# Effect of Phospholipids on Conformational Change and Heat Stability of Ovalbumin. Circular Dichroism and Nuclear Magnetic Resonance Studies

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The interactions between phospholipids and protein have been studied by nuclear magnetic resonance and circular dichroism techniques. Egg phosphatidylcholine (PC) did not affect the protein structure of ovalbumin (OA). However, lysophosphatidylcholine (LPC) changed the conformation of OA to increase the $\alpha$-helix and to reduce the $\beta$-sheet content by interaction with the protein. The heat stability of OA was enhanced by coupling with LPC and linoleic acid. The results of $^{31}P$ and $^{13}C$ NMR spectra and $T_2^*$ relaxation times of the complexes showed that the molecular motion of acyl chain and glycerol carbons of LPC was restricted by interaction with the protein. The motion of the headgroup, however, remained unaffected. These results indicated that LPC bound to the protein through hydrophobic interaction and led to conformational change of protein structure. It is therefore suggested that the heat stability of emulsion composed of the complexes was closely correlated with its protein structure and the dynamic state of the complexes.

## INTRODUCTION

The interaction between phospholipids (PLs) and proteins has been an active research topic for a number of years and has been widely investigated. The main research interest is to gain a better understanding of the structure and function of biological membranes. A typical membrane is known to contain both proteins and PLs. In addition to the structural element in cells, proteins and PLs contribute significantly to the physical properties of many aspects of technological interest (e.g., emulsions and foams). For example, the bilayer lipid membrane assists in stabilizing the lipid phase of milk by forming a film on the surface of fat globules (Keenan et al., 1983). Low-density lipoprotein is a major component of hen's egg yolk and is considered to be the most important contributor to the emulsifying properties of egg yolk (Vincent et al., 1966). Furthermore, protein structure, function, and properties are affected in various ways by PLs. It is obvious that the investigation of PL–protein interaction is important to the understanding of their physiochemical and biological properties in food systems as well as in cells.

Several studies have been made on the interaction of PLs and food proteins by using various techniques (Ohtsuru et al., 1976; Schenkman et al., 1981; Brown et al., 1983; Beckwith, 1984; Hirotsuka et al., 1984; Hanssens et al., 1985; Nakamura et al., 1988; Cornell, 1991). We have recently reported the interfacial adsorptivity of egg phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and ovalbumin (OA) by means of nuclear magnetic resonance (NMR), which provides valuable information about their interfacial adsorptivity (Chiba and Tada, 1988, 1989, 1990a,b; Mine et al., 1992a). We found that the interfacial adsorptivity of OA was promoted by interacting with LPC and free fatty acid (FFA). The bilayer vesicles composed of LPC and FFA were destroyed by coupling with OA (Mine et al., 1992b,c). The free energy of binding of PL to protein consists of two main contributions, (a) those from electrostatic interactions and (b) those from hydrophobic interactions (Oakes, 1973; Ohtsuru et al., 1976; Cornell and Patterson, 1989). Korver and Meder (1974) reported that the soya lysolecithin destabilized the native conformation of $\beta$-lactoglobulin similar to those caused by heat denaturation or increase in pH. The interaction between sodium dodecyl sulfate (SDS) and caseins is similar to that between lysolecithin and casein, suggesting that structure either deaggregated or became looser (Barratt and Rayner, 1972). The nature of binding of LPC to the proteins is not made clear. On the other hand, the emulsifying property and heat stability of egg yolk are improved by pancreatic phospholipase A-2 (Dutilh and Groger, 1981); consequently, the higher emulsifying property and heat stability of the modified egg yolk lipoprotein may be closely correlated with the formation of the complex composed of LPC, FFA, and proteins. It is not clear why the emulsion composed of enzyme hydrolytic egg yolk gains heat stability and higher emulsifying properties. Our interest in this study was focused on elucidation of the nature of binding of LPC to OA and their influence on heat stability.

Circular dichroism (CD) is a powerful tool for monitoring the conformational change of protein and has been frequently utilized in studying the conformation of protein-lipid systems. NMR studies can provide direct information about (a) the binding site, (b) changes in protein structure, and (c) the equilibrium and exchange rates between bound and free PL molecules. In addition, NMR relaxation time measurement constitutes a very sensitive probe for the study of the molecular environment, and $T_2^*$ values are also known to be sensitive to intermolecular interactions (Ramsammy et al., 1983).

In the present work, we performed a conformational analysis of the interaction of LPC, FFA, and OA and elucidated the binding property of LPC to the protein by means of CD and NMR measurement. We also discussed the heat stability of these complexes in relation to protein structure.

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**Table I. Effect of the Heat Treatment on the Emulsions Composed of OA, PC/OA Complex, and LPC/Linoleic Acid/OA Complex**

| | | mean droplet size[a] (μm) |
|---|---|---|
| OA | untreated | 6.6 |
| | heated at 80 °C[b] | 18.4 |
| PC/OA | untreated | 2.4 |
| | heated at 80 °C | 5.3 |
| LPC/$C_{18-2}$[c]/OA | untreated | 0.7 |
| | heated at 80 °C | 0.9 |

[a] The data are expressed as the mean volume droplet size. [b] Heated in a water bath at 80 °C for 20 min. [c] Linoleic acid.

## MATERIALS AND METHODS

**Materials.** Ovalbumin (OA) was prepared from fresh egg white by crystallizing out in aqueous sodium sulfate and was recrystallized from aqueous ammonium sulfate five times (Kekwick et al., 1936). PC, 98% pure, was obtained from egg PC (PC-98S, Q.P. Corp., Tokyo). LPC, 98% pure, was obtained from egg LPC (LPC-1, Q.P.) which was obtained by phospholipase A-2 action of egg PC. The fatty acid composition of LPC at glycerol *sn*-1 was analyzed by GLC after transmethylation with the following results: $C_{16-0}$, 67.0%; $C_{16-1}$, 1.3%; $C_{18-0}$, 24.9%; $C_{18-1}$, 5.2%; $C_{18-2}$, 0.6%; others, 1.0%. Linoleic acid, purity 99%, was purchased from SRL Corp. The peroxide value of the linoleic acid was under 5 mequiv/kg. Triolein (Wako Pure Chemicals, Tokyo) was purified by silica column chromatography (hexane–diethyl ether, 97:3). Other reagents were purchased from Wako.

**Preparation of Complexes.** The PC/OA or LPC/linoleic acid/OA complexes were prepared as described previously (Mine et al., 1992b).

**Emulsification and Evaluation of Heat Stability.** Emulsion was prepared as described previously (Mine et al., 1992b). The buffer used was 20 mM HCl, and the ratio of oil/protein (w/w) was 6. The heat stability of the emulsions were evaluated as follows; the emulsion was kept at 80 °C for 20 min. After heat treatment, the emulsion solution was cooled at room temperature by ice water. The size distribution of the emulsion droplets before and after heat treatment was measured by a laser light scattering photometer (submicron particle sizer, Pacific Scientific Nicomp, Model 370-HPL). The data were expressed as the mean volume droplet size.

**Circular Dichroism Measurement.** Circular dichroism (CD) measurements were made with a JASCO J-720 spectropolarimeter using a 1-mm cell at 25 °C. The relative proportions of secondary structure were calculated according to the modified CD curve-fitting method (Yang et al., 1986). The CD spectrum was simulated using the reference spectra for the α-helix, β-structure, and random coil determined by Yang et al. from eight proteins with known structure. The simulation was done in the wavelength region 240–190 nm at 1-nm intervals. Molecular ellipticity was expressed in terms of the mean residue ellipticity (116.9). The buffer used was 20 mM HCl, and the concentration of OA was kept at 0.0154%.

**Measurement of NMR Spectra.** $^{13}C$ NMR spectra were recorded on a Varian VXR-4000S spectrometer at 20 °C. The $^{13}C$ NMR spectra at 100.6 MHz fitted with a probe (10 mm, 45–165-MHz frequency) were acquired with 32K data points, 25 000-Hz spectral window, 45° pulse (12.7 μs), 20 rotations/s spinning, and 0.8-s pulse delay. Proton was completely decoupled by 8400-Hz decoupler modulation frequency, and chemical shifts were referenced to TMS at 0 ppm. $T_2^*$ relaxation times were calculated approximately from the line widths of the carbon signals ($\upsilon_{1/2}$, $T_2^* = 1/(\pi\upsilon_{1/2})$. $^{31}P$ NMR spectra were recorded on the same spectrometer as described previously (Mine et al., 1992b). NMR samples were 3.1 mL in 10-mm precision tubes.

## RESULTS

**Heat Stability of the Emulsion Composed of the PLs/OA Complex.** Table I shows the effect of heat treatment on emulsion composed of OA, PC/OA complex, and LPC/linoleic acid/OA complex. The molar ratio of PLs/OA was 20. The LPC/linoleic acid/OA complex formed smaller droplets compared to OA or PC/OA complex. The stability of emulsion composed of OA was affected by the heat treatment and leads to oil exudation by heating at 80 °C for 20 min. The mean droplet size of emulsion composed of PC/OA complex increased by heat treatment. On the other hand, the emulsion composed of LPC/linoleic acid/OA complex showed heat stability, and the mean droplet size of the emulsion was hardly changed by heating. The difference in heat sensitivity of these emulsions may be closely correlated with conformational change of OA caused by the heat treatment. To examine the conformational changes of OA by interacting with PLs and following heat treatment, we studied CD analysis.

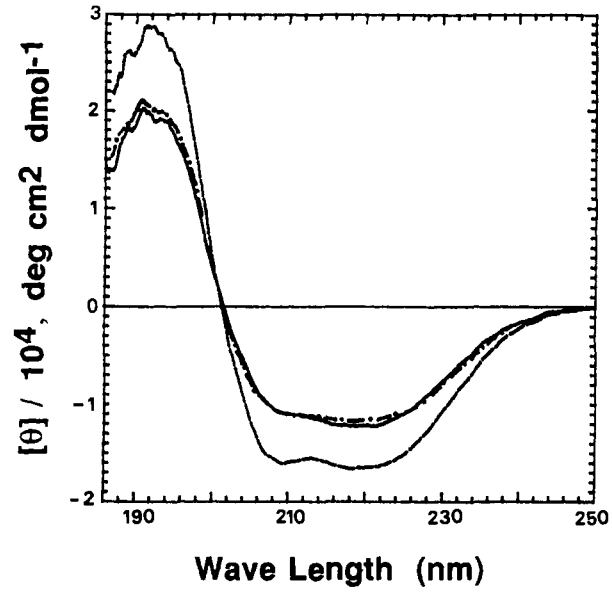


**Figure 1.** Circular dichroism spectra of OA(—), PC/OA complex (– · –), and LPC/linoleic acid/OA complex (- - -). The binding molar ratio of PLs/OA was 20.

**Circular Dichroism Measurement of PLs/OA Complex.** Figure 1 shows typical CD spectra of OA, PC/OA, and LPC/linoleic acid/OA complex. The molar ratio of PLs/OA was 20. The spectrum of the LPC/linoleic acid/OA complex differed from the other two spectra, showing increased α-helix content and reduction of β-structure on OA by interacting with LPC and linoleic acid. The sonication did not alter the conformation of OA. Linoleic acid did not affect the protein structure of OA employed in this experiment (data are not shown). The CD measurement was carried out with the PLs/OA complex composed of various molar ratios of PLs bound to the protein, and the observed spectra were analyzed by the procedure described under Materials and Methods. Figure 2 shows the effect of PLs on conformation of OA. In the absence of PLs, the α-helix and β-structure contents of OA were 30.9% and 34.9%, respectively. PC did not affect the protein structure of OA. However, LPC changed the conformation of OA and increased with the α-helix content of the protein. The increasing ratio of helical content in the protein was increased with an increase of binding ratio of LPC to the protein. The β-structure decreased with increasing LPC concentration. We further investigated the effect of heat treatment of LPC/linoleic acid/OA complexes on their protein structure, and the results are summarized in Table II. The α-helix content of OA was decreased from 30.9% to 19.2% by heating at 80 °C for 20 min. The β-structure content of the protein was increased by heat treatment. On the other hand, the α-helix content of OA was increased from 30.9% to 44.4%

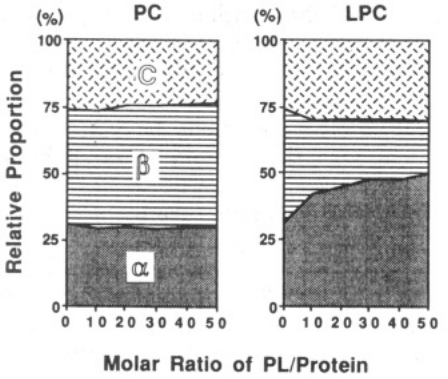


**Figure 2.** Changes in the secondary structure of OA as a function of PLs concentration. ($\alpha$) $\alpha$-Helix; ($\beta$) $\beta$-structure; (C) random coil. Each area gives the fraction of the corresponding structure.

**Table II. Secondary Structures of Native and Heated OA and LPC/$C_{18-2}$/OA Complex Derived from the CD Spectra**

| | | % secondary structure parameter | | | |
|---|---|---|---|---|---|
| treatment | | $\alpha$-helix | $\beta$-sheet | $\beta$-turns | random coils |
| OA | native | 30.9 | 34.9 | 7.8 | 26.5 |
| | heated at 80 °C[a] | 19.2 | 44.0 | 4.5 | 32.3 |
| LPC/$C_{18-2}$[b]/OA | native | 44.4 | 14.0 | 11.2 | 30.4 |
| | heated at 80 °C | 36.1 | 22.3 | 6.3 | 35.3 |

[a] Heated in a water bath at 80 °C for 20 min. [b] Linoleic acid.

by coupling with LPC and linoleic acid, and the secondary structure of LPC/linoleic acid/OA complexes were less influenced by heat treatment compared with OA. The heat stability of the emulsion composed of LPC/linoleic acid/OA complex may be closely correlated with its protein structure and the dynamic state of the complexes. To see more details, we observed the $^{13}C$ and $^{31}P$ NMR spectra of LPC and LPC/linoleic acid/OA complexes to get information about the binding site of LPC to OA.

**NMR Measurement.** Figure 3 shows the $^{31}P$ and $^{13}C$ NMR spectra of LPC micelles, LPC/linoleic acid liposome, and LPC/linoleic acid/OA complex dispersed in 20 mM HCl. The binding molar ratio of LPC/OA was 20. The $^{31}P$ and $^{13}C$ NMR spectra of LPC have been assigned (Birdsall et al., 1972; Chapman and Morrison, 1966; Mine et al., 1992b). The line widths of $^{31}P$ NMR and the $T_2^*$ values for choline C1–C3, glycerol C4–C6, and the $CH_3$ group of the hydrocarbon chain are shown in Table III. In an aqueous dispersion of LPC micelles (Figure 3a), the choline, glycerol carbons, and hydrocarbons were clearly resolved and showed sharp signals. Aqueous dispersions of an equimolar mixture of LPC and long-chain FFA form bilayers (Mine et al., 1992c). In the bilayers composed of LPC and linoleic acid, the line width of $^{31}P$ NMR was broader and $T_2^*$ values for choline C3, glycerol C4–C5, and the $CH_3$ group of the hydrocarbon chain were low in comparison with those of LPC in globular micelles. In the case of the LPC/linoleic acid/OA complex, glycerol C4–C6 and hydrocarbons were much broadened and the $T_2^*$ relaxation time was reduced by interacting with OA, resulting from restriction of molecular motion. The $T_2^*$ value for choline C3 was decreased, but that of choline C1–C2 scarcely changed. The line widths of $^{31}P$ NMR of LPC in the complexes were little influenced by interacting with the protein. These results shows that the acyl chains, glycerol, and a part of the choline carbons of LPC interact with the protein, while the motion of the headgroup remains unaffected. We also studied the binding energy

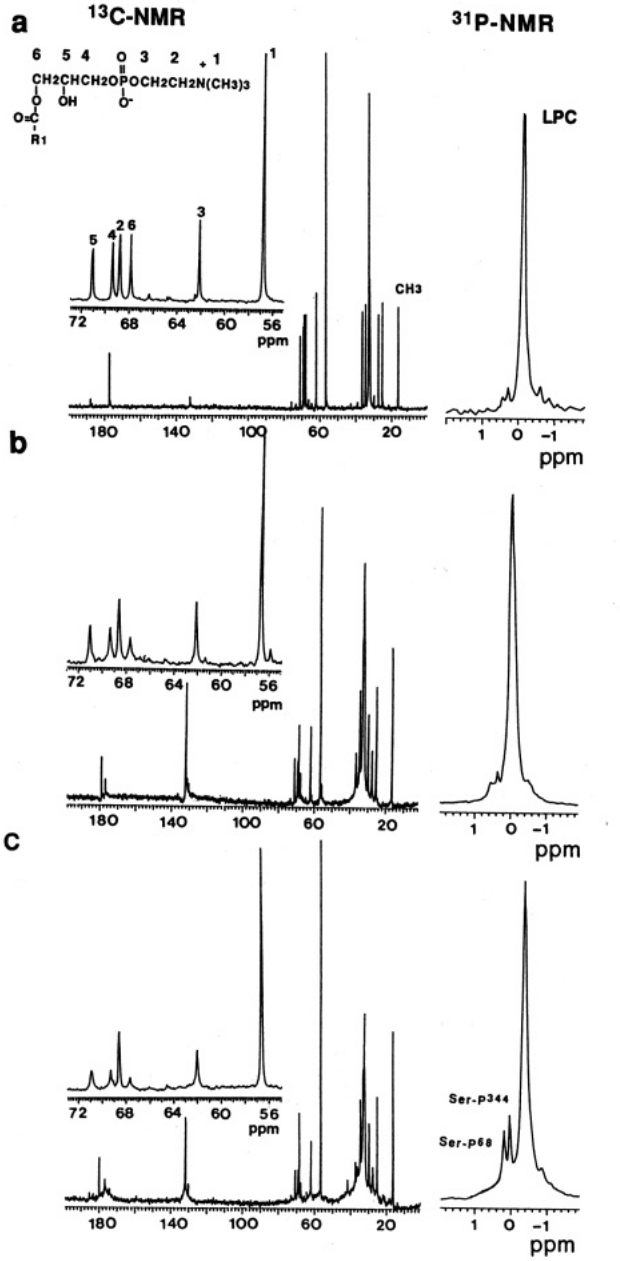


**Figure 3.** $^{13}C$ and $^{31}P$ NMR spectra of LPC (a), LPC/linoleic acid liposome (b), and LPC/linoleic acid/OA complex (c) dispersed in 20 mM HCl. Chemical shifts for choline, glycerol, and methyl group carbons of LPC were assigned as shown. All spectra were obtained at 20 °C with 20 160 accumulations and 5.0-Hz line broadening for $^{13}C$ NMR spectra and with 3000 accumulations and 10-Hz line broadening for $^{31}P$ NMR spectra.

**Table III. Line Widths of $^{31}P$ NMR and $T_2^*$ Relaxation Times for Choline, Glycerol, and Acyl Chain Carbons of LPC**

| | $^{31}P$ NMR (Hz) | $^{13}C$ NMR (ms) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | choline | | | glycerol | | | acyl |
| | | C1 | C2 | C3 | C4 | C5 | C6 | $CH_3$ |
| LPC | 20 | 26 | 19 | 28 | 23 | 21 | 32 | 48 |
| LPC/$C_{18-2}$ | 33 | 28 | 21 | 13 | 19 | 15 | 19 | 36 |
| LPC/$C_{18-2}$/OA | 27 | 25 | 18 | 17 | 17 | 12 | 16 | 31 |

of LPC and linoleic acid to OA. Reduction of electrostatic interaction, hydrogen bond, and hydrophobic interaction was observed with 0.6 M KCl, 1.5 M urea, and 8 M urea in the subphase, respectively (Kauzmann et al., 1959). At a subphase in 20 mM HCl, over 90% reduction in LPC/linoleic acid/OA binding was observed with 8 M urea in the subphase (data are not shown).

## DISCUSSION

We have previously shown that OA forms a complex with PL by sonication and the interfacial adsorptivity of the protein is promoted by coupling with LPC and FFA (Mine et al., 1992b). The results presented here show that the heat stability of the emulsion composed of LPC/linoleic acid/OA complex is correlated with its protein structure. It is well-known that charged surfactants induce conformational changes in proteins. Sodium dodecyl sulfate (SDS) is known to bind to most protein in a nonspecific cooperative manner, destbailizing the native conformation. In most cases, interaction between proteins and PLs led to major conformational change of protein. The protein is found to be either associated to the polar interface of the PL bilayer and monolayers by electrostatic interaction or incorporated in the lipid layers by a combination of hydrophobic and electrostatic attractive forces. Increase of $\alpha$-helix of proteins is a common indication of PL complexation by proteins (Cornell, 1982; Brown et al., 1983; Hanssens et al., 1985). According to our result, PC did not affect the protein structure of OA in spite of interaction with the protein; however, LPC affected the conformation of OA and increased $\alpha$-helix content and reduced $\beta$-sheet content of OA by coupling with the protein. Jirgensons (1973) has proposed the *reconstructive denaturation* that is caused with increase of $\alpha$-helix content of polypeptide chain by the interaction with anionic detergents such as SDS. The promotion of the $\alpha$-helix formation by SDS has been explained as the effect of shielding of the interior of the macromolecules by the hydrophobic chains of the detergents. The formation of $\alpha$-helix of OA by interacting with LPC and FFA is presumed to be caused when the $\beta$-sheet structure of OA is converted to helical structure by shielding the interior of the protein by the hydrophobic chains of LPC and FFA. It is interesting that the LPC/linoleic acid/OA complex showed heat stability. The stability of the emulsion composed of just OA was affected by heat treatment, and the emulsions composed of the complexes showed heat stability. The destruction of the emulsion by heat treatment was assumed to be due to the denaturation of the constructive components at the interface. The heat stability of OA was caused by coupling with LPC and FFA. Thus, it was suggested that the heat stability of the emulsion was correlated with the conformational changes of the protein structure caused by LPC and FFA.

NMR is a valuable technique for binding studies. When molecules have freedom of motion, relaxation times are long and absorption lines are narrow. When molecular motion is restricted, relaxation times are short and line widths increase. Broadened lines can result, for example, when solvent viscosity is very high or when molecular associations occur. Most globular proteins do not exhibit high-resolution spectra because the carbons and protons are constrained. Consequently, the line widths of carbons or protons in small molecules such as PL bound to proteins are greatly increased to values characteristic of macromolecules. Thus, NMR can show which carbons or protons of lipid are immobilized by binding to proteins. The sharp signals of LPC in 20 mM HCl are thought to be caused by the motional freedom of a headgroup in micelles. The changes of the line widths of $^{31}P$ NMR and $T_2^*$ values of choline C3, glycerol, and acyl chain carbons were interpreted as the interaction between LPC and FFA which formed bilayer vesicles. We previously reported that vesicles composed of LPC and FFA were destroyed and reconstituted by coupling with the protein (Mine et al., 1992c). The results of $^{31}P$ and $^{13}C$ NMR spectra and $T_2^*$ relaxation times of the complexes showed that the molecules of all acyl chain and glycerol carbons of LPC were restricted by interaction with the protein, but the motion of the headgroup remained unaffected.

From the present work, it can be concluded that the LPC was bound to the protein through hydrophobic interaction and caused the increased of the $\alpha$-helix and the reduction of $\beta$-sheet structure by coupling with the protein. The heat stability of the emulsion containing just OA was weak, but it was much improved by coupling with LPC and FFA. The reason for heat stability of emulsion composed of the complexes was closely correlated with the conformational change of the protein. Our results in this experiments also suggest that the heat stability of the emulsions composed of the modified egg yolk with phospholipase A-2 is correlated with protein structure caused by interacting with LPC and FFA. The transitions of functional properties of proteins incited by LPC and FFAs are of interest for a better understanding of the structural properties and their relation to biological function.

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