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The effect of protein stability on protein–monoglyceride interactions

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Abstract

We have previously shown that proteins such as β-lactoglobulin and lysozyme insert into monoglyceride monolayers and are able to induce an L_β to coagel phase transition in monoglyceride bilayers. These studies gave a first indication that protein stability could be an important factor for these interactions. This study therefore aims at further investigating the potential role of protein stability on protein–monoglyceride interactions. To this end we studied the interaction of stable and destabilized α-lactalbumin with monostearoylglycerol. Our results show that protein stability is important for the insertion of proteins into a monostearoylglycerol monolayer, such that the lower the stability of the protein the better the protein inserts. In marked contrast to β-lactoglobulin and lysozyme we found that destabilized α-lactalbumin does not induce the L_β to coagel phase transition in monoglyceride bilayers. We propose that this is due to an increased surface coverage by the protein which could result from the unfolding of the protein upon binding to the interface.

Keywords: Protein stability; Protein–monoglyceride interactions; β-Lactoglobulin

1. Introduction

Monoglycerides in the gel phase are able to form densely packed bilayer structures which can enclose large aqueous compartments (Lutton, 1965; Krog, 1990; Pezron et al., 1990). These monoglyceride systems are extensively used for the production of low-fat products such as margarine. Proteins are also an important part of these products and are located in the aqueous phase but may also interact with the monoglycerides and thereby influence the properties of the monoglycerides.

In our previous studies (Leenhouts et al., 1997; Boots et al., 1999, 2001) we showed that β-lactoglobulin and lysozyme are able to insert into densely packed monoglyceride monolayers. Furthermore, we have shown that when added to gel phase monoglyceride bilayers these proteins induce an L_β to coagel phase transition of the monoglycerides. β-Lactoglobulin and lysozyme were chosen because they are well-studied water-soluble globular proteins of similar size but are

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sequentially, structurally and functionally unrelated. Despite the lack of an apparent homology between these two proteins we observed no protein specificity in the interaction with monoglyceride bilayers. However, the insertion into monoglyceride monolayers was protein dependent. Lysozyme was found to insert significantly better than β-lactoglobulin. When comparing the two proteins the lower stability of lysozyme (Lee and Lee, 1987) could be an explanation because this would better allow hydrophobic residues buried in the core of the protein to interact with the monolayer. In order to verify this hypothesis we decided to extend our study to include a protein whose stability can be easily influenced. For this we used α-lactalbumin which is a structural analogue of lysozyme. In contrast to lysozyme, however, α-lactalbumin has a calcium binding site and the presence or absence of calcium dramatically affects its stability (Griko et al., 1994). In this study, we show that protein stability is important for the interaction of proteins with monoglyceride monolayers. Surprisingly, α-lactalbumin did not induce the L_β to coagel phase transition, implying that protein stability is also a very important for the interaction of proteins with monoglyceride bilayers.

2. Materials and methods

2.1. Materials and general analytical methods

Bovine α-lactalbumin, 1-monostearoyl-*rac*-glycerol and dicetylphosphate were obtained from Sigma Chemical Company (St. Louis, MO, USA) and were used without further purification. All other materials were from Merck (Darmstadt, Germany). α-Lactalbumin stock concentrations were determined spectroscopically at 280 nm using an $E^{1\%}$ of 20.3 calculated according to Mach et al. (1992)).

2.2. 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. The buffer used was 20 mM Tris, pH 7 with either 1 mM $CaCl_2$ or 1 mM EDTA. These concentrations of $CaCl_2$ and EDTA are sufficient to either saturate or decalcify α-lactalbumin (Hendrix et al., 2000). An appropriate amount of a lipid stock solution, dissolved in $CHCl_3$/MeOH (3:1 v/v), was spread on the subphase to give the desired initial surface pressure. Thirty microliter of a 10 mg/ml protein stock solution was injected under the monolayer through a separate hole in the Teflon dish. The final protein concentration in the subphase was 4 μM. Addition of more protein did not result in larger increases of the surface pressure. The subphase was continuously stirred with a magnetic bar.

2.3. Isothermal titration and differential scanning calorimetry

DSC and ITC experiments were performed using an MCS from MicroCal (Northampton, MA, USA). For DSC 70 μM α-lactalbumin solutions were prepared in 20 mM Tris, pH 7 with either 1 mM $CaCl_2$ or 1 mM EDTA. The samples were scanned between 15 and 90 °C at a rate of 60 °C/h. For ITC, lipid dispersions of 5 mM monostearoylglycerol containing 10 mol% diacetylphosphate in 1 mM Tris, pH 7 and 1 mM EDTA were prepared as described earlier (Boots et al., 2001). Higher monoglyceride concentrations resulted in solutions which were too viscous to be used in the ITC whereas higher buffer concentrations resulted in a poor signal-to-noise ratio in the ITC experiments. The used buffer concentration was found to be sufficient to keep the pH constant. All ITC experiments were performed at 20 °C. All solutions were degassed under vacuum before usage. For each experiment, the lipid dispersion was placed in the 1.345 ml reaction cell. A 117 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.

2.4. 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. (1982). A CM10 electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV was used for examining the replicas.

2.5. ^2H NMR

NMR spectra were recorded on a Bruker Avance 500 WB spectrometer. ^2H NMR spectra (76.8 MHz) were obtained using a high power 7.5 mm probe. A quadrupolar echo technique (Davis et al., 1976) was used with a 3 μs $\pi/2$ pulse, a 25 μs τ delay, a 15 s recycling delay and quadrature detection. The long recycling delay was used to avoid the possible saturation of the CD_2-resonances which are relaxing very slowly in the coagel phase (Chupin et al., 2001). Between 500 and 2000 free induction decays were accumulated. An exponential multiplication with a line broadening factor of 300 Hz was used before performing the Fourier transformation. All ^2H 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.

3. Results and discussion

In order to determine the effect of protein stability on protein–monoglyceride interactions we studied the effect of the presence and absence of calcium on the interaction of α-lactalbumin with monoglycerides. We adopted this approach because the more traditional approaches such as adding denaturants like urea or applying heat may also affect the monoglycerides (Chupin et al., 2001). α-Lactalbumin has a very high affinity for calcium at and above neutral pH (Griko and Remeta, 1999; Hendrix et al., 2000) and removing it requires either the addition a strongly calcium chelating compound, such as EDTA, or lowering of the pH. Although both these conditions strongly destabilize α-lactalbumin the effect on the structure of the protein is not the same. Above neutral pH in the presence of EDTA the protein is still compact and can be described as a destabilized native form while lowering the pH leads to the formation of a molten globule (Griko et al., 1994). Since we only aim at studying the effect of destabilization on the interaction with monoglycerides the destabilized protein should be as close to the native state as possible. We therefore decided to use EDTA and perform the experiments at pH 7 which is the pH value used in our previous experiments with β-lactoglobulin and lysozyme (Leenhouts et al., 1997; Boots et al., 2001).

First we checked the effect of calcium on the stability of α-lactalbumin with DSC. In the presence of 1 mM $CaCl_2$ we observed the thermal unfolding of the protein at 64 °C while in the presence of 1 mM EDTA this occurred at 34 °C (not shown). Both values correlate well with the reported values (Griko et al., 1994; Hendrix et al., 2000).

As can be seen in Fig. 1 calcium has a clear effect on the insertion of α-lactalbumin into a monostearoylglycerol monolayer. In the presence of calcium the protein is able to insert into the liquid condensed monolayer at initial surface pressures below 39 ± 0.7 mN/m. In the absence of calcium, i.e. in the presence of EDTA, the observed surface pressure increase is larger at all tested initial surface pressures. Moreover, α-lac-

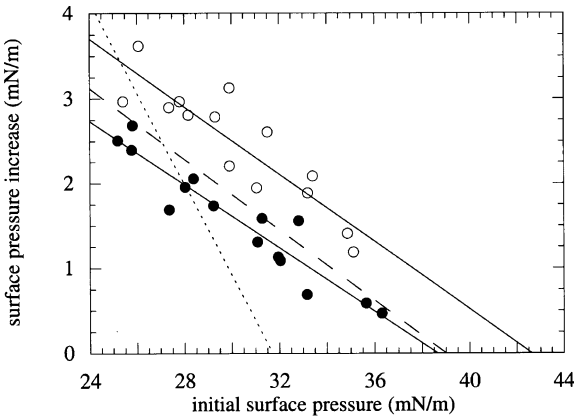

Fig. 1. α-Lactalbumin induced surface pressure increase as function of the initial surface pressure of a monostearoylglycerol monolayer in the presence (filled circles) and absence (open circles) of calcium. The buffer used was 20 mM Tris, pH 7 with either 1 mM $CaCl_2$ or 1 mM EDTA. The α-lactalbumin concentration in the subphase was 4 μM. For comparison the curves for β-lactoglobulin (dotted line, taken from (4)) and lysozyme (dashed line, taken from (6)) are also depicted.

talbumin is able to insert at higher initial surface pressures under these conditions and the limiting insertion pressure was found to be 43 ± 1.5 mN/m, which is very near the collapse pressure of 45 mN/m of a monostearoylglycerol monolayer (Leenhouts et al., 1997). For comparison we have also depicted the curves for β-lactoglobulin (Leenhouts et al., 1997) and lysozyme (Boots et al., 2001) in Fig. 1. The limiting insertion pressure of lysozyme is 39 ± 1.6 mN/m and this curve almost overlaps with the curve of α-lactalbumin in the presence of calcium. On the other hand, β-lactoglobulin only inserts below an initial surface pressure of 32 ± 0.3 mN/m. The insertion of β-lactoglobulin and lysozyme was not affected by the presence of calcium (not shown). These results together with the DSC results of α-lactalbumin and the reported (Lee and Lee, 1987) denaturation temperatures of lysozyme (74 °C) and β-lactoglobulin (90 °C) show that the relation between protein stability and monolayer insertion is such that the lower the stability of the protein is the better it inserts into a densely packed monoglyceride monolayer and vice versa. The reason why the small difference in stability between α-lactalbumin in the presence of calcium and lysozyme is not reflected in our monolayer results is probably because the difference is too small to have a detectable effect at very high initial surface pressures ($\pi_I > 32$ mN/m).

We next asked the question whether destabilization of α-lactalbumin also affects the interaction with gel phase monoglyceride bilayers. We therefore studied the interaction of α-lactalbumin with monostearoylglycerol bilayers using ITC. These experiments were performed in the presence of a negatively charged amphiphile because uncharged monoglyceride bilayers are not stable enough for these experiments (Boots et al., 1999). The experiments were conducted in the absence of calcium because negatively charged amphiphiles will strongly interact with calcium, which would complicate the interpretation of the results. As can be seen in Fig. 2, destabilized α-lactalbumin does interact with a monoglyceride bilayer. The binding can be described by a stoichiometry of 1874 ± 270 lipids per protein and a dissociation constant of 2.1 ± 0.36 μM both of which are in the same order

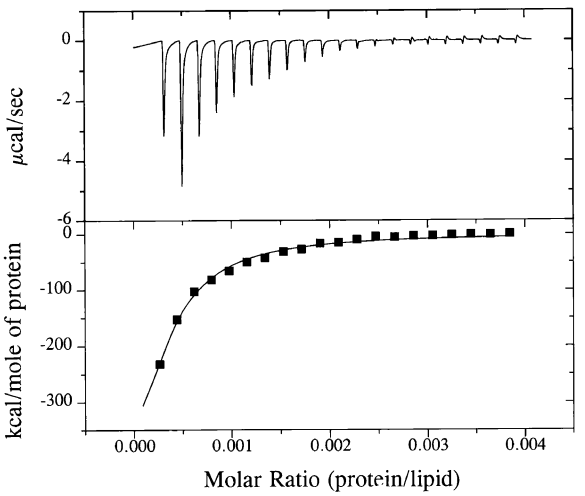

Fig. 2. Binding of α-lactalbumin a 10 mol% dicetylphosphate containing monostearoylglycerol dispersion at pH 7 and in the absence of calcium. The top panel shows the calorimetric trace while the lower panel shows the heat of reaction as evaluated from the peak areas. The solid line corresponds to the theoretical binding isotherm. The buffer used was 1 mM Tris, pH 7 with 1 mM EDTA. The total lipid concentration (monostearoylglycerol + dicetylphosphate) in the cell was 5 mM. The α-lactalbumin concentration in the syringe was 117 μM.

of magnitude as the values determined previously for β-lactoglobulin and lysozyme (Boots et al., 2001). Moreover, the binding of destabilized α-lactalbumin is accompanied by an exothermic enthalpy of -678 ± 133 kcal/mol of protein. This is very large compared with other protein–lipid interactions (Seelig, 1997) but does compare to the values of -1120 ± 28 and -578 ± 57 kcal/mol found for β-lactoglobulin and lysozyme, respectively, interacting with the same bilayer under the same condition of pH (Boots et al., 2001). This suggests that the strongly destabilized protein also induces the L_β to coagel phase transition of the monoglycerides (Boots et al., 2001). However, inspection of the resulting protein–monoglyceride complex with ^2H NMR and freeze fracture EM showed that this is not the case. The ^2H NMR spectrum of the monoglycerides after the addition of α-lactalbumin (Fig. 3A) cannot result from the coagel phase. The observed anisotropic signal is characterized by two doublets with quadrupolar splittings of 49 and 12 kHz for the methylene deuterons and terminal methyl deuterons, respectively, which is indicative for the L_β phase (Chupin et al., 2001). For comparison, the quadrupolar splittings of these deuterons in the coagel phase are

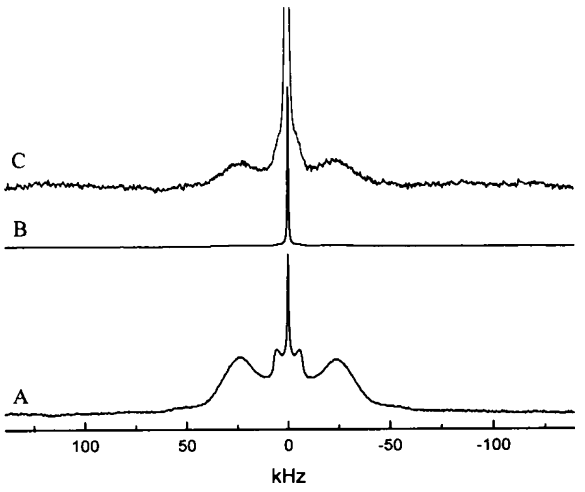

Fig. 3. ^2H NMR spectra of dispersed $^2H_{35}$-monostearoylglycerol containing 10 mol% dicetylphosphate at pH 7 and 20 °C in the presence (A) and absence (B) of α-lactalbumin. Spectrum C shows an enlarged part of spectrum B. The molar lipid-to-protein ratio was 100:1.

120 and 35 kHz, respectively. Interestingly, in contrast to what we previously found with β-lactoglobulin and lysozyme (Boots et al., 2001) ^2H NMR also showed that the L_β phase is stable for at least 1 week in the presence of α-lactalbumin. Freeze fracture EM (Fig. 4) showed unilamellar vesicles with a facetted structure and size distribution similar to those found in the absence of protein (Boots et al., 2001). No aggregated vesicles or stacks of monoglyceride bilayers were found in these samples. These results mean that α-lactalbumin does not induce coagel formation but instead stabilizes the gel phase. An explanation for this can be found in the mechanism we proposed for the monoglyceride phase transition induced by β-lactoglobulin and lysozyme (Boots et al., 2001). In this mechanism, the proteins bind to two or more vesicles and as a result bring the bilayers close together. The resulting close proximity of the vesicles allows the stacking of the bilayers which results in coagel formation. Since we observe single vesicles with freeze fracture EM α-lactalbumin apparently binds to only one vesicle. The most likely explanation for this is that the surface coverage of α-lactalbumin is much higher than that found for β-lactoglobulin (Leenhouts et al., 1997). The small differences in the stoichiometries suggests that an increased surface coverage results from the unfolding of α-lactalbumin upon binding to the interface which could result from the strongly decreased stability of the protein. This explanation does not address the large ΔH of the interaction which is too large to be explained by protein unfolding or protein–lipid interactions (Boots et al., 1999). This means that the monoglycerides must be affected by the interaction but in this case without the occurrence of a phase transition. The ^2H NMR spectrum of the monoglyceride in the absence of α-lactalbumin (Fig. 3B) is indeed different and shows a distinct isotropic signal with an anisotropic signal underneath (Fig. 3C). As can be seen this small anisotropic signal corresponds to the spectrum of the monoglycerides in the L_β phase (Fig. 3A). The isotropic signal must also arise from monoglycerides in the L_β phase and it is most likely that it originates from the smallest vesicles present in the sample in which tumbling has averaged the quadrupolar splittings

Fig. 4. Dicetylphosphate (10 mol%) containing monostearoylglycerol vesicles after the addition of α-lactalbumin in the presence of 1 mm EDTA at pH 7 as visualized by freeze fracture EM. Bar = 500 nm.

(Boots et al., 2001). The addition of α-lactalbumin shifts the spectrum to the anisotropic signal (Fig. 3A). The simplest explanation for this would be that the protein decreases the rate of isotropic movement of the monoglycerides either by slowing down vesicle tumbling or by decreasing the lateral diffusion of the monoglycerides within the bilayer. Vesicle tumbling could be slowed down by the surface adherence of the protein but is unlikely to result from vesicle aggregation because such aggregates were not observed by EM. A decrease in the rate of lateral diffusion could be caused by the surface penetration of the protein which might cause changes in the organization of the lipids within the gel state, thereby causing the large ΔH. One such possibility is that the protein changes the acyl chain tilt.

The results of our present study show the importance of protein stability for the interaction between proteins and monoglycerides, and demonstrate that destabilized proteins can largely prevent coagel formation which could be of relevance for the various applications of saturated monoglycerides.

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