

## Interaction of Phospholipids in Monolayers with $\beta$ -Lactoglobulin Adsorbed from Solution

The interaction of  $\beta$ -lactoglobulin adsorbed from solution with phospholipids in monolayers has been studied by ultraviolet and circular dichroism spectroscopy of films transferred from the air/water interface of a Langmuir film balance. The lipid monolayers consisted of mixtures of palmitoyl-oleoylphosphatidylcholine (POPC) and palmitoyl-oleoylphosphatidylglycerol (POPG).  $\beta$ -Lactoglobulin exhibited considerable adsorption to a film of POPC/POPG (65/35 mol %) at pH 4.4, but only a small amount of protein was detected in the mixed films when the subphase pH was 7. When the pressure of the lipid was below about 28 mN/m, a rise in film pressure upon injection of  $\beta$ -lactoglobulin into the subphase suggested that a portion of the protein molecule inserted into the monolayer. Circular dichroism spectroscopy showed no detectable difference between the conformation of  $\beta$ -lactoglobulin in films and solution. The results are all consistent with an electrostatic mechanism where, at pH 4.4, positively charged  $\beta$ -lactoglobulin interacts with negatively charged lipids in the monolayer to form a coating about one molecule thick.

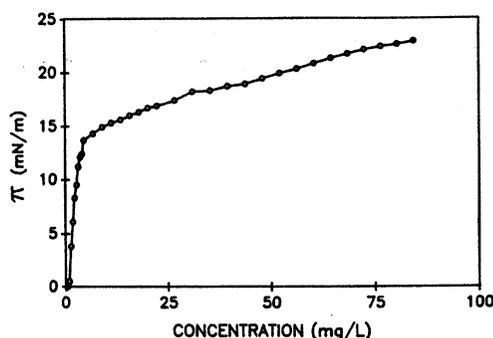
The lipid phase of milk is stabilized in part by a protective layer of proteins forming a coat on the fat globule surface (Walstra, 1983), the fat globule membrane (FGM). One picture of the FGM structure is one of electrostatic binding of the proteins to the phospholipids of milk concentrated in a layer at the fat globule surface (Keenan et al., 1982). During homogenization, the fat globules are reduced in size, with an accompanying increase in the oil/water interfacial area. This freshly exposed lipid surface is potentially able to adsorb serum (non-membrane) proteins to give a stable, highly dispersed fat phase. Much research effort has been expended in determining the emulsifying properties of food proteins, but little quantitative information on the amount and conformation of proteins adsorbed at the oil/water interface has been available, due to the difficulty in dealing with heterogeneous systems.

In this paper we report quantitative information on the amount and conformation of  $\beta$ -lactoglobulin (BLG) bound to phospholipids in a monolayer, obtained with a Langmuir film balance and specially developed tech-

niques for ultraviolet (UV) (Cornell, 1984) and circular dichroism (CD) spectroscopy (Cornell, 1979).  $\beta$ -Lactoglobulin is the major whey protein in milk, phosphatidylcholine is a major zwitterionic phospholipid in milk fat, and phosphatidylglycerol was chosen to represent the charged phospholipids because of its stability in monolayers. The pH of the subphase solution ranged from that of acid whey (4.4) to that of market milk (about 7); the calcium concentration range studied (0–30 mM) includes the maximum total (free and bound) found in milk.

### MATERIALS AND METHODS

The phospholipids were from Avanti Polar Lipids, Birmingham, AL; the tristearin and  $\beta$ -lactoglobulin were from Sigma, St. Louis, MO. The spreading solvent for the lipids was ACS reagent-grade chloroform, treated to remove surface-active impurities (Cornell, 1982). Walpole's acetate (pH 4.4) (Sober, 1970) and Tris-HCl (pH 7.0) were used at 1–5 mM concentration to buffer the subphase. The low buffer concentration was necessary to minimize light scattering in the films used for ultraviolet and circular dichroism spectroscopy.

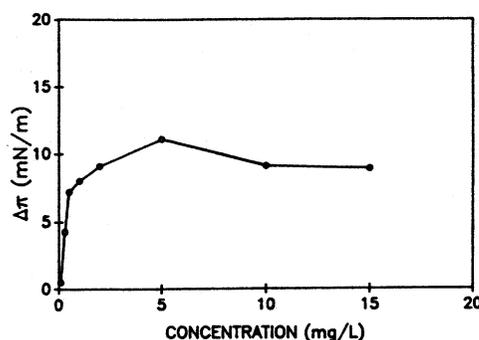


**Figure 1.** Surface pressure (decrease in surface tension) of  $\beta$ -lactoglobulin in Walpole's acetate, pH 4.4, as a function of concentration.

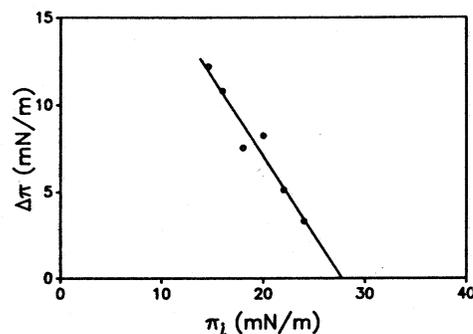
A miniature Teflon trough ( $15 \times 11 \times 0.7$  cm) with a dipping well (total depth 2 cm) for film transfer was used for this work. The capacity of the trough was about  $150 \text{ cm}^3$  with an air/water interface area  $165 \text{ cm}^2$ . A magnetic stirrer was used to mix the peptide in the subphase. The miniature trough was mounted in the cradle of a previously described (Cornell, 1982) Langmuir balance from which the large trough had been removed. The barrier drive, Wilhelmy plate, constant-pressure control, and film transfer were all provided by the large film balance system. A complete discussion of film balance techniques has been given (Gaines, 1966).

Spectroscopic observations were made on films transferred to eight quartz plates ( $20 \text{ mm} \times 12.5 \text{ mm} \times 1 \text{ mm}$ ,  $L \times W \times T$ ). One end of each plate was wrapped with Teflon tape and then inserted into a spring-loaded clamp and mounted in a rack and pinion "elevator" for lowering and raising the plate set through the air/water interface. When in place, the plates were immersed in the subphase across the width of the film balance with their faces parallel to the sides of the trough. The experimental protocol for generating the mixed monolayer was identical to steps 3 and 4 below except that the quartz plates were immersed in the subphase prior to spreading the lipid. At the end of a run, the films were removed from the surface of the water and transferred to the quartz substrate by slowly lifting the plates through the air/water interface (at a rate of 3–5 mm/min). The film was kept under constant pressure during transfer by the advancing compression barriers of the miniature trough. Details of the spectroscopic equipment and methods have been discussed (Cornell, 1979, 1984); for this work, the CD instrument was an Aviv Model 60DS spectropolarimeter.

The principal objective of this work was to estimate the amount and conformation of protein interacting with phospholipids at the oil/water interface as a function of solution conditions. Preliminary experiments were run to determine the optimum film pressure, protein concentration, etc., for UV and CD spectroscopy as follows: (1) The surface pressure (reduction in surface tension) vs the concentration of  $\beta$ -lactoglobulin in aqueous solution was determined by the Wilhelmy plate Cahn-RG microbalance technique. This established the maximum surface pressure that BLG could achieve over the range of protein concentrations studied. (2) With use of a fresh subphase, a lipid monolayer film was formed by spreading (chloroform solvent) onto the surface of water, to a pressure above the maximum achieved by the protein at the air/water interface. After a period of equilibrium, protein was injected into the subphase beneath the lipid monolayer and the pressure of the lipid monolayer was monitored for 30 min. Any pressure changes in the film usually occurred in the first few minutes of a run, with little if any change noted toward the end of the period. A series of such runs at varying protein concentration established a pressure rise–protein concentration profile for that particular lipid monolayer/subphase. These profiles were usually in the shape of a sharply rising pressure at low concentration followed by a plateau at higher concentration. (3) The pressure rise in a lipid monolayer observed upon injecting protein (using a protein concentration well into the plateau region established above) was determined on a series of monolayers at different initial pressures. These data gave a plot of pressure rise vs initial pres-



**Figure 2.** Rise in surface pressure of a phospholipid (POPC/POPG, 65/35 mol %) monolayer upon injecting BLG into the subphase. The initial pressure of the lipid film was about 22 mN/m. Subphase: Walpole's acetate, pH 4.4.



**Figure 3.** Increase in lipid monolayer pressure upon injecting BLG into the subphase as a function of the initial lipid monolayer pressure. Protein concentration in the pH 4.4 subphase was 5 mg/L.

sure that could be extrapolated to zero pressure rise at a "critical" pressure referred to as the "critical insertion pressure". (4) Experiments were run above and below the critical insertion pressure and films were transferred to quartz plates for UV and CD spectroscopy. Steps 1 and 2 were performed with a Teflon cup–Wilhelmy plate combination. Steps 3 and 4 required either closely controlled or high initial lipid film pressure. For these steps we use the miniature Langmuir trough described above, adding lipid to about 10 mN/m and then compressing the film to the desired "initial" pressure. Films formed this way showed little pressure change with time (prior to injection of protein into the subphase) and had the "correct" specific areas even for pressures approaching 40 mN/m. The subphase solution conditions were selected to assist in elucidating mechanisms of lipid–protein interaction and to simulate the pH and calcium concentrations of market milk and acid whey.

## RESULTS

The behavior of  $\beta$ -lactoglobulin at the air/water and phospholipid/water interfaces is illustrated in Figures 1–3. The data in Figure 1 represent a single run where the surface pressures were read after each injection of protein into the subphase (circles); the data shown are typical for this protein/subphase combination. In Figures 2 and 3, each circle represents an individual run where protein was injected into a freshly prepared lipid monolayer/aqueous subphase. The sets of runs represented by the data in Figures 2 and 3 were performed only once for each protein/subphase combination. The scatter of points in Figure 3 and the fact that the points in Figure 2 do not lie on an idealized adsorption asymptote may represent the error in these techniques.

The surface pressure developed by the film formed on solutions of the protein in Walpoles acetate, pH 4.4, is shown in Figure 1. An initial steep rise in the surface pressure (surface tension lowering) occurs up to about 14 mN/m at 5 mg/L concentration after which the increase

in film pressure with concentration is much less steep. The pressure continued to rise with no plateau apparent up to the limit of protein concentration (about 80 mg/L) studied here.  $\beta$ -Lactoglobulin exhibits much more surface activity at a charged lipid/water interface than at the air/water interface as shown in Figure 2. Here the initial steep rise in the surface pressure of the phospholipid monolayer (initially poised at about 18 mN/m) is complete at a protein concentration of about 1 mg/L, and a plateau in the surface pressure increase ( $\Delta\pi$ ) is reached between 3 and 5 mg/L of protein. The initial pressure of the lipid monolayer (18 mN/m) was higher than the film pressure generated by the protein at the air/water interface when its concentration was below about 50 mg/L. Lipid pressure this high should exclude the formation of pure protein patches at the air/water interface. The rise in pressure of the lipid monolayer upon injecting protein beneath the oil/water interface is thus taken to mean that a portion of the BLG molecule, either side group or backbone, is interacting with the lipid in the film with concomitant insertion into the monolayer. The increased packing of the molecules in the monolayer is observed in an increase in the film pressure. At sufficiently high initial lipid pressure, insertion of the protein into the lipid monolayer is prevented, as shown in Figure 3. The linear extrapolation of  $\Delta\pi$  vs  $\pi$  to  $\Delta\pi = 0$  (no insertion) suggests that insertion into a POPC/POPG monolayer at pH 4.4 is prevented when the initial pressure of the lipid film is above about 28 mN/m. Although insertion of the protein is prevented above 28 mN/m, interaction of the protein with the lipid does occur as indicated by the ultraviolet spectrum of transferred films (not shown). The ultraviolet spectra of lipid monolayer-protein complexes showed that the amount of protein associated with the lipid film was nearly the same for the low and high initial pressure lipid films, about 0.4 absorbance unit for 16 monolayers. The circular dichroism spectrum of BLG-lipid monolayers (low initial lipid pressure), shown in Figure 4, is very similar to the CD spectrum of BLG in solution, suggesting that there is little if any conformation change in the protein upon interacting with the monolayer lipids. This is in contrast to earlier reports where BLG was first spread as a dilute film at the air/water interface and then compressed. These films were found to contain increased amounts of  $\beta$ -structure compared to the molecule in aqueous solution (Cornell, 1982; Loeb, 1971).

The data for the amount of BLG associated with the lipid films are summarized in Table I. The protein content was determined from the ultraviolet spectra (185–350 nm) of monolayers transferred to quartz plates using what is essentially Beer's law for films (Cornell et al., 1989). In samples where molecular motion is restricted, such as monolayer films, the ultraviolet absorption of the chromophore will depend upon its orientation in the light beam of the spectrophotometer. This is of course in contrast to solutions in which the light beam samples an average orientation of the rapidly tumbling molecule. The effect of sample orientation on the ultraviolet spectrum of spread monolayers of BLG has been determined (Cornell, 1984). In that work, the protein was first spread at the air/water interface, presumably with some degree of unfolding of the globular structure, and then compressed and transferred to quartz plates. The molar (residue) absorptivity at 193 nm was determined to be about 12 000 L/mol-cm for the spread film compared to 9600 L/mol-cm for isotropic solutions (McDiarmid, 1965). Since the degree of net orientation of the protein in the films

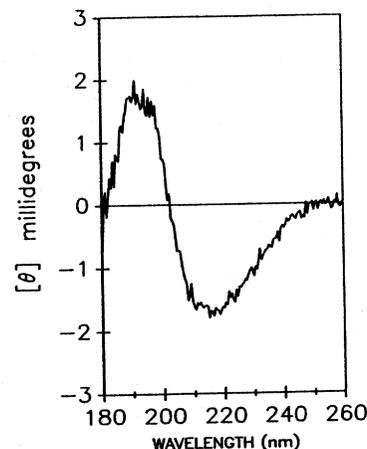


Figure 4. Circular dichroism spectra of BLG-POPC/POPG complex transferred to quartz plates. The initial lipid pressure was about 22 mN/m; a 7 mN/m rise in film pressure indicated that insertion of a portion of the protein molecule occurred.

Table I. Protein Concentration in  $\beta$ -Lactoglobulin-Phospholipid Monolayers

lipid subphase	units	POPC/POPG (65/35 mol %)	
		low $\pi$	high $\pi$
Walpole's acetate, pH 4.4	mg/M <sup>2</sup>	2.3–2.8	2.4–3.0
	molecule/cm <sup>2</sup>	(7–9) $\times 10^{12}$	(0.8–1) $\times 10^{13}$
	POPG/BLG ratio	5–6	6–7
Tris, pH 7.0	mg/M <sup>2</sup>	0.3–0.4	0.3–0.4
	molecule/cm <sup>2</sup>	(1.0–1.2) $\times 10^{12}$	(1.0–1.3) $\times 10^{12}$
	POPG/BLG ratio	38–45	45–58

studied here is unknown, we have used both absorptivities in calculating their protein content. The resulting range is enclosed in brackets in Table I. The range of 2.3–3.0 mg/m of protein absorbed to lipid films spread on pH 4.4 media is well above the value of 1 mg/m normally associated with milk proteins spread at the air/water interface and accompanied by some degree of unfolding (Mitchell et al., 1970) where most of the backbone resides in the plane of the monolayer. The range of 2.3–3.0 mg/m shown in Table I suggests a protein molecule that has remained in its compact, globular form in the aqueous phase. Values of this order have been reported for many proteins adsorbed at the air/water interface (migrating from bulk solution), where the measurement techniques were ellipsometry (Benjamins et al., 1975) and surface radioactivity (Graham and Phillips, 1979).

The effect of calcium on the interaction of BLG with the monolayer lipids is illustrated in Figure 5. Protein was injected beneath a high-pressure (>35 mN/n) PC/PG film into the subphase containing 0.1–30 mM calcium. The ultraviolet spectra (180–350 nm) were run on the transferred films, and the absorbance at 191 nm was compared to the absorbance obtained from films transferred from calcium-free subphase. The protein concentration in the subphase was 5 mg/L in all cases. The results are expressed as percent lipid-BLG complex versus calcium concentration.

## DISCUSSION

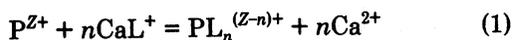
A plausible mechanism of lipid-protein interaction at the oil/water interface suggested by the work reported here consists of the following steps: (1) approach of a protein molecule to the surface of the lipid monolayer, driven by diffusion and abetted by electrostatic attraction; (2) binding of the protein to the oppositely charged lipid; (3) insertion of a portion of the protein into the

lipid monolayer in cases where the lipid film is below a critical pressure.

The electrostatic mechanism is supported by the results obtained with the lipids spread on subphases of different pH (Table I). At pH 4.4 BLG is below its isoelectric point of 5.5 (Whitney et al., 1976) and carries a net positive charge. Phosphatidylcholine is isoelectric over a wide pH range while acidic phospholipids (of which phosphatidylglycerol is an example) will be fully ionized at pH >4 (Hauser and Phillips, 1979). Thus, the charge on the POPC/POPG monolayers resides almost exclusively in the PG molecule at pH 4.5. Under these conditions electrostatic attraction between the lipid and protein is to be expected. Conversely, in a neutral subphase both species are negatively charged and should exhibit little (electrostatic) affinity for each other.

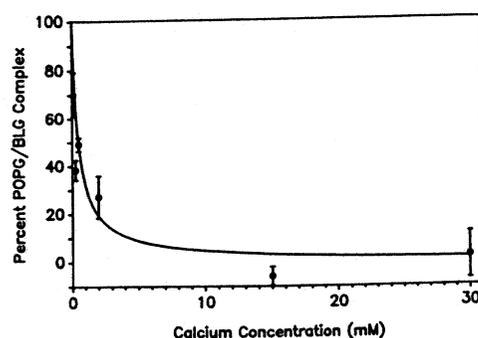
The net charge per BLG molecule is +8 to +10 at pH 4.4 (Basch and Timasheff, 1967); hence, the charged lipid/protein ratio of 5–7 suggests that the lipid–protein film carries a small net positive charge. Although the NET charge is +8 to +10 at pH 4.4, there are 21 residues (3 Arg, 2 His, 15 Lys, plus the N terminus) (Whitney et al., 1976) that one would expect to be positively charged at acid pH. Inspection of the three-dimensional structure of BLG (Papiz et al., 1986) shows that there is at least one orientation of the molecule where five to seven positive charges could be brought into the proximity of the negatively charged monolayer.

Calcium ion is known to bind to phosphatidylglycerol in a 1/1 complex (Lau et al., 1981) and might be expected to interfere with lipid–protein binding. This expectation is borne out by the results shown in Figure 5 where even a submillimolar level of calcium displaces an appreciable fraction of the BLG bound to the lipid monolayer. The maximum calcium concentration studied here (30 mM) approximates the total (bound and free) concentration in milk (Fox, 1985). The free calcium concentration (unbound, uncomplexed  $\text{Ca}^{2+}$ ) in milk is about 2 mM (Holt, 1985), but even at this level, complexation with POPG has reduced the amount of BLG–lipid complex to about 25% of its value found in the calcium-free monolayer experiments. The concentration of BLG used in these experiments (5 mg/L), however, is considerably less (by 600-fold) than the 3 g/L found in fresh whole milk (Gordon and Kalan, 1974). At this much higher concentration of BLG, one might expect that the protein would displace most of the calcium bound to the lipid monolayer. All of this can be summarized by eq 1,



where P is protein carrying charge Z, Ca is calcium, L is the lipid  $\text{PG}^-$ , and  $n$  represents the number of PG lipids bound per protein molecule. The protein  $\text{P}^{Z+}$  and calcium  $\text{Ca}^{2+}$  are in solution; the protein and calcium–lipid complexes  $\text{PL}_n^{(Z-n)+}$  and  $\text{CaL}^+$  are components of the monolayer.

One might expect from eq 1 that the equilibrium could be readily driven to the right, in the direction of increased protein–lipid binding and reduced calcium binding, simply by increasing the protein concentration in the subphase. We have demonstrated this, increasing the protein concentration by a factor of 10 (to 50 mg/L) at a calcium concentration of 2 mM, with an increase in the amount of lipid–protein complex observed to about 36%. Assuming equilibrium 1 above is for  $n$  identical noninteracting binding sites, it can be demonstrated by a simple mass action expression that this relative insensitivity to protein concentration implies that  $n$  is “large”. The



**Figure 5.** Effect of calcium on the lipid monolayer–BLG complex. The percent lipid–protein complex was determined with UV spectroscopy of transferred films. The absorbance of the film collected from calcium-free water was taken as the 100% reference. Standard errors of three to four replicates are indicated by bars.

assumption of  $n$  identical and independent binding sites is not generally applicable however, and the determination of multiple equilibria constants can be rather involved (Tanford, 1961; Steinhardt and Reynolds, 1969). Furthermore, the scatter in the data of Figure 5 is too great to allow an analysis in terms of multiple binding sites. Increasing the protein concentration much above 50 mg/L resulted in destabilizing the lipid monolayer film; hence, a direct test of the lipid–protein binding under the conditions prevailing in milk, 2 mM Ca and 3 g/L BLG, has not proved possible. Nevertheless, since at 3 g/L BLG is some 600 times more concentrated than in most systems studied here, it seems reasonable to expect that protein rather than calcium binding to charged lipids will prevail at pH 4.4.

**Registry No.** POPC, 6753-55-5; POPG, 81490-05-3; calcium, 7440-70-2.

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