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Conformational changes of β-lactoglobulin induced by anionic phospholipid

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Abstract

Conformational changes of β-lactoglobulin (β-LG) induced by anionic phospholipid (dimyristoylphosphatidylglycerol, DMPG) at physiological conditions (pH 7.0) have been investigated by UV–VIS, circular dichroism (CD) and fluorescence spectra. The experimental results suggest that β-LG–DMPG interactions cause β-LG a structural reorganization of the secondary structure elements accompanied by an increase in α-helical content, and a loosening of the protein tertiary structure. The interaction forces between β-LG and DMPG are further evaluated by fluorescence spectra. The fluorescence spectral data show that conformational changes in the protein are driven by electrostatic interaction at first, then by hydrophobic interaction between a protein with a negative net charge and a negatively charged phospholipid.

Keywords: β-lactoglobulin; DMPG; Conformational changes; UV–VIS; Circular dichroism; Fluorescence

1. Introduction

It is well known that membranes in living organisms are approximately half phospholipids and half protein. The interactions between phospholipids and protein have aroused a great interest in the past decade. A variety of lipid membrane structures, formed by synthetic lipids, has been used to mimic the properties of biomembrane, and they furnish unique opportunity to investigate the interaction between phospholipids and protein. The vesicle system, a self-closed bilayer aggregate, constitutes one of the most explored models of biomembrane.

β-lactoglobulin (β-LG, as shown in Fig. 1 [1]) is a major protein of the whey fraction in bovine milk, and also present in the milk of most mammals and marsupials [2,3]. At physiological conditions, bovine β-LG exists as a dimer, with each monomer consisting of 162 amino acid residues and is characterized by a molecular mass of 18 kDa [4]. The structure of bovine β-LG, determined by X-ray and NMR respectively, reveals that β-LG is a predominantly β-sheet protein, which is composed of eight antiparallel beta-strands arranged in a barrel (A–H) and one α-helix [5–8]. Despite abundant physical and biochemical studies on β-LG, its biological role is poorly established. However, it has been suggested that β-LG may be involved in the transport of some small hydrophobic and amphiphilic compounds (e.g. phospholipids) [9]. Therefore, studies of the interaction between β-LG and phospholipids have induced a great interest.

In this paper, we reported that the interaction between β-LG and DMPG, with lipid vesicles as a model of biomembrane, could induce conformational changes in β-LG at pH 7.0. CD and fluorescence spectroscopy are versatile and sensitive techniques to measure conformational transition in proteins. To the best of our knowledge, the interaction between β-LG and DMPG investigated by CD and fluorescence spectra has not been reported. Na_2SO_4 or Ca^{2+} was added in the complex of β-LG and DMPG, and the effect of dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylglycerol (DPPG) was compared with that of DMPG to evaluate the interaction forces between β-LG and DMPG.

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2. Materials and methods

2.1. Materials

β-Lactoglobulin (from bovine milk, contains β-lactoglobulins A and B), DMPG, DMPC and DPPG were purchased from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were of analytical grade quality and used as received. Pure water was used throughout obtained by means of Millipore Q water purification set.

2.2. Sample preparation

A concentrated solution of β-LG in 10 mM Tris–HCl (pH 7.0) was stored in the refrigerator at −20 °C. The final solutions, prepared from the stored solution, were kept at 4 °C and used within two days after preparation.

For the preparation of lipid vesicles, a dry film of lipid was produced under a stream of nitrogen from lipid solutions in $CHCl_3$/MeOH (2:1), then under a vacuum pump for 4 h to remove the remnant solvent. An appropriate amount of 10 mM Tris–HCl solution was added to the glass tube containing the dried lipid film to obtain required concentration of lipid suspension. The lipid suspension was maintained for about 15 min above the gel to liquid crystalline phase transition temperature (T_c, 25 °C for DMPG; 23 °C for DMPC, 41 °C for DPPG). It was then sonicated in water bath in a temperature higher than the T_c until a clear solution suitable for spectroscopic determinations was produced.

2.3. Spectra methods

UV–VIS absorption spectra were measured with a Cary 500 Scan UV–VIS–NIR Spectrophotometer (Varian Co., USA). A protein concentration of 0.2 $mg \cdot ml^{-1}$ was used. The corresponding solvent (buffer with or without lipid vesicles) spectrum was subtracted from each sample spectrum.

Circular Dichroism (CD) spectra were recorded with a 62A DS CD spectrometer (AVIV Co., USA) with a 1.0 cm path length rectangular quartz cell. A protein concentration of 1 $mg \cdot ml^{-1}$ was used for near-UV CD spectra. The samples (0.04 $mg \cdot ml^{-1}$) for far-UV CD spectra were directly diluted with buffer from those for near-UV CD spectra. The baseline was corrected by subtracting the corresponding solvent (buffer with or without lipid vesicles) spectrum from each sample spectrum. Four scans were averaged per spectrum. All CD spectra were subjected to five smoothing algorithms.

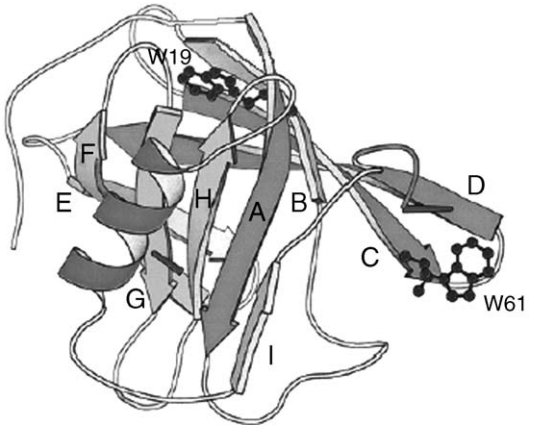

Fig. 1. Ribbon diagram of a single unit of bovine β-LG (adapted from [1]). The locations of Trp19 and Trp61 are indicated.

Fluorescence spectra were collected with a LS55 Luminescence Spectrometer (Perkin Elmer instruments; made in U.K.). Cuvette of 1 cm path length and a protein concentration of 0.2 $mg \cdot ml^{-1}$ were used. Background of light scattering was corrected by subtracting from each sample emission spectrum the corresponding solvent (buffer with or without lipid vesicles) spectrum.

All experiments were conducted in room temperature (20±1 °C).

3. Results and discussion

3.1. UV–VIS absorption spectra

The major absorbance of β-LG in the near UV (shown in Fig. 2a), with a maximum at about 280 nm, attributes to tryptophan (Trp) in the amino acid residue [10]. When binding to DMPG, the absorbance spectrum of β-LG has no obvious change except a small hypochromicity effect (shown in Fig. 2b).

3.2. CD spectra measurements

CD spectra provide an excellent means of probing the interaction between protein and other molecules. CD spectra were used to monitor the effects of DMPG on the structural and conformational properties of β-LG.

The far-UV CD spectra have been widely used to understand the secondary structure of proteins and provide the information about the peptide bond absorption [11]. Fig. 3a illustrates the far-UV CD spectrum of β-LG in 10 mM Tris–HCl buffer of pH 7.0, which is characterized by a largely β-sheet structure and a fraction of α-helix with a wide minimum in the 212–218 nm region. The shape of our spectrum is in agreement with previous reports [1,12–14]. Upon binding to DMPG, an obvious change was observed in the far-UV CD spectrum of β-LG (as shown in Fig. 3b, c, d). With increasing the concentration of the DMPG, the wide minimum gradually widened and deepened. Two minima at 209 and 222 nm appeared, which is indicative of α-helix. Thus, we can conclude from the far-UV CD spectral changes observed for β-LG that α-helical content in the protein is increased by an addition of DMPG. Our result is in accordance with that observed by two-dimensional infrared spectroscopy [9]. This result should not be surprising due to the high helical propensity of some residues included in β-strands in this protein. The increase in α-helix content can also be induced by some aqueous surfactant solutions [14]. Another cationic lipid DDAB has also been found to induce the β–α transition in the β-LG [14].

The near-UV CD spectra can characterize the tertiary structure of proteins mainly due to constrained asymmetries in

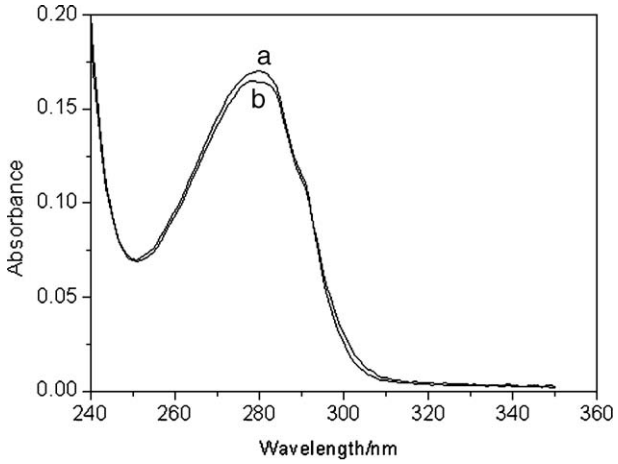

Fig. 2. Near-UV absorption spectra of 0.2 mg·ml^{-1} β-LG in pH 7.0 Tris–HCl buffer in the presence of (a) 0 and (b) 0.6 mg·ml^{-1} DMPG.

the environment of the aromatic amino acids [11]. The main chromophores in the near-UV CD in proteins are tryptophan, tyrosine, and phenylalanine [15,16]. There are two tryptophans (Trp 19 and Trp 61) in β-LG, which have been indicated in Fig. 1. As shown in Fig. 4a, the near-UV CD spectrum of β-LG in pH 7.0 displays two sharp peaks at about 293 and 285 nm. They have been ascribed to the Trp19 absorbance alone since Trp61 is located on the protein surface and not considered to be a significant source of the CD Trp band, while the indole ring of Trp19 is buried with the protein core, making it easily detectable [7,17,18]. Obvious changes can be observed in the near-UV CD spectrum of β-LG once binding to DMPG, which has been shown in Fig. 4b, c, d. The two peaks disappear gradually with continuous addition of DMPG. The intensity of the two sharp peaks indicates Trp19 is located in a chiral environment. The loss of the two peaks due to the interaction of DMPG shows that Trp19 has moved to a less chiral environment. This indicates that the tertiary structure of protein surrounding Trp19 has been loosened by the interaction of DMPG.

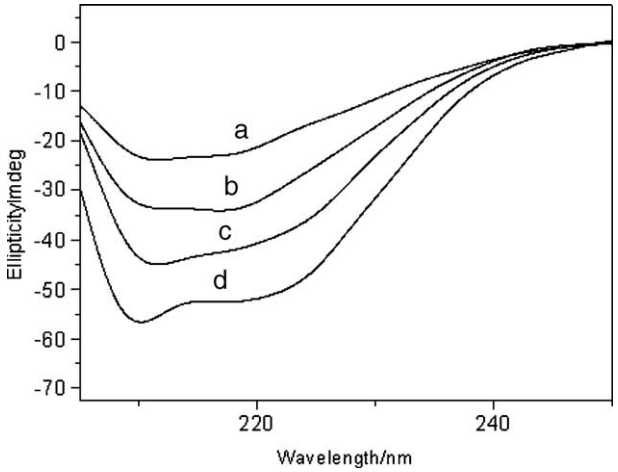

Fig. 3. Far-UV CD spectra of 0.04 mg·ml^{-1} β-LG in pH 7.0 Tris–HCl buffer in the presence of (a) 0, (b) 0.03, (c) 0.06 and (d) 0.12 mg·ml^{-1} DMPG.

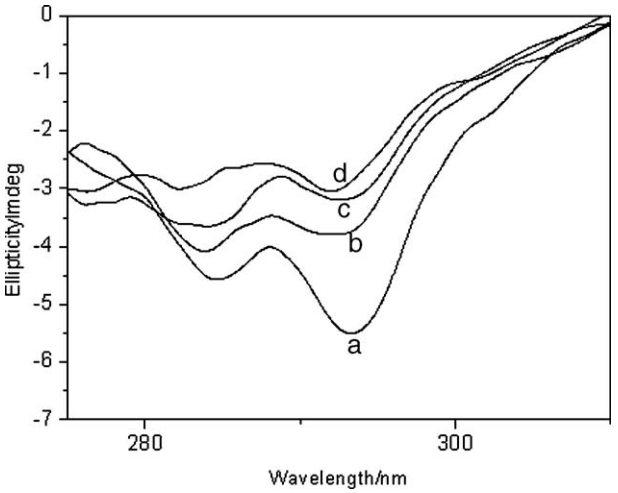

Fig. 4. Near-UV CD spectra of 1 mg·ml^{-1} β-LG in pH 7.0 Tris–HCl buffer in the presence of (a) 0, (b) 0.75, (c) 1.5 and (d) 3 mg·ml^{-1} DMPG.

3.3. Fluorescence spectra

Fluorescence spectroscopy is a useful technique to follow tertiary structure transitions in proteins because the intrinsic fluorescence of tryptophanyl residues is particularly sensitive to the polarity of microenvironments along the transition [14]. As is known that the transfer of Trp from an aqueous to a lipid medium leads to a blue shift in wavelength and an increase in intensity of the emission maximum. This is the case in our experiments. As shown in Fig. 5, exposure to increasing the concentration of DMPG causes the protein a slightly blue shift (339 to 331 nm) in the fluorescence emission maximum of Trp and a substantially increase in fluorescence intensity. The increase in intensity indicates the disappearance of tertiary interactions that quench the fluorescence in the native state. Trp is exposed to a less constrained environment. In native β-LG, Trp19, situated at the bottom of the calyx formed by the antiparallel β-strands, contributes about 80% to the total

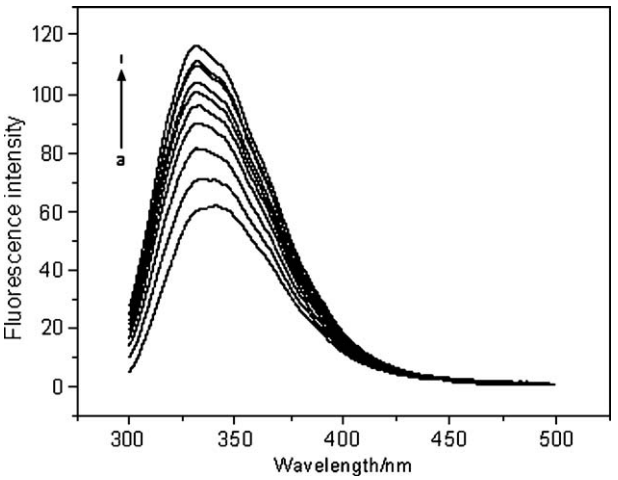

Fig. 5. Fluorescence emission spectra of 0.2 mg·ml^{-1} β-LG in pH 7.0 Tris–HCl buffer with excitation at 285 nm in the presence of (a) 0, (b) 0.075, (c) 0.15, (d) 0.225, (e) 0.3, (f) 0.375, (g) 0.45, (h) 0.525, (i) 0.6 and (j) 0.675 mg·ml^{-1} DMPG.

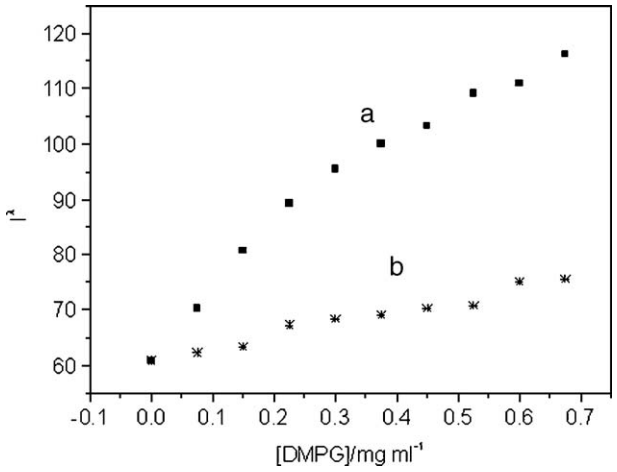

Fig. 6. Fluorescence intensity of 0.2 mg·ml^{-1} β-LG in pH 7.0 Tris–HCl buffer with excitation at 285 nm in the absence of (a) and in the presence of (b) 0.3 M Na_2SO_4 as a function of the DMPG concentration.

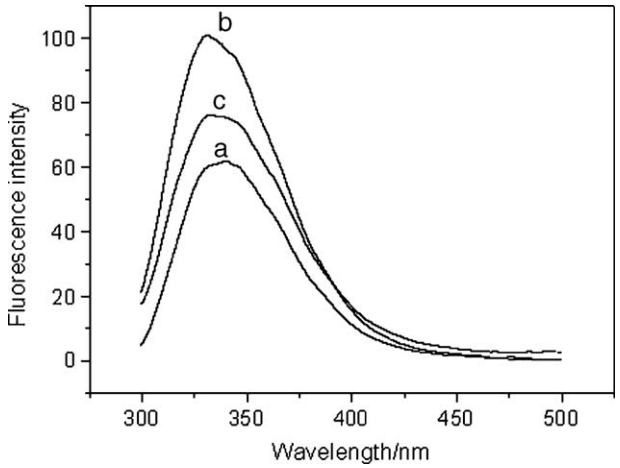

Fig. 7. Fluorescence emission spectra of (a) β-LG, (b) β-LG+DMPG and (c) β-LG+DMPG+Ca^{2+} in pH 7.0 Tris–HCl buffer with excitation at 285 nm. β-LG: 0.2 mg·ml^{-1}; DMPG: 0.375 mg·ml^{-1}; Ca^{2+}: 6 mM.

fluorescence [19] and a disulfide bond may quench Trp61 emission [20]. Therefore, the less restriction of Trp61 by disulfide bond, when β-LG bound to DMPG, directly led to an increase of the fluorescence intensity of β-LG. It indicated that the protein is partially unfolded by the interaction of DMPG. A blue shift of the emission maxima indicates that at least part of the Trp residues transfers into a more hydrophobic environment due to the interaction of β-LG with the acyl chain of phospholipid.

3.4. Interaction forces of β-LG with DMPG at pH 7.0

Hydrophobic interaction and electrostatic interaction between β-LG and phospholipids have been evaluated by some methods. Interaction between β-LG and phospholipids at the air/water interface by the film balance technique suggests that not only electrostatic but also hydrophobic interaction is needed for the interaction [21]. The studies on monolayer and lipid aqueous dispersion propose that the presence of a surface charge is important for the interaction of β-LG with the 1-monostearoylglycerol bilayers [22,23]. One and two-dimensional infrared spectroscopy also imply that the surface charge of the membrane seemed to be the main factor for inducing protein conformational changes by DMPG [9]. In this paper, fluorescence spectra were used to evaluate the interaction forces between β-LG and DMPG.

At pH 7.0, above the isoelectric point of β-LG (pI=5.2), the protein is negatively charged, while DMPG molecules are in the anionic form. How can they interact with each other? The reason may be as follows. One is the protein interacts with DMPG through hydrophobic force due to their same charge. The other possible explanation might be that a region rich on positively charged residues on the protein surface will be orientated toward the phospholipid [21]. The negative charge headgroup of DMPG interacts with the protein surface rich on positive charged residues; that is, electrostatic interaction operates in the complex of protein and phospholipid. Of course, hydrophobic interaction may also exist because a blue shift of the emission maximum is found. We prefer the latter one. It has been proved that β-LG has a high dipole moment 730D, as a result of an inhomogeneous charge distribution [24].

In order to prove our assumption, the following experiments were performed. Firstly, the effects of salts on the interaction between β-LG and DMPG are evaluated. Generally, the addition of salt results in the reduction of the electrostatic interaction. Salt solutions (0.1–1 M) and neutral pH are known to affect the structure and property of β-LG, the effect being dominated by anions. However, we did not find that 0.3 M Na_2SO_4 could affect the fluorescence spectra of β-LG in our experiment (data not shown), in accordance with previous report [13]. As shown in Fig. 6, with the same concentration of the DMPG, the fluorescence intensity of β-LG in the presence of 0.3 M Na_2SO_4 is lower than that in the absence of the salt. In order to quantitatively interpret the effect of salt, the fraction of bound β-LG, α, is calculated from the changes in the fluorescence emission intensity at 285 nm. The fraction of bound (α) can be estimated by defining $\alpha = (I^{\lambda} - I^{\lambda}_{free})/(I^{\lambda}_{bound} - I^{\lambda}_{free})$, where I^{λ} is fluorescence intensity of lipid/β-LG mixtures and I^{λ}_{free} is the fluorescence intensity in the absence of lipid. I^{λ}_{bound} denotes the fluorescence intensity of

Table 1
The fraction of bound β-LG (α) in the absence of and presence of Na_2SO_4

	α_0	α_1	α_2	α_3	α_4	α_5	α_6	α_7	α_8	α_9
Without Na_2SO_4	0	0.056	0.118	0.168	0.205	0.232	0.251	0.286	0.296	0.327
With Na_2SO_4	0	0.0089	0.0154	0.0384	0.0449	0.0488	0.0564	0.059	0.0848	0.0873

α_{0-9} was corresponding to α in the different concentration of DMPG: 0, 0.075, 0.15, 0.225, 0.3, 0.375, 0.45, 0.525, 0.6 and 0.675 mg·ml^{-1}.

β-LG totally bound. The value of I^{λ}_{bound} is 230.14, which can be extrapolated from a double-reciprocal plot [25]. The values of α at different concentrations of lipid in the absence and presence of Na_2SO_4 have been shown in the Table 1. It is clear that the values of α are reduced greatly by the addition of Na_2SO_4. Therefore, it can be concluded that electrostatic force plays an important role in the interaction.

Ca^{2+} is known to form 1 : 1 complex with phosphatidylglycerol [26] and proven to interfere with lipid-protein binding in the monolayer experiments [27]. Thus, secondly, Ca^{2+} was added in the complex of β-LG and DMPG to study the interaction force. Fig. 7 illustrates that fluorescence intensity of the complex of β-LG and DMPG decreases upon an addition of 6 mM Ca^{2+}, which can neutralize the negative charge in the headgroup of DMPG. Actually, the fluorescence spectrum of β-LG is found to be independent of 6 mM Ca^{2+}. This further proves the presence of a surface charge is important for the lipid membrane to bind with β-LG. Finally, the other two phospholipids (DMPC, DPPG) were tested in order to compare with DMPG (data not shown). We found that the fluorescence spectra of β-LG did not have any significant change in the presence of DMPC, while the presence of DPPG had almost the same effect as DMPG. Zwitterionic phospholipids such as PC, does not induce significant perturbations in native structure of the protein [28] and anionic phospholipid has a higher affinity for β-LG [29]. It has been pointed out that a blue shift of the emission maximum is indicative of hydrophobic interaction. It is surprising that we did not find a blue shift of the emission maximum when DMPC, which has the same acyl chain with DMPG, was present. It has been reported that it is impossible for phosphatidylcholine (PC) to bind the native β-LG. However, when the helix content of the protein is increased, PC can bind to β-LG [30]. Thus, we infer that electrostatic interaction first induces an increase of helix, and then hydrophobic interaction occurs when β-LG interacts with DMPG. We can conclude from the experiments above that electrostatic interaction plays a main role in the interactions between β-LG and DMPG.

4. Conclusions

We have shown clearly that the native structure of β-LG can be modified due to the interaction of DMPG. By combined UV–VIS, CD and fluorescence spectra, the conclusions can be drawn as follows.

(1). It is suggested by the far-UV CD data that binding to DMPG can cause the increase of α-helix content in β-LG. The secondary structure elements in this protein are reorganized.
(2). By combining the near-UV CD and fluorescence spectroscopy, it can be concluded that the tertiary structure is loosened and the protein is partially unfolded owing to the binding of DMPG.
(3). As for the interaction forces, electrostatic interaction is indispensable for the interaction between β-LG and DMPG.

Acknowledgments

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