via a spectrum which is indicative for monoglycerides in the gel phase. This means that the mechanism of coagel formation has two steps and we therefore propose the following mechanism (Fig. 11). Firstly, the addition of protein results in the aggregation of the vesicles as seen at the rim of the lipid aggregate shown in Fig. 10A. After the aggregation the bilayers which are now close to each other form the stacks which are characteristic for the coagel. Since the dimensions of these stacks are larger than those of the initial vesicles this step probably also includes bilayer fusion. The proteins are responsible for the initial aggregation step because their presence destabilizes the vesicles. The aggregation of the vesicles cannot be the result of the charge of the protein neutralizing the negative charge of the bilayer because it is also observed at pH 7 where β-LG has an overall negative charge. Probably the proteins bring the bilayers close to each other by simultaneously binding to two or more vesicles. The resulting close proximity of the bilayers could facilitate the eventual formation of the coagel. However, the negative surface charge is likely to prevent this, in particular at very close range. The proteins may therefore also play a role in this step because they interact with the negatively charged amphiphiles in the bilayer. As a result of this interaction the negatively charged amphiphiles could become segregated from the monoglycerides which would destabilize the vesicle and facilitate interbilayer stacking characteristic for the coagel.

In conclusion, our studies have revealed several novel and unexpected aspects of the interactions of monoglycerides and soluble proteins. The results obtained deepen our molecular understanding of monoglyceride–protein interactions which is of relevance for the application of monoglycerides, including the crystallization of proteins from monoglyceride systems.

Acknowledgements

The authors would like to thank Professor Dr. A. Verkleij for the use of and helping with the freeze fracture EM. Supported by the Netherlands Organization for Scientific Research (NWO), CW/STW project 349-4608.

References

[1] E.S. Lutton, J. Am. Oil Chem. Soc. 42 (1965) 1068–1070.
[2] N. Krog, K. Larson, Chem. Phys. Lipids 2 (1968) 129–143.
[3] I. Pezron, E. Pezron, B.A. Bergenstahl, P.M. Claesson, J. Phys. Chem. 94 (1990) 8255–8261.
[4] B. de Kruijff, Nature 386 (1997) 129–130.
[5] E.M. Landau, J.P. Rosenbusch, Proc. Natl. Acad. Sci. USA 93 (1996) 14532–14535.
[6] E. Pebay-Peyroula, G. Rummel, J.P. Rosenbush, E.M. Landau, Science 277 (1997) 1676–1681.
[7] G. Rummel, A. Hardmeyer, C. Widmer, M.L. Chiu, P. Nollert, K.P. Lochner, I. Pedruzzi, E.M. Landau, J.P. Rosenbush, J. Struct. Biol. 121 (1998) 82–91.
[8] E.M. Landau, G. Rummel, S.W. Cowan-Jacob, J.P. Rosenbush, J. Phys. Chem. B 101 (1997) 1935–1937.
[9] Krog, N.J. (1990) in: Food Emulsions (Larsson, K., Friberg, S.E., Eds.), pp. 127–202, Marcel Dekker, New York.
[10] J.M. Leenhouts, R.A. Demel, B. de Kruijff, J.-W.P. Boots, Biochim. Biophys. Acta 1330 (1997) 61–70.
[11] J.-W.P. Boots, V. Chupin, J.A. Killian, R.A. Demel, B. de Kruijff, Biochim. Biophys. Acta 1420 (1999) 241–251.
[12] G. Cassin, C. de Costa, J.P.M. van Duynhoven, W.G.M. Agterhof, Langmuir 14 (1998) 5757–5763.
[13] W. Gou, J.A. Hamilton, Biophys. J. 64 (1995) 1383–1395.
[14] D. Buchnea, Lipids 6 (1971) 734–739.
[15] D. Tsiourvas, D. Kardassi, C.M. Palfos, S. Gehant, A. Skoulios, Liq. Cryst. 23 (1997) 269–274.
[16] H. Mach, C.R. Middaugh, R.V. Lewis, Anal. Biochem. 200 (1992) 74–80.
[17] R.A. Demel, Subcell. Biochem. 23 (1994) 83–120.
[18] M.J. Costello, R. Fetter, M. Hochli, J. Microsc. 125 (1982) 125–136.
[19] J.H. Davis, K.R. Jeffrey, M. Bloom, M.I. Volic, T.P. Higgs, Chem. Phys. Lett. 42 (1976) 390–394.
[20] T. Wiseman, S. Williston, J.F. Brandts, L. Lin, Anal. Biochem. 179 (1989) 131–137.

[21] J. Seelig, Biochim. Biophys. Acta 1331 (1997) 103–116.
[22] J.H. Davis, K.R. Jeffrey, Chem. Phys. Lipids 20 (1977) 87–104.
[23] J. Seelig, Q. Rev. Biophys. 10 (1977) 353–418.
[24] L.J. Smith, M.J. Sutcliffe, C. Redfield, C.M. Dobson, J. Mol. Biol. 229 (1993) 930–944.
[25] M.Z. Papiz, L. Sawyer, E.E. Eliopoulos, A.C.T. North, J.B.C. Findlay, R. Sivaprasadarao, T.A. Jones, M.E. Newcomer, P.J. Kraulis, Nature 324 (1986) 383–385.
[26] L.L. Lee, J.C. Lee, Biochemistry 26 (1987) 7813–7819.
[27] D.R. Kodali, T.G. Redgrace, D.M. Small, D. Atkinson, Biochemistry 24 (1985) 519–525.