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Equilibrium and Dynamic Spectroscopic Studies of the Interaction of Monomeric β-Lactoglobulin with Lipid Vesicles at Low pH

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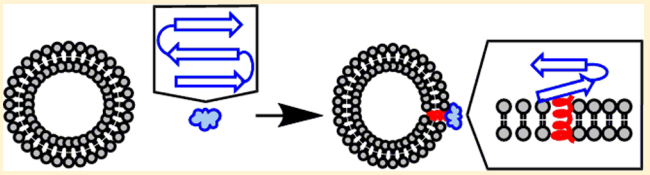
Supporting Information

ABSTRACT: β-Lactoglobulin (βLG) is a member of the lipocalin protein family that changes structure upon interacting with anionic surfactants and lipid vesicles under higher-pH conditions at which βLG is dimeric. In this study, a β-sheet to α-helix transformation was also observed for monomeric βLG obtained at pH 2.6 when it was mixed with small unilamellar vesicles (SUVs) of zwitterionic lipids, but being mixed with anionic lipids produced little change. The dynamics and extent of this change were quite dependent on the lipid character, phase, and vesicle size. With 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), at ∼50 °C and pH 2.6, the βLG converted to a substantially helical form upon addition of ∼10 mM lipid in a two-step kinetic process having time constants of ∼1 and ∼25 h, as monitored by circular dichroism (CD). Fluorescence changes were simpler but implied a rapid initial change in the Trp environments followed by a slower process paralleling the change in secondary structure. Polarization attenuated total reflectance Fourier transform infrared results indicate the formed helices are at least partially inserted into the lipid bilayer and the sheet segments are on the surface. Thermal behavior showed that the secondary structure of the lipid-bound βLG had two phases, the first being characteristic of the protein−lipid vesicle interaction and the second following the DSPC phase change after which the protein apparently dissociated from the vesicle. Large unilamellar vesicles had a weaker interaction, as judged by CD, which may correlate to the partial exposure of the hydrophobic parts of the SUV bilayer. Other zwitterionic lipids bound βLG with much slower kinetics and often required sonication to induce interaction, but these also showed dissociation upon lipid phase change. These thermal and kinetic behaviors suggest a mechanism for the interaction of monomeric βLG with zwitterionic lipids different from that seen previously for the dimeric form.



Protein folding and misfolding have been studied on many levels, one aspect of which involves interaction between proteins and membranes. The genome encodes a great number of membrane-bound or active proteins. Furthermore, many extracellular proteins interact with membranes and/or membrane proteins as part of their function, one important example of which is signaling and transport processes. Optical spectroscopic methods are useful for studying complex protein−membrane systems and yield global, or averaged, structural data, which can be useful for monitoring folding changes and conformational equilibria. Optical spectra also can track dynamics because of their relatively fast response.

Bovine β-lactoglobulin (βLG), a major component of cow's milk, is a member of the lipocalin or lipocalycin family.[1] In its native state, βLG is a soluble, globular protein with a predominantly β-sheet structure consisting of one major α-helix and a β-barrel of eight continuous antiparallel β-strands shaped into a flattened cone or calyx.[2,3] This can provide a pocket for binding fatty acids and other nonpolar molecules, but the prime biological function of βLG remains unknown.[4] At physiological pH values, a ninth β-strand forms H-bonds to the like strand in another βLG, creating a native state dimer, which dissociates to a monomer form below pH ∼3,[5] and retains this monomer native conformation even at pH values as low as 2.[6,7] The monomer and dimer share the same folded secondary structure.[8] Although the βLG secondary structure consists mainly of β-sheet components in its native state, structure prediction algorithms suggest that various segments of its sequence have a strong propensity to adopt helical structures.[9] Non-native partially folded structure was observed during an early stage of βLG folding as shown by kinetic studies detecting an α-helical intermediate form in refolding βLG from a denatured, disordered form (random coil).[10,11] It has been shown that native state folding of βLG provides a good model for studying the mechanism of an α-helix to β-sheet (α−β) transition in proteins,[11,12] which can be an important mechanistic step for understanding the folding of a number of protein molecules, including misfolding related to amyloid diseases.[13−16] On the other hand, equilibrium studies have shown that native state βLG unfolds, undergoing a β-sheet to α-helix transition upon being transferred to solutions containing alcohols,[17] ionic surfactants,[18] and lipids.[19]

Our previous βLG−lipid interaction studies have shown that anionic lipid vesicles and anionic surfactant micelles can induce such a β-to-α conformational change in dimeric βLG at neutral

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pH or somewhat reduced pH (4.6) and that the extent of the transformation depended on concentration and pH.[20−22] We demonstrated that electrostatic interaction between the negative lipid and positive protein was a prerequisite for this conformational change but also found that hydrophobic interaction with the lipid bilayer played a secondary role in developing the final conformation. These studies also demonstrated that the helices, at least in part, insert into the membrane and lead to vesicle leakage at neutral pH. The resulting proposed interaction mechanism was a multistep process in which the extent of βLG interaction with the lipid vesicles was shown to depend on electrostatics as well as lipid packing or phase characteristics.[20]

In this study, we turn from the native state dimer to address the equilibrium and kinetic aspects of interaction of the monomer form at low pH with lipid vesicles. Our results show an entirely different process, which depends primarily on hydrophobic interaction in studies of the interaction of βLG with zwitterionic lipids, as exemplified here by distearoylphosphatidylcholine (DSPC), although results with other lipids will be additionally discussed. Lowering the pH to stabilize the monomer βLG form alters the nature of the lipid vesicles, such that the previously studied, negatively charged phosphatidylglycerol (PG) lipids, which have pK_a values in the range of 3−3.5,[23] become protonated. These were found to have no effect on the conformational change in βLG at pH 2.6. By contrast, the zwitterionic (PC) lipids, a main component of the eukaryotic membrane, do lead to a structural change in βLG at low pH.

While PC lipids are affected by low pH, only a small fraction of their phosphate groups become protonated at pH 2.5.[24] The lipid phase transition is also shifted by varying the pH value.[24] Thus, these monomeric studies effectively eliminate electrostatics as a driving force and let us focus on the role of hydrophobic forces in the protein−lipid interaction. Recently, it has been reported that the membrane binding of proteins with lipid vesicles can be strongly dependent on curvature when electrostatic forces are reduced, so that the interaction will be predominately driven by a hydrophobic mechanism.[25] These studies showed that the weaker the electrostatic interaction, the greater the dependence on hydrophobicity and membrane curvature.[25] All of these aspects are important for the monomer βLG−lipid interaction, so that the previously reported charge-driven mechanism for interaction at neutral pH is changed for the monomer with zwitterionic lipids.

One hypothesis is that interaction of monomer βLG and zwitterionic lipid SUVs at low pH will be mainly driven by hydrophobic effects and the previously dominant electrostatic interactions at higher pH will be largely eliminated. Varying the lipid acyl chains for these low-pH studies actually has dramatic effects on the interaction, in contrast to our experiences with previous studies at higher pH.[20−22] In this case, the simplest results were obtained with DSPC, which are the focus of this paper. The kinetics of these interactions were much slower than those seen previously with negatively charged lipids and higher pHs, so that the kinetics of the change in secondary and tertiary structure could be monitored by repeated scans of the CD and fluorescence spectra measured over the accessible spectral regions.

MATERIALS AND METHODS

Materials. β-Lactoglobulin A from bovine milk (Sigma-Aldrich, L 7880) was used without further purification. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-di(9,10-dibromo)-stearoyl-*sn*-glycero-3-phosphocholine (Br-DSPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Organic solvents (methanol and chloroform, spectral grade) and sodium phosphate (analytical grade) were purchased from Sigma. Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc.

Preparation of Lipid Vesicles. Small unilamellar vesicles (SUVs) were freshly prepared just before being used following literature techniques.[26] The weighed amount of dried lipid was dissolved in a chloroform/methanol mixture [2:1 (v/v)] in a 25 mL round-bottom flask, and the lipid solution was dried under a mild stream of N_2 gas while being gently shaken and rotated to form a thin film on the wall of the flask. It was dried under vacuum overnight to remove all the organic solvents. The film was then hydrated by adding 20 mM phosphate buffer (pH 2.6) at 60 °C. The hydrated suspension was vortexed and then sonicated with several 30 s pulses from a probe sonicator (Microson XL2000, Misonix) to form a clear SUV solution. The temperature of the solution was kept at ∼60 °C by immersion in a water bath during the sonication step. The final radii of these SUVs were determined with a DynaPro Titan (Wyatt, CA) dynamic light scattering (DLS) instrument to be 20−30 nm for several preparations for both pH 7 and 2 at low temperatures. The large unilamellar vesicles (LUVs) were prepared by extrusion at 60 °C with a membrane having a pore size of 100 nm. DLS measurements indicated the LUVs had a radius of ∼60−70 nm. The sizes of SUVs and LUVs were relatively stable up to 50 °C, more so at low pH, because of their higher-temperature phase transition, but the size of the SUVs increased at higher temperatures (∼65 °C) to be equivalent to that of LUVs, as shown in Figure S1 of the Supporting Information. The required amount of βLG stock solution was slowly added to the aqueous lipid vesicle solution, and the solutions were mixed well. βLG and vesicles were allowed to equilibrate at 50 °C overnight to form a stable protein−lipid complex for the equilibrium studies.

Circular Dichroism. The CD spectra were measured under equilibrium conditions on a J-810 spectrometer (Jasco) using a 1 mm path length quartz cell that was maintained at 50 °C during the measurements using a thermoelectric controller accessory (CDF-426S/15, Jasco). The protein concentrations were 0.1 and 0.2 mg/mL, while lipid concentrations were varied from 0 to 12 mM. The CD spectra were obtained by averaging eight scans obtained with a scan rate of 50 nm/min, a bandwidth of 1 nm, and a response time of 2 s. The final spectra were obtained by subtracting a background spectrum, measured for the same cell but containing just the vesicle solution without protein, from the sample spectrum. The fractional secondary structure for βLG in the absence and presence of lipid vesicles was estimated from the CD spectra by use of the SELCON 3 method, which is part of the CDPro software package.[27] The thermal stability of the βLG−lipid complex was monitored by measuring complete CD scans in 5 °C steps from 5 to 90 °C with thermoelectric control.

Fluorescence Measurements. Fluorescence measurements were recorded on a Fluoromax-3 spectrofluorimeter (Jobin-Yvon Inc.). The excitation wavelength was 295 nm, and the emission spectrum was scanned from 300 to 450 nm with a slit width of 5 nm. The concentration of βLG was kept at 0.2

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mg/mL (same as for CD). In all experiments, background spectra of lipid in buffer were subtracted from the sample spectra. The temperature was controlled by use of a circulating bath (Thermo Neslab) under software control.

For fluorescence quenching experiments, small aliquots of a 5 M acrylamide stock solution were added to the βLG in the absence or presence of lipid vesicles, and fluorescence emission spectra were recorded at an excitation wavelength of 295 nm. The data were analyzed according to the Stern–Volmer equation $F_0/F = 1 + k_{sv}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, and k_{sv} is the Stern–Volmer quenching constant.[28] Additional quenching experiments were conducted by adding Br-DSPC to the DSPC lipids in a 1:1 ratio. While quenching of the fluorescence was observed in this labeled, mixed lipid system, the effect was relatively small.

FTIR Measurements. Fourier transform infrared (FTIR) spectra were recorded on a Vertex 80 spectrometer (Bruker) using 4 cm^{-1} resolution and averaging of 512 scans. The samples were prepared by dissolving βLG and DSPC SUVs separately in deuterated phosphate buffer (pD 2.6) and then mixing them to concentrations of ∼1 mg/mL and 50 mM, respectively (chosen to match saturation conditions found in CD titration) (see Results). The samples were incubated at 50 °C overnight and placed in a sealed homemade cell consisting of two CaF_2 windows separated by a 100 μm Teflon spacer. Temperature-dependent FTIR experiments were conducted by measuring spectra in 5 °C steps from 5 to 95 °C as regulated by the flow from a spectrometer-controlled circulating bath (HAAKE DC50 K20). Background spectra, measured as buffer alone or buffer with only lipid vesicles in a similar sample cell, were subtracted from the corresponding sample spectra.

ATR-FTIR Measurements. Polarized attenuated total reflectance (ATR)-FTIR experiments were conducted on the Vertex 80 spectrometer with a MIRacle accessory (PIKE Technologies, Inc., Madison, WI), incorporating a single-bounce diamond crystal ATR plate. A 12 μL sample of the protein–lipid vesicle solution was pipetted onto the crystal plate and dried under a gentle stream of nitrogen gas to form a dry multilayer film. Spectra were recorded with both parallel (0°) and perpendicular (90°) polarization by use of a wire grid polarizer placed in front of the sample. The dichroic spectra were recorded by subtracting the weighted spectrum recorded with parallel polarized light from the spectrum recorded with perpendicular light, using a weighting coefficient, R_{iso}. For this experiment, R_{iso} was determined by integrating the area of the lipid carbonyl band at ∼1740 cm^{-1} from both 0° and 90° polarizations and ratioing them to give $R_{iso} = A_{90}/A_0$.[20,29,30]

RESULTS

Equilibrium and Kinetic Interaction of Monomeric βLG by DSPC Lipid Vesicles. CD and fluorescence were used to identify secondary and tertiary structural changes in monomeric βLG (the low-pH form) upon interaction with zwitterionic lipid vesicles (DSPC). As shown in Figure 1a, the far-UV CD spectrum of βLG (0.2 mg/mL) in phosphate buffer at pH 2.6 (---) exhibits a broad, weak minimium around 216 nm, which is typical of β-sheet structure. With the addition of zwitterionic lipid DSPC (—), the CD spectrum of βLG has a characteristic β-sheet to α-helix transition, developing two, more intense minima at ∼208 and 222 nm. The inset of Figure 1a shows the ellipticity change at 222 nm for 0.011 mM βLG (∼0.2 mg/mL) as a function of DSPC concentration and shows

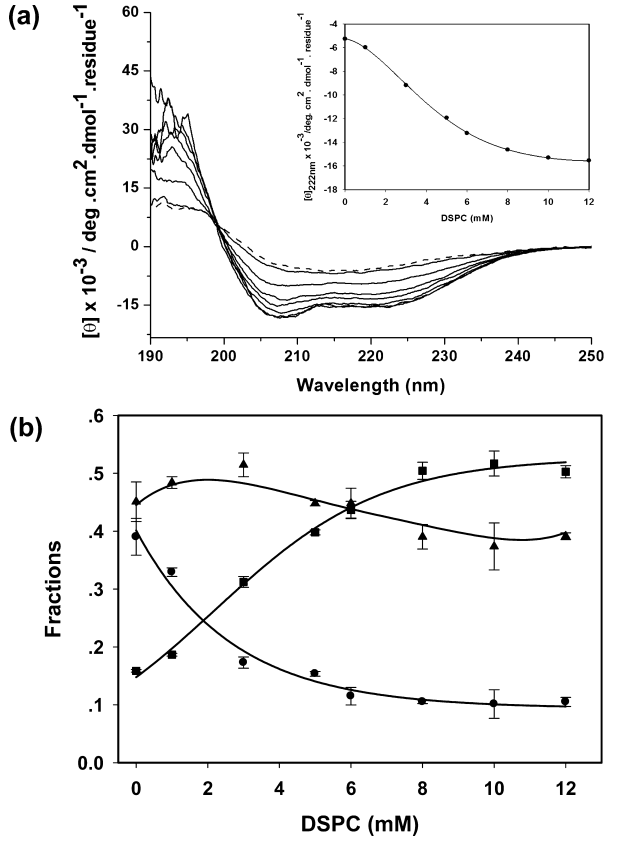

Figure 1. DSPC-dependent conformational transition of βLG measured by far-UV CD (in molar ellipticity per residue unit). (a) Far-UV CD spectra of 0.2 mg/mL βLG in the absence (---) and presence of concentrations of DSPC increasing from 1 to 12 mM at pH 2.6 (—). The inset shows the ellipticity change at 222 nm as a function of DSPC concentration. (b) Helical fractions (■), sheet fractions (●), and unordered fractions (▲) are estimated as a function of DSPC concentration at pH 2.6 using SELCON 3 in the CDPro program. Error bars represent standard deviations of the fractional secondary structure from three independent trials.

an increase in negative intensity that stabilizes at ∼10 mM DSPC. The negative ellipticity at 222 nm of βLG at pH 2.6 increased almost linearly upon addition of DSPC to a final concentration of ∼6 mM and then slowly reached a plateau upon addition of more DSPC. Estimated changes in secondary structure (determined using SELCON 3[27]) as a function of DSPC concentration are presented in Figure 1b. The DSPC-induced α-helix fraction of 0.2 mg/mL βLG reached a maximal value (∼50%) when the DSPC concentration was more than 6 mM, which was correlated with a decrease in the β-sheet fraction to a minimal value (∼10%). The far-UV CD spectrum of βLG at a lower concentration, 0.005 mM (0.1 mg/mL), was also measured upon addition of DSPC at pH 2.6. The estimated change in secondary structure for this lower βLG concentration (see Figure S2 of the Supporting Information) was maximal at a lower DSPC concentration, consistent with the DSPC-induced α-helix fraction reaching a maximum when the millimolar concentration ratio between βLG and DSPC was ∼1:900. The highest lipid concentrations resulted in considerable noise for $\lambda < 200$ nm, because of light loss, but the trends indicate a process consistent with the same sort of transition from native β-sheet structure to non-native α-helical structure at all concentrations studied.

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This conversion of secondary structure is relatively slow at 50 °C (especially as compared to the results of our previous neutral-pH studies with negatively charged lipids).[20−22] The CD shows a sharp drop in the first hour followed by a continued but slower change as illustrated in Figure 2. The

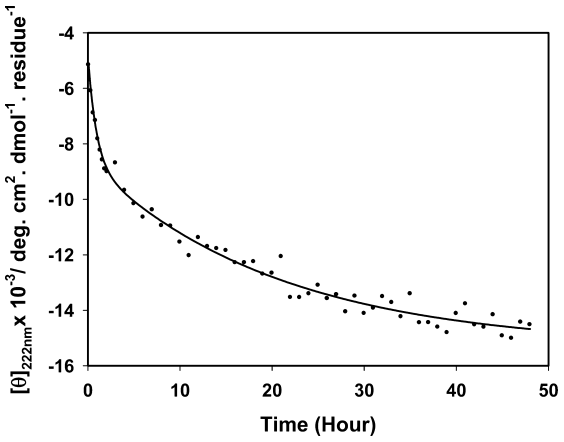

Figure 2. CD-detected kinetics for mixing SUVs of 10 mM DSPC with 0.2 mg/mL (0.011 mM) βLG at pH 2.6 monitored via a change in the ellipticity at 222 nm for samples held at 50 °C. The solid line indicates a fit with a double-exponential decay function (eq 1, —), with a k_1 of 1.1 ± 0.23, a k_2 of 0.05 ± 0.017, an an R^2 of 0.98.

transition shown, resulting from the interaction between βLG and SUVs from 10 mM DSPC at 50 °C, could be fit with a double-exponential decay function

$$\theta(t) = \theta_0 + \theta_1 \exp(-k_1 t) + \theta_2 \exp(-k_2 t) \quad (1)$$

that yielded fast and slow component rate constants, k_1 and k_2, of 1.1 ± 0.23 and 0.05 ± 0.017 h^{-1}, respectively. By contrast, if βLG is mixed with DSPC LUVs at the same concentrations, relatively little CD change occurs, indicating only a minor increase in helicity (see Figure S3 of the Supporting Information) and a significant dependence on the curvature of the bilayer.

As a probe of the change in tertiary structure, fluorescence spectra of βLG at various DSPC concentrations were measured (Figure 3a). Without DSPC, the fluorescence spectra of βLG at low pH (---) had a maximum (λ_{max}) at ∼331 nm, indicating the emitting Trp residues were in a shielded hydrophobic environment. After the addition of various concentrations of DSPC SUV (—), the fluorescence intensity increased (as summarized in the inset of Figure 3a). This may be due to less quenching resulting from the increased motional flexibility of Trp61, which in the native state is probably quenched by the Cys66−Cys160 disulfide bond.[31] However, the fluorescence band λ_{max} had a blue shift (to ∼324 nm), with a residual shoulder at ∼336 nm, which suggests that the protein does not fully unfold and the Trp residues do not become solvent-exposed. This could be consistent with burial of at least one of the Trp residues (Trp61 or Trp19) in the lipid bilayer. As shown in Figure 3b, the blue shift of the maximal intensity also reached a maximum at ∼6 mM added DSPC (for 0.2 mg/mL βLG), suggesting the tertiary structural changes are correlated to the secondary structural changes.

This fluorescence-detected kinetic change for the mixing of 0.2 mg/mL βLG with 3 mM DSPC at 50 °C can be monitored in terms of the change in the relative fluorescence intensity, as

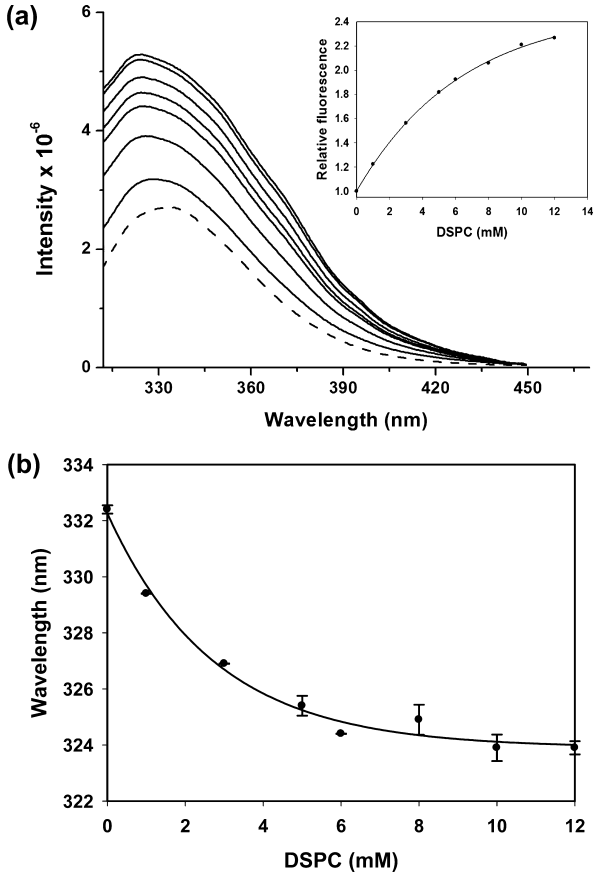

Figure 3. DSPC-dependent change in the tertiary structure of βLG measured by (a) the fluorescence of 0.2 mg/mL βLG (λ_{exc} = 295 nm) in the absence (---) and presence (—) of increased concentrations (as in Figure 1) of DSPC lipid vesicles. The inset shows that intensities of λ_{max} in the normalized fluorescence increase as a function of lipid concentration. (b) λ_{max} peak shift of the normalized fluorescence as a function of lipid concentration.

monitored at 331 nm and starting ∼1.5 min after mixing (Figure 4), and can be fit with single-exponential function

$$F(t) = F_0 + a[1 - \exp(-kt)] \quad (2)$$

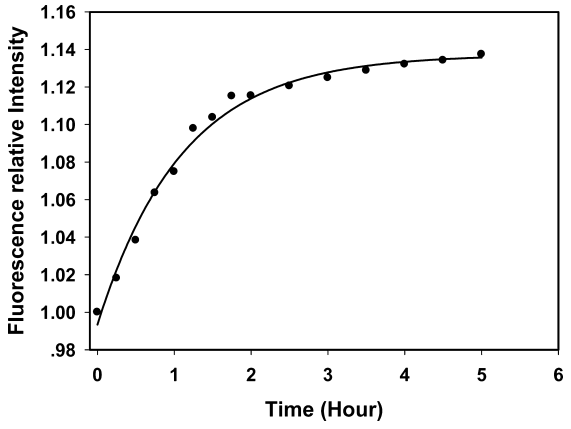

Figure 4. Change in the relative fluorescence intensity at 331 nm of 0.2 mg/mL βLG being mixed with 3 mM DSPC SUVs at 50 °C as a function of time, fit by a single-exponential function (eq 2, —), with a k of 0.91 ± 0.44 and an R^2 of 0.99.

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that rises to a maximum with a rate constant k of 0.91 ± 0.44 h^{-1}, which is consistent with the kinetics (~1.1 h^{-1}) observed for the fast component of the CD intensity change. The parallel kinetics imply that the emitting Trp is transferred to a more hydrophobic environment when the secondary structure rearranges. However, there is also an initial burst step for both the frequency shift and intensity with the initial change from βLG in buffer to βLG mixed with lipid (see Figure S4a,b of the Supporting Information), which could be due to other fast structural changes of βLG or more likely to the change in the solvent environment (index or scatter) following the fast mixing of protein and lipid.

While these results are consistent with our observation that βLG inserts into a lipid bilayer at higher pH values, as controlled by the electrostatic driving force, we were not able to conduct the kind of leakage experiments as previously reported for higher pH values.[20,21] However, we did measure acrylamide quenching, which showed the Trp residues to be relatively protected upon binding to the SUVs (see Figure S5 of the Supporting Information), consistent with results obtained for higher-pH vesicles. Our efforts to use Br-DSPC lipids to quench Trp fluorescence and determine insertion were inconclusive.

Orientation of Binding of βLG with Lipid Vesicles. Polarized ATR-FTIR was used to monitor the preferred orientations of βLG segments interacting with DSPC vesicles after they were deposited to form multilayer films on a surface. The differential polarized ATR-FTIR spectrum for βLG in DSPC (1:950 molar ratio) at pH 2.6 is shown in Figure 5. The

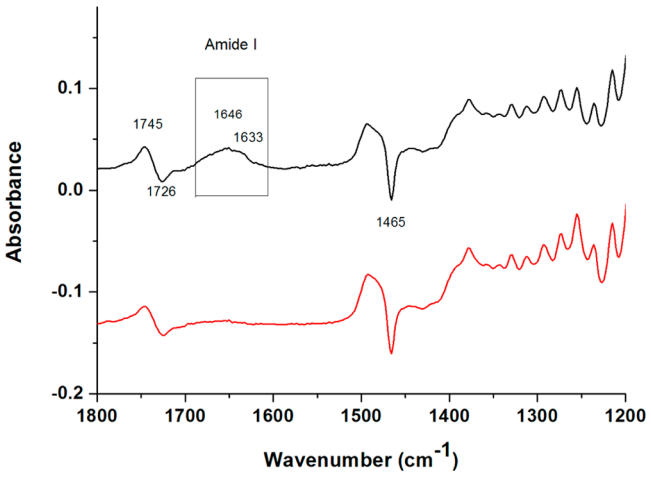

Figure 5. Polarized ATR-FTIR difference spectra (90° − 0° polarization) of βLG in the DSPC bilayer with a molar ratio of 1:950 (top, black) and just DSPC (bottom, red) at pH 2.6.

difference spectrum (90° − 0°) was obtained using a weighting coefficient R_{iso} of 1.31. The bands in the region from 1350 to 1180 cm^{-1} corresponding to positive difference peaks arise from wagging motions of the methylenes in the lipid acyl chains, and the negative difference peak at 1465 cm^{-1} is due to their CH_2 scissoring bands. Assuming the lipid chains were oriented perpendicular to the plate and these scissor mode vibrations are normal to the chain extension direction, the negative bands can be assumed to indicate dipole transitions oriented parallel to the surface of the membrane and positive bands to indicate a perpendicular orientation.[32] For the amide I region, the polarization difference spectra at the ATR plate for the βLG−DSPC complex film at pH 2.6 showed two weakly positive peaks at 1646 and 1633 cm^{-1}, which can be assigned to the βLG α-helix and β-sheet components, respectively. For the α-helix component, the positive peak at 1646 cm^{-1} corresponds to a C═O stretching mode polarized parallel to the helix axis, and by this result, the helix axis appears to be primarily oriented perpendicular to the lipid membrane surface, which is consistent with insertion of at least a significant fraction of the helical segments into the membrane. For the β-sheet component, the C═O stretching vibration is perpendicular to the β-sheet strand direction, so that the positive amide I difference peak at 1633 cm^{-1} indicates the β-strands are oriented more parallel to the bilayer surface and possibly lie on the membrane−solvent interface at pH 2.6.

Thermal Stability of the Lipid−βLG Complex. The thermal stabilities of βLG only and the lipid-bound βLG complex at pH 2.6 as monitored by far-UV CD are shown in Figure 6. With an increase in temperature, the ellipticity at 204

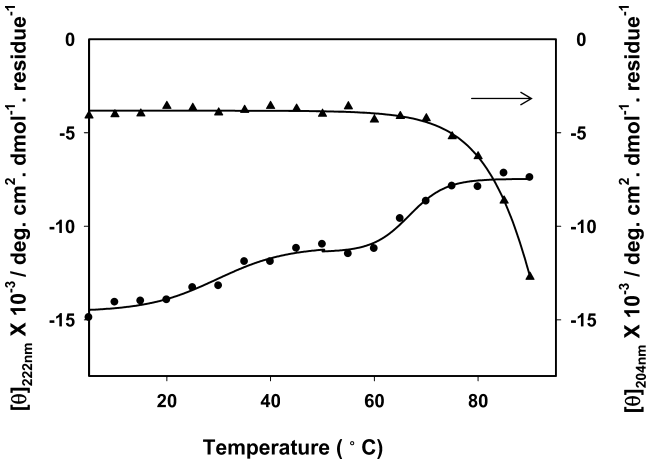

Figure 6. Thermal stability measured by monitoring the far-UV CD ellipticity at 222 nm for 0.2 mg/mL βLG with 10 mM DSPC (●) and at 204 nm for the 0.2 mg/mL βLG in just buffer at pH 2.6 (▲) (corresponding CD spectra are available in panels a and b, respectively, of Figure S6 of the Supporting Information).

nm of βLG alone (▲) was initially stable and then sharply became more negative well above 70 °C, consistent with the protein undergoing a β-sheet to unordered transition (see panels a and b of Figure S6 of the Supporting Information for corresponding βLG and βLG−DSPC CD spectra, respectively). The transition did not reach a stable high-temperature state, because the CD continued to change, as the temperature was increased to 90 °C. This indicates that the T_m of βLG at pH 2.6 is higher than that at neutral pH, which is ~68 °C based on our previous study.[20] By contrast, with an increase in temperature, the ellipticity of the βLG−lipid complex at 222 nm (●) decreases, becoming less negative, and undergoes a two-stage transition. The result was fit with two sigmoidal functions having T_m values of 27 ± 3.6 °C (R^2 = 0.96) and ~67 ± 1.3 °C (R^2 = 0.97), respectively (Figure 6 with the corresponding spectra in Figure S6b of the Supporting Information).[33]

The thermal transition of βLG only and the lipid-bound βLG complex as monitored by FTIR at higher concentrations was roughly consistent with the CD results (see Figure S7 of the Supporting Information for the spectra and Figure S8 of the Supporting Information for the variation). For βLG alone, the intensity for the β-sheet component (1633 cm^{-1}) (▲)

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fluctuated with the change in temperature but was relatively stable and then decreased sharply above 75 °C, consistent with the unfolding process monitored by CD. The FTIR of the α-helical component band (1644 cm^{-1}) for the βLG−DSPC complex showed a transition at a temperature lower than that seen in CD with a T_m of 60 ± 2.1 °C (R^2 = 0.94), determined by fitting to a sigmoidal thermodynamic function.[33] On the basis of the FTIR spectrum (shown in Figure S7b of the Supporting Information), the βLG−DSPC complex exhibited a typical β-sheet component after the transition and between 65 and 75 °C, but then it went through a second β to unordered transition above 80 °C. Consistent with the thermal CD result, the decrease in the fraction of helical structure correlated with the thermal transition of the DSPC lipid and may result from dissociation of the protein−lipid complex leading to unfolding rather than being due to simply unfolding of the protein while it is still associated with the lipid. It should also be noted that the SUVs grew in size, presumably because of fusion with other SUVs, to become equivalent in size to LUVs at 65 °C (see Figure S1 of the Supporting Information).

The transition temperature for the change in the ordering of the lipid bilayer in the vesicles was determined by monitoring the IR frequency for the CH_2 asymmetric and symmetric stretching modes of the aliphatic chains. For DSPC, the asymmetric CH_2 mode frequency increases from 2917.6 to 2923.8 cm^{-1} and the symmetric CH_2 mode increases from 2850.1 to 2853.4 cm^{-1} as the temperature is increased with a transition temperature of 61 ± 0.8 °C at low pH. After the lipid had been mixed with protein, its transition did not change much, shifting to 64 ± 1.5 °C as monitored with the asymmetric CH_2 mode and to 65 ± 0.7 °C for the symmetric mode. All the data were fit with a sigmoidal thermodynamic function (as shown in Figure S9 of the Supporting Information).

DISCUSSION

Driving Force for the Association of βLG with DSPC. The interactions between proteins and lipids involve several steps: initial binding to the surface, induction or stabilization of a specific lipid-bound secondary structure, modulation of membrane biophysical properties by protein binding, and finally either partial or full insertion of the protein into the membrane bilayer.[34] This interaction is driven by the difference in the stability of the protein in buffer as opposed to its stability when it is associated with the lipid vesicle, which offers a complex, nonhomogeneous environment involving a charged or highly polar surface and a hydrophobic interior. The association of water-soluble proteins with lipids thus depends on the surface attraction caused by the overall electrostatic force,[35] stabilization by hydrophobic interaction,[36] or both.[37] The electrostatic forces will be determined by the relative charge of the lipid and protein and can be modified by pH, while the hydrophobic aspect can be enhanced by unfolding the protein to expose inner residues or by disrupting the surface to promote access to the core of the bilayer, which as recently shown can be enhanced by the increased curvature in small vesicles (SUVs as compared to LUVs).[25] In this process, the protein structure may undergo dramatic changes, including a disruption of tertiary structure and/or a formation of specific lipid-bound secondary structure, each of which can depend on a change in the lipid physical state.

Our previous studies showed that the main driving force of the β-to-α transition in dimeric βLG at pH 6.8 and 4.6 as induced by anionic lipid vesicles was due to electrostatic interaction, because zwitterionic lipids alone did not induce significant changes in the secondary or tertiary structure of βLG at those pH values.[20−22] Monomeric βLG at pH 2.6 has a net positive charge because it has a pI of 5.3, and zwitterionic lipid DSPC has only a small protonated fraction (~2.6% of the phosphate groups are reported to be protonated).[24] Our CD results showed a clear β-to-α transition upon addition of DSPC SUVs to monomeric βLG at low pH (Figure 1). The α-helical fraction increased to ~50% at the expense of the β-sheet fraction, which is consistent with previous results for the interaction of dimeric βLG with anionic lipids at higher pH values.[20] By contrast, the protonated anionic lipid DSPG at low pH did not induce a conformational change in βLG (data not shown). In addition, the DSPC (as well as DMPC) SUVs rapidly lost the βLG after undergoing a phase transition, and LUVs of DSPC did not significantly bind βLG or alter its secondary structure, as monitored by CD. The DSPC SUVs were shown to be stable in size, as measured by DLS, up to 50 °C, which is the temperature used for our binding measurements, but at 65 °C, they fused and became equivalent in size to the LUVs (Figure S1 of the Supporting Information).

To explore the sensitivity of the interaction between monomeric βLG and DSPC lipid vesicles to curvature, the far-UV CD spectrum showed that relatively little α-helix was induced in βLG by DSPC LUVs (Figure S3 of the Supporting Information). This observation combined with the loss of binding after the lipid phase transition and fusion of the SUVs to form LUVs suggests the binding is dependent on the curvature of the bilayer. The higher curvature of the SUVs can expose the core of the bilayer to interaction with hydrophobic residues, which, in the absence of electrostatic binding, can become the dominant interaction and can promote the insertion of the hydrophobic part of the protein into the bilayer, as recently demonstrated.[25]

In comparison to our previous studies,[20−22] the CD kinetic results show that interaction of monomeric βLG with the DSPC lipid at pH 2.6 and 50 °C had a much slower rate of structural change and required more concentrated lipids to achieve the final maximal helical structure (Figures 1 and 2), compared to the case for the neutral-pH anionic lipids. CD-monitored dynamics at lower temperatures, for the mixing of βLG with DSPC at 30 °C (see Figure S10 of the Supporting Information), also showed a two-stage kinetic process with rates similar to but somewhat slower (k_1 ~ 0.93 ± 0.35 h^{-1}, and k_2 ~ 0.02 ± 0.68 h^{-1}) than what was observed at 50 °C (k_1 ~ 1.1 ± 0.23 h^{-1}, and k_2 ~ 0.05 ± 0.017 h^{-1}). However, much less helical structure was induced at 30 °C (Figure S10 of the Supporting Information); consequently, in the results shown above (Figures 1 and 2) the βLG−DSPC samples were incubated at 50 °C to induce more interaction and faster kinetics. This temperature dependence of the degree of helicity gained suggests a dynamic aspect to the hydrophobic interaction, which could be due to the increased level of exposure of the bilayer core correlated to the motion of the headgroups, or it could suggest that increased lipid dynamics closer to the phase change favors insertion of helical segments.

The fluorescence intensity change when βLG is mixed with DSPC exhibited kinetic behavior similar to that of the CD fast component (Figure 4), but there was an initial fast mixing step in the dead time (Figure S4a,b of the Supporting Information). The overall kinetic change suggests that the change in the solvent environment at 50 °C from an aqueous monomer low-

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pH state to an α-helical DSPC membrane-bound state may cause βLG to first form an intermediate (molten globule state), in which the protein may be partially unfolded and expose the hydrophobic component, and then slowly insert into the highly curved SUV lipid bilayers, induced by the hydrophobic effect, to develop more helical structure. A molten globule intermediate state has also been previously proposed to explain the lack of coincidence for midtransition points of the changes in secondary and tertiary structure in βLG.[38−40]

We found that other zwitterionic lipids, for example, DMPC, DOPC, and POPC, can also induce a change in the secondary structure in low-pH βLG, but vesicles of these lipids required sonication to effect the protein−lipid interaction and promote structural change. This suggests a more complex mechanism, probably one in which sonication causes incorporation of protein into the bilayer followed by unfolding and restructuring. The desolvation of the protein on mixing under sonication may overcome a kinetic barrier to protein−vesicle initial interaction. Because the protein hydrophobic clusters can be more easily exposed in a molten globule state, being in a lipid environment can stabilize hydrophobic residues in the restructuring process.[41] Given the loss of electrostatic driving force at this low pH, the main interaction between the positively charged βLG and the zwitterionic lipid vesicles becomes hydrophobic. Thus, the effectively mechanical mixing of lipid and protein provided by sonication, possibly partially unfolding the protein, while breaking and re-forming the SUVs, could better expose the protein to the hydrophobic core of the lipid bilayer structure. In some sense, the sonication of lipids has an even greater advantage for promoting hydrophobic lipid−protein interaction than the increased level of access to the bilayer core seen in the comparison of SUVs and LUVs discussed above. The interactions between the monomeric βLG and zwitterionic lipids also depend on the character of the lipids, such as the length and saturation of the hydrocarbon chain and their effect on chain ordering and the temperature of the phase transition. DOPC and POPC, which have unsaturated hydrocarbon chains and lower-temperature phase transitions, induced lower α-helical fractions than did the saturated lipids (data not shown).

Insertion and Orientation of βLG into Lipid Bilayers. Fluorescence can provide useful information about the solvent exposure or accessibility of tryptophan residues in the proteins or proteins associated with lipid vesicles. The λ_{max} blue shift (∼9 nm) and reduced level of quenching of the fluorescence for βLG mixing with different DSPC concentrations (Figure 3) indicated the emitting tryptophan residues in βLG were shielded by insertion into the hydrophobic lipid bilayers. Stern−Volmer plots of the quenching of the Trp fluorescence in βLG with and without DSPC by acrylamide showed that increasing the concentration of the quencher resulted in a near-parallel decrease in the fluorescence intensity in both media (Figure S5 of the Supporting Information). It indicates the solvent accessibility of the fluorophore did not change substantially through βLG expansion as induced by interaction with DSPC, which is consistent with our previous study.[20] The polarized ATR-FTIR results demonstrate that the α-helical components of the βLG−DSPC complex are preferentially perpendicular to the membrane surface and suggest that the residual β-sheet components are preferentially parallel to the membrane surface (Figure 5), indicating partial insertion of βLG into DSPC lipid bilayers, which is consistent with previous high-pH results.[20] If the Trp residues were part of the sequences that were inserted, that would be consistent with our results. Mutation studies could address this in the future.

Stability of the βLG−DSPC Complex. FTIR can be used to study thermal transitions in both protein secondary structure and lipid vesicle structure.[42] The CD and FTIR thermal stability results showed the T_m of the βLG−DSPC complex was ∼60−65 °C, temperatures at which the native monomeric βLG is stable (Figure 6 and Figure S8 of the Supporting Information). However, the size of the DSPC SUVs at 65 °C increased to that characteristic of LUVs coincident with the phase transition of the DSPC at pH 2.6 (Figure S1 of the Supporting Information), resulting in the protein−DSPC complex dissociating. However, the source of the initial thermal transition seen in the CD of the protein−lipid complex at ∼27 °C is unclear. The FTIR thermal data have fluctuations that may correspond to this transition, but they are too weak to be reliable.

The increase in the frequency of both CH_2 asymmetric and symmetric modes indicates increased flexibility of the lipid alkyl chains[42] and is traditionally used to determine the phase transition, which is ∼60−65 °C for DSPC at pH 2.6 (Figure S9 of the Supporting Information). The binding of βLG to DSPC lipid vesicles does not significantly disturb the lipid phase transition equilibrium, which is consistent with the previous results that βLG does not affect the global organization or ordering of the vesicle, although it can insert into the hydrophobic lipid bilayers and cause leakage.[20,21]

Similarly, the βLG−DMPC complex had a transition at ∼35−45 °C that is close to the DMPC phase transition (∼30−35 °C) at pH 2.6 (Figures S11−S13 of the Supporting Information). Thus, the thermal destabilization of the βLG−lipid complex is correlated with the lipid bilayer phase transition and indicates that the protein dissociates from the lipid bilayer as the flexibility of the lipid aliphatic chains increases. This behavior is somewhat contrary to what is seen at high pH, where an increased α-helical component was found for lipid bilayers at the higher-temperature (liquid crystalline) phase as opposed to the lower-temperature gel phase. At low pH, our results suggest the gel phase can trap the protein, presumably at least partly in the hydrophobic bilayers, but this trapping appears to involve a barrier making the kinetics of the interaction much slower than those found previously at higher pH values. As the flexibility of the acyl chains increases at higher temperatures (above the lipid phase transition), the lipid releases the protein, so potential faster kinetics of binding to a liquid crystal phase cannot be observed when only hydrophobic forces are involved. At high pH values, the protein−lipid complex is somewhat stable even at high temperatures (higher than the lipid phase transition), which is presumably due to the added electrostatic attraction, which is not available at low pH values. Thus, the low-pH monomer and neutral-pH dimer have different mechanisms for both binding different lipid forms, zwitterionic versus negatively charged, and dissociating from them.

CONCLUSION

Zwitterionic phospholipids can induce a high degree of non-native α-helical structure in monomeric βLG at low pH values. The mechanism of this interaction, the kinetic process of the conformational transition, and the stability of the lipid-induced complex are different from those of the previous high-pH βLG−anionic lipid model. The monomeric protein may initially interact with the lipid to form an intermediate state (molten

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globule) between the native β-sheet dominant and lipid vesicle-bound α-helical states, which exposes hydrophobic residues to the lipid bilayer, and the high-curvature DSPC SUVs with the hydrophobic bilayer interior exposed can promote this interaction. The absorption and insertion of βLG into zwitterionic lipid bilayers at low pH values appear to be driven by desolvation and the consequent hydrophobic stabilization. As a consequence of a weaker driving force (at a distance) for interaction, the secondary and tertiary structural changes are relatively slow compared to the electrostatically driven interaction at neutral pH and are only stable in the gel phase of the bilayer. Once the protein is partially inserted into the gel phase lipid bilayer, βLG changes its structure presumably because of the hydrophobic environment. The protein−lipid vesicle complex dissociates when the lipid undergoes a phase transition to a more fluid form at increased temperatures. This study may improve our understanding of potential biological functions of βLG, perhaps as an intracellular transporter of fatty acids. The aggregation process of the protein can be accelerated by the transition through a molten globule state.[41] This intermediate state appears to be important in the low-pH βLG−lipid interaction, which may be related to a model for some amyloid diseases, as well.

■ ASSOCIATED CONTENT

Supporting Information

Vesicle size measurements, alternate concentration secondary structure and kinetics, LUV versus SUV effects, fluorescence kinetics and quenching, thermal variations of protein and lipid CD and IR spectra, and DMPC comparative results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

ATR, attenuated total reflection; CD, circular dichroism; FTIR, Fourier transform infrared; βLG, β-lactoglobulin; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LUVs, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SUVs, small unilamellar vesicles.

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