

Complex Formation in Sonicated Mixtures of β -Lactoglobulin and Phosphatidylcholine¹

ELEANOR M. BROWN*, ROBERT J. CARROLL, PHILIP E. PFEFFER and JOSEPH SAMPUGNA²,
Eastern Regional Research Center, Agricultural Research Service,
U.S. Department of Agriculture, Philadelphia, PA 19118

ABSTRACT

β -Lactoglobulin, the major whey protein of bovine milk, is secreted via the endomembrane system of the mammary gland. The primary structure of β -lactoglobulin shares certain characteristics with membrane proteins, although the soluble protein assumes a globular conformation. We have prepared complexes of β -lactoglobulin and phosphatidylcholines by dissolving both in a helix-forming solvent (chloroform-methanol). The complex is stable when transferred to aqueous solutions and sonicated to form vesicles. Both ionic and hydrophobic interactions appear to be involved in complex formation. We have used spectroscopy (circular dichroism, fluorescence, and nuclear magnetic resonance) and electron microscopy to study these complexes. At pH 3.7, the small, single bilayer vesicles produced by sonication are protected against aggregation by the presence of the protein. As determined by circular dichroism, the proportion of α -helix in β -lactoglobulin is increased by complexation with phosphatidylcholine. Circular dichroism and fluorescence spectra show the involvement of at least 1 tryptophan residue in the conformational change. At pH 7.2, β -lactoglobulin-phosphatidylcholine vesicles form aggregates as observed by electron microscopy and ³¹P nuclear magnetic resonance spectroscopy. These aggregated vesicles could be resuspended by raising the pH. The ability of the partially unfolded β -lactoglobulin to interact with lipids is believed to be important to its transport through the endomembrane system.

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The secretion of proteins involves their transport across membranes. The milk system, involving several classes of proteins and membrane complexes, is particularly difficult to study (1). The use of a model system allows us to focus on the interactions of one protein with a specific lipid membrane component. Our model consists of β -lactoglobulin, a secreted protein for which detailed primary (2) and secondary (3-5) structural information is available, and phosphatidylcholine, a typical membrane lipid. β -Lactoglobulin, the major whey protein of bovine milk, has been studied extensively because of its ability to form complexes with itself (6) and other proteins (7-9). As a secretory protein, β -lactoglobulin is known to pass through the membranes of the endoplasmic reticulum. Mercier and Gaye (10) have shown that a conformational change is associated with the transport of β -lactoglobulin across this membrane. These authors also found that, when β -lactoglobulin is synthesized in vitro in the presence of microsomes, its signal peptide is cleaved while the protein is passing through the membrane, and the protein then assumes its native conformation. In a microsome-free system, the signal peptide is not cleaved and is not accessible to enzymatic attack after the

protein folds (10). Although its physiological function has not yet been determined, β -lactoglobulin does share certain structural characteristics with membrane proteins. The average hydrophobicity of β -lactoglobulin is 1230 on the Bigelow scale (11), higher than most soluble proteins. The ratio of charged to nonpolar residues as calculated by the method of Barrantes (12) is 1.13, placing β -lactoglobulin between the integral (average value 0.59) and the peripheral membrane proteins (average value 1.37) in this characteristic. Segrest and Feldman (13) have suggested that residues 130-143 of β -lactoglobulin (2) may form an amphipathic helix, similar to those found in lipoproteins and some membrane proteins.

Protein-detergent models have been used to study membrane-like behavior. The interactions of β -lactoglobulin with sodium dodecyl sulfate (SDS) have been extensively studied (4, 14-16). The choice of dipalmitoyl phosphatidylcholine (DPPC) as the major lipid of interest here is based on the occurrence of 16:0 as one of the major acyl chains in bovine milk phospholipids (17). Additionally, studies of free fatty acid binding (18) and polymer bound alkane binding (19) showed β -lactoglobulin to have a strong preference for palmitate. Interactions of β -lactoglobulin with phosphatidylcholines were first reported by ourselves (20), more recently others have demonstrated similar interactions for another whey protein, α -lactalbumin (21, 22). The results reported herein describe the properties of the sonicated complex formed between solvent-treated β -lactoglobulin and phosphatidyl-

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²Dairy Research Inc., sabbatical fellow; permanent address: Department of Chemistry, University of Maryland, College Park, MD.

*Author to whom correspondence should be addressed.

cholines as studied by optical spectroscopy (ultra-violet (UV) absorption, circular dichroism (CD), fluorescence), ^{31}P nuclear magnetic resonance (NMR), and electron microscopy.

MATERIALS AND METHODS

β -Lactoglobulin was isolated from bovine milk and purified by the method of Aschaffenburg and Drewry (23); homogeneity was established by SDS-polyacrylamide gel electrophoresis. DPPC and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma* and used without further purification. Each lipid gave a single spot when chromatographed on silica gel plates using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65:25:4). All other chemicals were of reagent grade.

Sample Preparation

Homogeneous lipid-protein solutions were prepared by diluting 1 vol of β -lactoglobulin (≤ 10 mg/ml) in 0.14 M KCl with 5 vol of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1). The precipitated protein was then redissolved by dropwise addition of 3% HCl in CH_3OH . Dry lipid was added and the mixture vortexed to give a clear solution. Solvent was removed under a stream of dry N_2 , leaving a lipid-protein film. Final traces of solvent were removed by storing the sample overnight under high vacuum. The films were dispersed in 0.02 M Tris in 0.14 M KCl, pH 7.2, or 0.02 M acetate in 0.14 M KCl, pH 3.7, and adjusted to the buffer pH with 0.1 M KOH. Values for pH were chosen to be above and below pH 5.2, the isoionic point of β -lactoglobulin. Small unilamellar vesicles were formed by sonicating the aqueous mixtures at 50 C under N_2 for 30-60 min with a Heat Systems W185 sonifier equipped with a microtip (24). Centrifugation at 100,000 g for 1 hr removed titanium particles and multilamellar or aggregated vesicles. Both DPPC and DMPC, gave similar results in these experiments when the sonication temperature was above the lipid transition temperature. All illustrations used here were obtained with DPPC.

Analytical

The concentration of β -lactoglobulin in suspensions at pH 3.7 was determined from the absorbance at 278 nm ($E_{1\text{ cm}}^{1\text{ mg/ml}} = 0.96$) (3) after correction for light scattering by the extrapolation of $\log A$ vs $\log \lambda$ between 400 and 320 nm, where the protein does not absorb; such corrections were typically ca. 0.01 A. At pH 7.2, the protein-lipid complexes aggregated, and Peterson's modification (25) of the Lowry method (26) was used to determine protein content.

*Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Lipid concentrations were determined by the method of Ames and Dubin (27) for total phosphate. Sucrose density gradient (10-25%) centrifugation (16 hr at 200,000 g) was used to estimate the ratio of lipid to protein in the vesicle complex.

Optical Spectra

A Cary (Model 14) spectrophotometer was used to record absorption spectra. A Jasco 41-C spectropolarimeter calibrated by the 2-point method of Chen and Yang (28) was used for CD measurements. The solutions used were visually clear, and the extrapolated baseline ($\log A$ vs $\log \lambda$) was not greater than 0.01 A at 200 nm in the 0.05-cm cell used for far-UV CD spectra. Fluorescence measurements were made with an Aminco Bowman spectrofluorimeter operated in the ratio mode. Inner filter effects were minimized by using a 2.9-mm pathlength cell and diluting samples having $A_{280\text{ nm}}^{1\text{ cm}} > 0.15$. All reported spectra are the average results of at least 5 determinations for which the variability was not greater than 10% at 210 nm.

Nuclear Magnetic Resonance

The ^{31}P resonance decoupled spectra were obtained at 24.15 MHz with ca. 15W decoupling power and a 0.5 KHz bandwidth. The proton decoupler was centered at 47.80 kHz, corresponding to a δ of 5.0 ppm on the proton resonance scale relative to external TMS. A JEOL FX 60Q spectrometer equipped with temperature control and field stabilization via a deuterium lock was operated in the Fourier transform mode. Free induction decays were obtained from 10,000 to 100,000 transients with a delay time of 0.48 sec and a pulse angle of 90° .

Electron Microscopy

Vesicles were deposited on 200 mesh copper grids, negatively stained with phosphotungstic acid, at the appropriate pH, and viewed in a Zeiss 10-B electron microscope operating at 60 kV.

RESULTS

Sonicated vesicles provide a well characterized system (29) suitable for optical and magnetic resonance spectroscopy. These techniques allow us to observe both the effect of the lipid on the protein and of the protein on the lipid. Measurements were made at pH 3.7 and 7.2 to allow variation in the charge distribution on β -lactoglobulin while maintaining the pH-dependent monomer-dimer equilibrium of the protein (30).

Centrifugation of mixtures of native β -lactoglobulin and phosphatidylcholine resulted in separation of a multilamellar lipid pellet and a lipid-free protein solution. The behavior of sonicated mix-

tures of DPPC with native β -lactoglobulin was pH-dependent, but did not appear to involve lipid-protein interactions. At pH 7.2, the supernatant resulting from sonication and centrifugation of a mixture containing 4 mg/ml DPPC and 1 mg/ml β -lactoglobulin contained all of the protein, and 90-95% of the lipid. The supernatant was clear and the UV and CD spectra were identical with those of β -lactoglobulin in the absence of lipid. At pH 3.7, the initial supernatant contained all of the protein and 55-60% of the lipid. The turbidity of this supernatant increased with time so that when the sample was recentrifuged 16 hr later, only 20% of the lipid remained suspended. Essentially all of the protein remained in the supernatant and had the spectral properties of native β -lactoglobulin. These data show that native lactoglobulin did not interact with DPPC. On the other hand, when the lipid was added to β -lactoglobulin dissolved in the $H_2O/CHCl_3/CH_3OH/HCl$ system, interactions did occur. The physical behavior of such complexes after removal of the solvent, resuspension in buffer, sonication, and centrifugation depended on whether the pH was above or below the isoionic point (pH 5.2) of the protein. Immediately after centrifugation, all samples were essentially clear, indicating that the lipid was present primarily as a suspension of single bilayer vesicles. Except in the case of the lipid-protein mixture at pH 7.2, most of the lipid was suspended and only very small pellets, consisting primarily of titanium from the sonication probe, were formed. At pH 3.7, the turbidity of a DPPC vesicle suspension without protein increased more rapidly than did that of an equal concentration of DPPC sonicated with the solvent-treated protein (Fig. 1). At pH 7.2, the DPPC vesicle suspension without protein was stable, but the inclusion of solvent-treated β -lactoglobulin led to pelleting of both lipid and protein. From sucrose

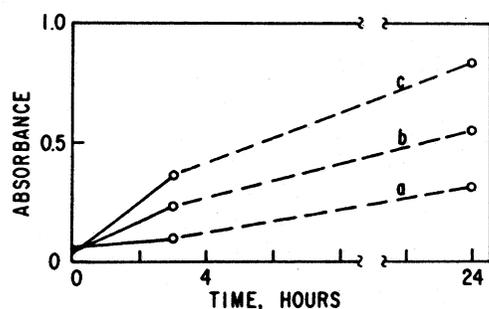


FIG. 1. Time-dependence of the turbidity of the supernatant from sonicated, centrifuged lipid, and lipid-protein samples: (a) 4 mg/ml DPPC, 1 mg/ml β -lactoglobulin, pH 3.7; (b) 4 mg/ml DPPC, pH 7.2; and (c) 4 mg/ml DPPC, pH 3.7. Sonication and centrifugation of 4 mg/ml DPPC with 1 mg/ml β -lactoglobulin at pH 7.2 left little protein or lipid in the supernatant.

density gradient centrifugation, the lipid-protein ratio for sedimenting complexes was determined to be 20 ± 5 , in good agreement with the binding capacity of β -lactoglobulin for SDS (15). Significant changes in salt concentration interfered with formation of these complexes, in that at low ionic strength, stable vesicles were not formed, while at higher ionic strength the protein did not bind the lipid.

Absorption and CD Spectra

Sonication did not significantly affect the spectral properties of native β -lactoglobulin. The shape of the UV-absorption band (Fig. 2) and the correlation between absorptivity and protein concentration were maintained. Solvent-treated β -lactoglobulin is relatively insoluble; it tends to adhere to the glass and is not easily resuspended in aqueous buffer at pH 3.7 or 7.2. That portion which does dissolve tends to renature, as can be seen in the absorption spectrum (Fig. 2) and the near-UV CD spectrum (Fig. 3). When sonicated and centrifuged in the presence of DPPC (2:1,w/w) at pH 3.7, virtually all of the solvent-treated protein was in the supernatant, along with most of the lipid and the suspension formed was reasonably clear (Fig. 2).

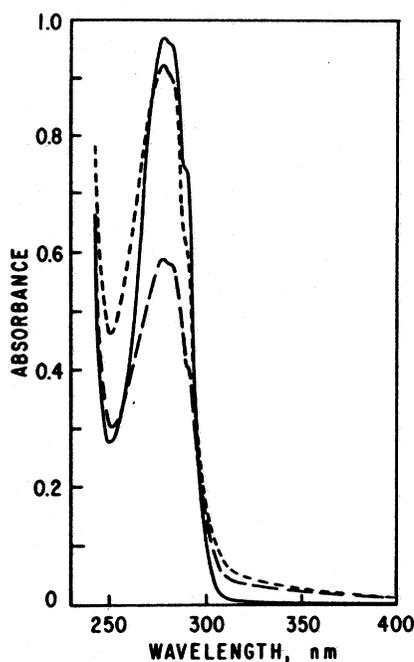


FIG. 2. Absorption spectra of sonicated, centrifuged samples of β -lactoglobulin in 0.02 M acetate, 0.14 M KCl, pH 3.7: (—) native protein 1 mg/ml, (---) an equivalent aliquot denatured as in the text, and (- - -) with 2.5 ml DPPC included.

The CD spectrum of native β -lactoglobulin was in agreement with published spectra (3,4,14). The far-UV CD spectrum of native β -lactoglobulin (Fig. 3a) shows a negative dichroic peak in the 215-218 nm region typical of β -structures (3,4). The conformation of native β -lactoglobulin as determined by CD (3,4,14) and infrared (5) consists of about 10% α -helix with the remainder equally divided between aperiodic and β -structures. The shoulder at 230 nm in the spectrum of the re-suspended solvent-treated protein indicates an increase in aperiodic structure and a decrease in β -structure (31). Evidence for ca. 10% α -helix (32) can be found in spectra of both native and solvent-treated β -lactoglobulin when dissolved in aqueous buffer. The double minimum at 222 and 208 nm in the spectrum is characteristic of α -helical structures and is seen only for proteins with at least 25% helix (32). The far-UV CD spectrum of β -lactoglobulin in the lipid-protein complex thus clearly indicates an increase in α -helical structures to at least 25-30%. If, despite our precautions, there are minor light-scattering effects, they would tend to reduce the apparent intensity of the CD signal (33) so that this estimate of helical structure may be considered as a minimum value.

The near-UV CD spectrum (Fig. 3b) of native β -lactoglobulin exhibits negative peaks at 293 and 285 nm, and a shoulder at 266 nm. Both the 293 and 285 nm peaks have been attributed to tryptophan

(3,4). The near-UV CD spectrum of β -lactoglobulin in the lipid-protein complex consists of a broad, negative peak centered at 285 nm with a shoulder at 266 nm. The disappearance or at least diminution and nonresolvability of the 293 nm peak combined with the 285 nm minimum suggest that 1 of the 2 tryptophan residues per monomer is affected by the formation of the lipid-protein complex.

Fluorescence

The intrinsic fluorescence of β -lactoglobulin in the DPPC vesicle complex at pH 3.7 is 10% greater than that of the native protein. The emission maximum occurs at 333 nm for the native protein, as it does for the complex (Fig. 4).

NMR

Nonsonicated DPPC liposomes exhibit an axially symmetric proton decoupled ^{31}P NMR spectrum characterized by a broad peak with a shoulder ca. 40 ppm downfield, due to the chemical shift anisotropy of phosphate phosphorus in an environment with restrictive motion (34). Sonicated DPPC vesicles (Fig. 5a) exhibit only a narrow line 14 ppm downfield from the high-field maximum of the liposome pattern. The spectra of liposomes and vesicles are not appreciably affected by changes in pH nor by the presence of native β -lactoglobulin. The ^{31}P NMR spectrum of sonicated β -lacto-

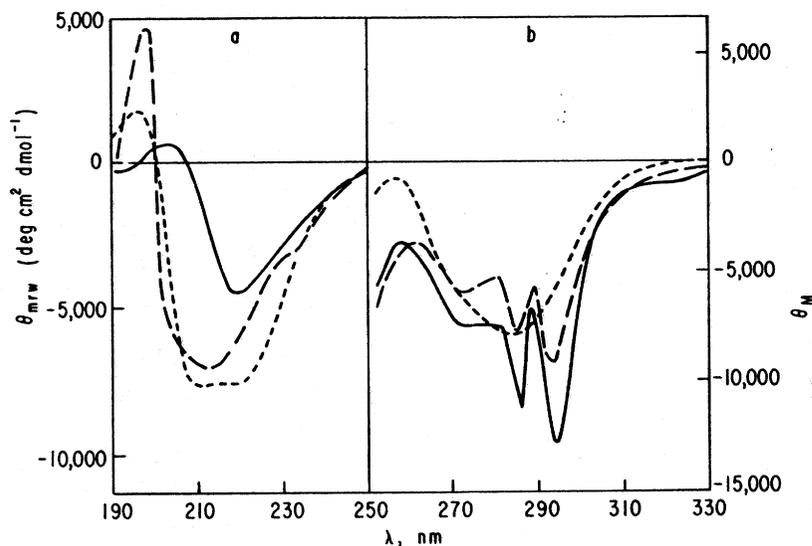


FIG. 3. The far-UV (a) and near-UV (b) CD spectra of (—) native β -lactoglobulin, (—) denatured, redissolved β -lactoglobulin, and (---) complex of denatured β -lactoglobulin with DPPC vesicles. All samples in 0.02 M acetate, 0.14 M KCl, pH 3.7. θ_M based on molecular weight of 18,000, θ_{MRW} based on a mean residue weight of 113. All spectra shown are averages of at least 3 separate determinations. Pathlengths of 1.0 cm in the near-UV and 0.05 cm in the far-UV were chosen to assure $A < 2.0$ at the λ recorded.

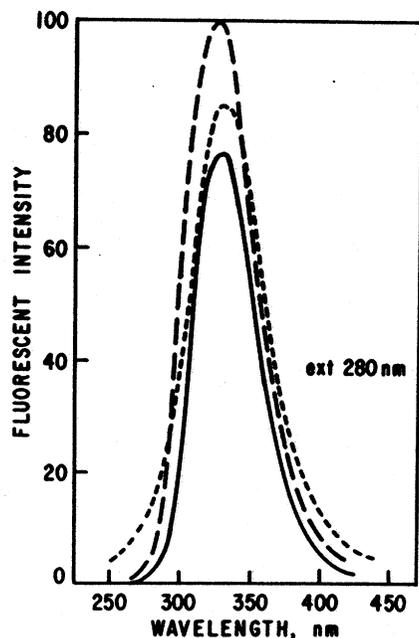


FIG. 4. Fluorescence emission spectra of sonicated, centrifuged samples of β -lactoglobulin in 0.02 M acetate, 0.14 M KCl, pH 3.7; (—) native protein, (---) denatured protein, and (- - -) protein-DPPC vesicle complex. Protein concentration 0.15 mg/ml.

globulin-DPPC complex at pH 3.7 closely resembled that of DPPC vesicles without protein; at pH 7.2 the ^{31}P NMR spectrum of the sonicated complex (Fig. 5b) contained elements of both vesicle and liposome spectra, but was not simply the sum of these. The restricted motion of the phosphorous seen here could be attributed to either fusion or aggregation of vesicles. When titrated to pH 11 where the solvent-treated protein becomes more soluble, the sample became less turbid and the ^{31}P NMR spectrum (Fig. 5c) of sonicated vesicles appeared, indicating that the earlier spectrum was due to the aggregation of vesicles, not fusion.

Electron Microscopy

Electron microscopy of the sonicated lipid-protein complex at pH 3.7 (Fig. 6) shows vesicles in the 200-600 Å range; while a few clusters are seen, they are generally not aggregated. Vesicles of DPPC at this pH appear to fuse so that only a few, much larger (1000-1500 Å) vesicles are seen in the micrographs (Fig. 6b). At pH 7.2, all of the vesicles were in the 200-600 Å range. Those containing lipid-protein complexes are flattened and aggregate to form stacks (Fig. 6c). DPPC vesicles at this pH appear as a uniform dispersion (Fig. 6d). β -Lacto-

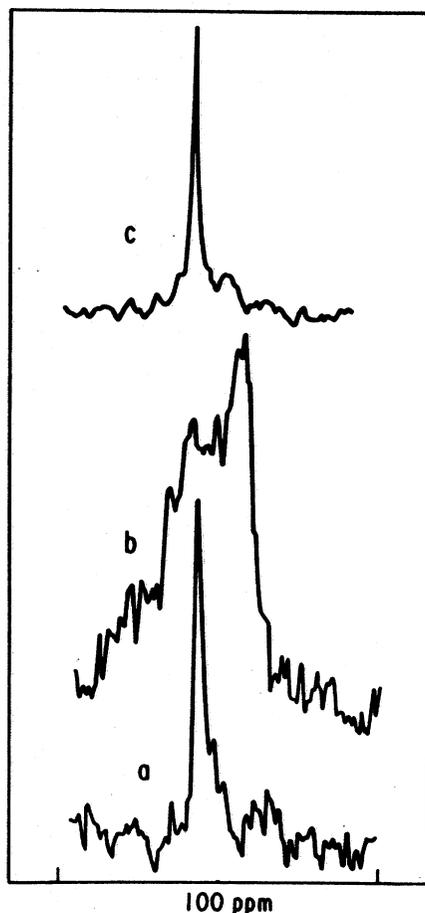


FIG. 5. 24 MHz proton decoupled ^{31}P NMR spectra obtained from sonicated samples of (a) DPPC at pH 7.2; (b) 1:25 (w/w) mixture of β -lactoglobulin with DPPC at pH 7.2; and (c) after titration of (b) to pH 11.

globulin which has been solvent-treated and sonicated at pH 3.7 appears as 42 Å particles, suggesting that little aggregation occurs. This dimension is in close agreement with the reported size of the β -lactoglobulin monomer (30)

DISCUSSION

The results presented here show that sonicated vesicles containing a complex of β -lactoglobulin and DPPC can be formed. Treatment of the protein with a helix-forming solvent is necessary as the native protein does not complex with the lipid. While this may appear to be drastic treatment for a water-soluble protein, it is similar to the conditions used in the isolation of some membrane proteins (35). Additionally, the lipid must be mixed with the protein while it is in the helical conformation because of β -lactoglobulin's tendency to renature

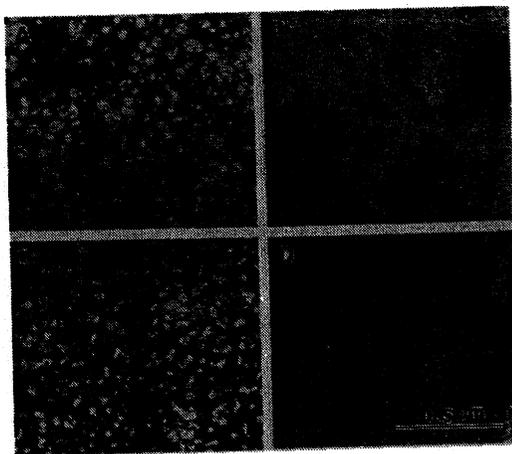


FIG. 6. Electron micrographs of phosphotungstic acid-stained vesicles: (A) DPPC with β -lactoglobulin, pH 3.7; (B) DPPC, pH 3.7; (C) DPPC with β -lactoglobulin, pH 7.2; (d) DPPC, pH 7.2. The lipid concentration is 0.1 mg/ml.

when transferred to an aqueous medium. This ability of the protein to renature when returned to a more native environment suggests that other proteins treated similarly may also be studied in their native conformations. Sonication must be done at a temperature above the lipid transition point, but the resulting complex can then be studied at room temperature.

Cosonication of native protein with phospholipid, a less drastic procedure than that used here, was found to bind immunoglobulin G to phosphatidylcholines (36). However, we have shown that sonication does not alter the conformation of β -lactoglobulin and that DPPC vesicles formed in the presence of native β -lactoglobulin behave as though there was no protein present. Additionally, cosonication of native β -lactoglobulin with DPPC does not lead to any significant encapsulation. However, the small capture volume of phosphatidylcholine vesicles ($0.1 \mu\text{l}/\mu\text{M}$) (37), and the low concentration of β -lactoglobulin ($<0.06 \text{ mM}$) used here may account for this.

Increases in α -helix are a common indication of lipid complexation by serum lipoproteins (38) or lipid-binding polypeptides (33). The helical content of β -lactoglobulin increases from 10% to ca. 45% in 0.02 M SDS (14) and to nearly 60% in acidic CH_3OH (3). Our $\text{H}_2\text{O}/\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HCl}$ solvent system should induce at least 50% helix; unfortunately, the high concentration of CHCl_3 prevents the measurement of the far-UV CD spectrum in this solvent system. The 25-30% helical content observed for a β -lactoglobulin-phosphatidylcholine complex is less than the solvent-induced helix, but is more than twice that of the native

protein. Application of the Chou-Fasman (39) procedure to the amino acid sequence of β -lactoglobulin (2) results in an overestimation of helical content, that is, the potential for forming more helix than can be seen in the CD spectrum of the native protein. Some of these potentially helical regions are buried in the interior of the native protein so that the addition of lipid does not by itself cause their exposure and subsequent binding. However, once these regions are exposed by the organic solvent, they can participate in lipid binding which then stabilizes their helical conformation. The CD spectrum of β -lactoglobulin-DPPC vesicle complex shows a helical content consistent with the predictions of Chou and Fasman (39).

The emission maximum for either native β -lactoglobulin, or the β -lactoglobulin-DPPC vesicle complex occurs at 333 nm; however, the latter occurs with a 10% increase in intensity. In systems where the protein-phospholipid interaction increases the hydrophobicity of the environment of 1 or more tryptophan residues, a blue shift in the intrinsic fluorescence is likely to be seen; for mellitin, the shift is from 352 to 333 nm (40), for glucagon it is from 350 to 338 nm (35), and for very low-density lipoprotein it is from 350 to 345 nm (38). Precedent does exist in hydrophobic myelin protein where the maximum is at 329 nm with or without lipid (41). The binding of long-chain fatty acids to β -lactoglobulin increases the fluorescence intensity by 8% but does not shift the emission maximum (18). The formation of a β -lactoglobulin-DPPC vesicle complex apparently does not increase the hydrophobicity of the environments of tryptophans 19 and 61. However, an emission maximum of 333 nm suggests relatively hydrophobic environments for these residues in the native protein. The increased fluorescence yield is believed to be an effect of the conformational change seen in the CD data. While this conformational change does not affect the average hydrophobicity of the tryptophan environment, it may increase the distance between them and a carboxyl or other charged residue, thus decreasing fluorescence quenching.

At pH 3.7, observations obtained by both electron microscopy and ^{31}P NMR confirmed the presence of small, uniform vesicles in sonicated mixtures of denatured β -lactoglobulin and DPPC. The protein conformation as determined by CD in this system shows that β -lactoglobulin is bound to the lipid vesicles. At pH 7.2, evidence for aggregated protein-lipid vesicles is seen in both electron microscopy and ^{31}P NMR data. Titration to a higher pH disaggregates the vesicles possibly with the dissolution of denatured β -lactoglobulin from the outer surface, similar to the observations of Koter et al. (42) on the removal of calcium from calcium-aggregated vesicles.

In view of the CD data, the amphipathic helix model (13) of protein-lipid interaction seems reasonable. According to this model, the initial interaction is between charged amino acid residues in the protein and the polar head group of the lipid, after which the hydrophobic side of the helix is buried in the hydrocarbon chains of the lipid. Among the charged amino acid residues, glutamic acid is an excellent helix initiator (39), and the helix forming potential of positively charged lysine and arginine is enhanced by partial submersion in the lipid (13). Ionizable amino acids comprise 29% of the residues in β -lactoglobulin. Although the net charge per dimer of β -lactoglobulin can vary between +20 at pH 3.7 and -10 at pH 7.2 (43), the degree of lipid (19) and detergent (15) binding is similar at these pH values, indicating that hydrophobic interactions are the more important. The lipid-protein ratio of 20 ± 5 would be sufficient to coat a lipid vesicle (400 Å diameter) with a protein shell if the protein were completely extended. While complete unfolding of the protein is unlikely, a significant portion of the vesicle surface must be covered, or more protein would be expected to bind to the exposed lipid. The variation with pH of the net charge remaining on the protein after interaction with the lipid may account for the aggregation behavior of the complexes. At pH 3.7, one would expect all of the unused charges to be positive and for the protein coated vesicles to repel each other. At pH 7.2, both positive and negative charges should be available, if these are spread over the surface of the vesicles they may neutralize each other by attracting other such vesicles. At pH 11, the excess would be negative charge, only arginine (6 residues per dimer of β -lactoglobulin) would remain positively charged, leading to repulsion and possibly even removal of the protein from the lipid which would no longer be zwitterionic.

Initial ionic attraction to position lipid and protein molecules followed by hydrophobic interactions to stabilize a complex have been proposed to explain lipid-protein binding in other systems (13,18,35,40). Our results are consistent with this model in that the optical spectra, particularly CD, show hydrophobic interactions in the helix formation, while the ionic strength effects imply electrostatic interactions between the lipid and β -lactoglobulin (44).

Secretory proteins, in their native conformations, may not in general form lipid-protein complexes. However, with some degree of disruption, one should be able to obtain a form of the protein which will interact with lipids. Hydrophobicity and helix formation are important factors in the interactions of most membrane proteins and serum lipoproteins with lipids. For secretory proteins, the occurrence of helical structure in the final protein is less important than the potential for forming a

transitory helix while transversing the membrane. There is no extended portion, 10 residues or more, of β -lactoglobulin for which the helical potential (39) is less than 1.03. Thus, this is a good illustration for the Engelman and Steitz theory (45) of helical hairpin insertion. According to their theory, the signal peptide orients the process and assures that the peptide will not come out of the membrane on the side of synthesis. Secretion is then driven either by a monotonic decrease in the polar character of the peptide chain from the amino to the carboxy end, or by energy derived from folding the peptide on the exterior side of the membrane. In β -lactoglobulin, residues 60 to 100 (the peptide chain is 162 residues) form the most hydrophobic portion, suggesting that the first pathway is probably not operational. The leader sequence of β -lactoglobulin is cleaved before secretion is complete (10) and the protein starts to fold into its native conformation which has ca. 10% helical structure. This dynamic folding would provide energy to complete the export process. The native structure is then stabilized by the formation of disulfide bonds making the secretory process irreversible.

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