^{31}P NMR Study on the Interfacial Adsorptivity of Ovalbumin Promoted by Lysophosphatidylcholine and Free Fatty Acids

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The promotion of the interfacial adsorption of ovalbumin by phospholipids (PLs) and free fatty acids (FFAs) was investigated by observing the phosphate residues of PLs and proteins with ^{31}P NMR. The complex of ovalbumin and PLs showed separated phosphorus signals of Ser–P^{68} and Ser–P^{344} of ovalbumin and phosphate residues of PLs in the ^{31}P NMR. The interfacial adsorptivity was evaluated by the droplet size distribution. The line widths of the phosphorus signals of the complex in emulsion were well correlated with their interfacial adsorptivity and differed markedly between the highly adsorptive protein–lipid complexes and lesser ones. The interfacial adsorptivity of ovalbumin increased with the interaction of phosphatidylcholine (PC) or lysophosphatidylcholine (LPC) to form smaller droplets, and the formation of the microemulsion was further promoted by the addition of linoleic acid in the mixture. It was also found that the motional property of Ser–P^{68} of ovalbumin was much restricted at the interface when the complex formed a microemulsion. The results suggested that LPC and FFA mutually changed the structure of protein to increase the interfacial adsorption.

INTRODUCTION

Proteins and phospholipids (PLs) are the principal surface-active compounds of nature. They have a very important physical property in common, an inherent amphiphilic property. This dualistic character is important for the formation of composite biological structures, such as cell membranes and lipoproteins. Interactions between proteins and PLs have received intensive interest for many years.

In the field of food chemistry, physical and chemical properties of protein–PL complexes in emulsions are interesting problems. Phosphatidylcholine (PC) is one of the most common PLs for constructing biomembranes and exists in egg yolk and soybean in higher concentration. PC also plays an important role in the manufacture of foods as an emulsifier. Lysophosphatidylcholine (LPC), although it exists as a minor component in phospholipids, is also an interesting emulsifier which shows higher water solubility and emulsifying properties. Phospholipase A_2 specifically hydrolyzes the fatty acids at glycerol-*sn*-2 of the PC molecules, and LPC is widely utilized industrially for an emulsifier with higher surface activity (von Nieuwenhuyzer, 1981).

A number of papers have appeared on the interaction between PC and food proteins (Ohtsuru et al., 1976, 1979; Kanamoto et al., 1977; Schenkman et al., 1981; Brown et al., 1983; Beckwith, 1984; Hanssens et al., 1985; Cornell and Patterson, 1989; Ericsson, 1990). Hirotsuka et al. (1984) have shown that soy lecithin has a strong affinity for soy protein and the emulsification activity of the complex was increased by ethanol and heat treatments of the complex. Emulsifying properties of several proteins were greatly enhanced by sonicating proteins with egg yolk PC (Nakamura et al., 1988). On the other hand, very few investigations have been reported on the interaction between LPC and food proteins and on the interfacial adsorptivity of the complex of LPC and proteins.

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In general, proteins are known to expose hydrophobic amino acid side chains at low pH, and proteins such as β-lactoglobulin (Brown et al., 1983), α-lactalbumin (Hanssens et al., 1985), and bovine serum albumin (Schenkman et al., 1981) interact well with PC vesicles at low pH. PC is also associated with soybean protein by either hydrophobic interaction between a PC molecule and the protein or the combination of PC lamellae at the protein surface (Ohtsuru et al., 1976). The emulsifying property and heat stability of egg yolk lipoprotein are improved by the phospholipase A_2 (Hell et al., 1970; Dutilh and Groger, 1981); consequently, the higher emulsifying property of the modified egg yolk lipoprotein may be closely correlated with the formation of the complex composed of LPC, FFAs, and proteins. In addition, the dispersion of bulk fat globules into finely divided emulsion particles (<0.5 μm in diameter) occurs in gastrointestinal lipid digestion (Carey et al., 1983). It is well-known that biliary and dietary PC or choline play an important role in fat adsorption as an emulsifier of the triglyceride (O'Doherty et al., 1973; Tso et al., 1981). Phospholipase A_2 is also secreted from the pancreas when we eat dietary lipids. As phospholipase A_2 forms an equimolar mixture of LPC and FFA, there is a possibility that LPC enhances the interfacial adsorptivity of proteins by the interaction with FFAs to promote the absorption of dietary oil or protein digestion. Thus, it is interesting to investigate the formation of complexes of hydrolytic products of PC with proteins and the effect on the interfacial adsorption. The analysis of the dynamic state of protein molecules at the interface is also important to elucidate whether LPC and FFA form complexes with proteins to promote its interfacial adsorption or the mixture of LPC and FFA shows higher emulsifying property. However, there is much difficulty in the evaluation of the interfacial adsorptivity of the complex of protein and PL by conventional physicochemical techniques.

Phosphorus NMR of phospholipids has given new insight in lipid–protein interactions in membranes and provided useful information about lipid–protein interactions (Seelig, 1978; Yeagle, 1982). Previously, we inves-

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tigated the interfacial adsorptivity of PC and LPC by ^{31}P NMR and found that the headgroup motion of PC and LPC changed with the interfacial adsorptivities and with their emulsion stabilities (Chiba and Tada, 1989, 1990a,b). The line width of ^{31}P NMR spectra of LPC was well correlated with its interfacial adsorptivity. Moreover, stable emulsions were effectively formed by LPC in the presence of FFA (Chiba and Tada, 1990b).

Egg white proteins are extensively utilized as functional food products in food processing, and ovalbumin is the main constituent of egg white proteins. Previously, we observed a correlation of the emulsifying properties of ovalbumin with phosphorus signals of ^{31}P NMR (Mine et al., 1992). Thus, the NMR technique is expected to be applied in the evaluation of the effect of interaction between proteins and PL on the interfacial adsorptivity.

In this paper, the interfacial adsorptivity of complexes of ovalbumin and PLs was investigated by ^{31}P NMR to elucidate the role of LPC and FFA on the promotion of interfacial adsorption of proteins.

MATERIALS AND METHODS

Materials. Ovalbumin was prepared from fresh egg white by crystallizing in aqueous sodium sulfate and recrystallized from aqueous ammonium sulfate five times (Kekwick et al., 1936). *p*-Ovalbumin was prepared by pepsin digestion (Kitabatake and Doi, 1988). PC and LPC were obtained from egg PC (Q. P. Corp., PC-98N) and egg LPC (Q. P., LPC-1) which was obtained by phospholipase A_2 treatment of egg PC. The purity of PC and LPC was more than 98%. Triolein (Wako Pure Chemicals, Tokyo) was purified by silica gel column chromatography (hexane–diethyl ether 97:3) and evaporated to dryness under N_2 gas. FFAs were purchased from Wako.

Preparation of Vesicles and Micelles. Unilamellar PC vesicles (SUV-PC) were prepared as follows: PC (37 mg/mL) in 20 mM HCl or 20 mM NaOH was subjected to a microfluidizer (Model M-110F, Microfluidics Corp.). The pressure was raised to 8000 psi at 4 °C. After centrifugation (5000*g*, 30 min, 4 °C), a clear or slightly translucent dispersion was obtained. The sample was swept with nitrogen and put in vials; the vials were capped tightly and placed in a refrigerator at 4 °C. The size distribution of SUV-PC was 20–50 nm, as measured with a laser light scattering photometer (submicron particle sizer, Pacific Scientific Nicomp Model 370-HPL). The LPC globular micelles (25 mg/mL) in 20 mM HCl or 20 mM NaOH were prepared by sonicating (Branson sonifier Model 250, output 40 W) for 5 min at 60 °C. The LPC/FFA vesicles were prepared as follows: An aqueous dispersion of an equimolar mixture of LPC (25 mg/mL) and linoleic acid in 20 mM HCl or 20 mM NaOH was sonicated for 5 min at 60 °C. The size distribution of LPC/linoleic acid vesicles was 50–200 nm. The phosphorus signal of NMR in SUV-PC and LPC/linoleic acid vesicles was split by the addition of 5 mM $PrCl_3$, indicating the bilayer vesicle.

Preparation of Complexes. The SUV-PC, LPC micelles, and LPC/FFA vesicles were mixed with ovalbumin solution (45 mg/mL in 20 mM HCl or 20 mM NaOH) to yield various molar ratios. The total volume of this mixture was adjusted to 2 mL with 20 mM HCl or 20 mM NaOH. Next, the mixture was sonicated for 5 min at 25 °C. The ratio of PC or LPC bound to protein was measured as follows: The mixture was subjected to chromatography on a Sephacryl S-200HR column (2.8 × 42 cm) and eluted with 20 mM HCl or 20 mM NaOH. The fraction eluted with the complex was combined, and the PC or LPC bound to protein was extracted by chloroform–methanol (2:1 v/v). The amount of protein was measured according to the Lowry method (Lowry et al., 1951), and PC or LPC was determined by an Iatroscan-TLC/FID analyzer (Iatron Model TH-10, Tokyo; $CHCl_3$:CH_3OH:H_2O 70:30:3).

Emulsification and Evaluation of Emulsion. Emulsification was achieved by addition of triolein (135 μL) dropwise to the complex solution (2 mL) in 20 mM HCl with agitation by a disperser (physcotron equipped with generator shaft NS-10; Nition Rikaki Corp., Tokyo) at 12 000 rpm for 2 min. The size distribution of emulsion droplets was measured by a laser light scattering photometer. The data were expressed as the mean volume droplet size.

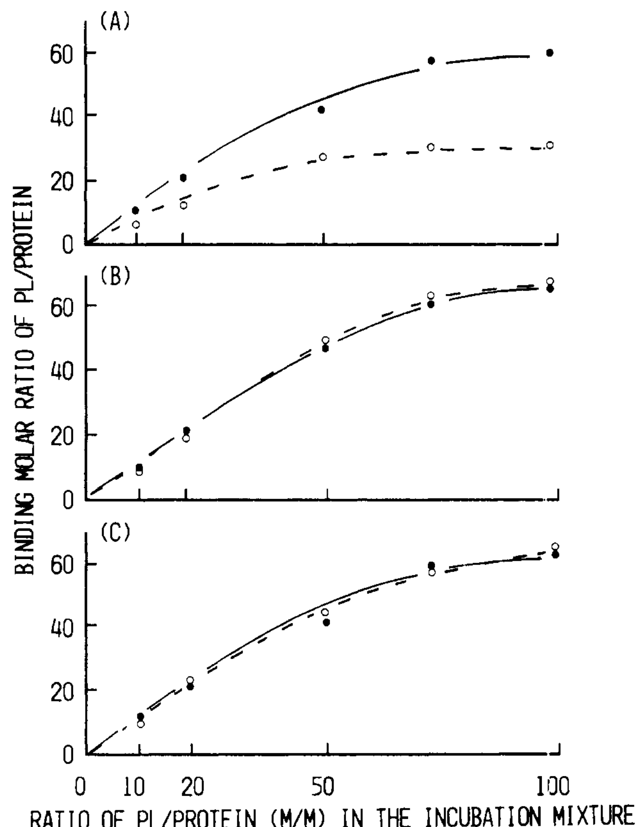

Figure 1. Changes of the molar ratio of PC or LPC bound to ovalbumin in 20 mM HCl (—), and 20 mM NaOH (- - -). (A) PC/ovalbumin; (B) LPC/ovalbumin; (C) LPC/linoleic acid/ovalbumin.

Measurements of ^{31}P NMR Spectra. ^{31}P NMR measurements were performed at 20 °C on a Varian VXR-4000s spectrometer at 161.0 MHz, fitted with the probe (10 mm, 45–165-MHz frequency), using a 45° pulse (25 μs), with 32K data points, a 40 000-Hz spectral window, 20 rotations/s spinning rate, and 2.0-s pulse delay. Proton was fully decoupled by 9900-Hz decoupler modulation frequency. NMR samples were 3.1 mL in 10-mm precision tubes. The line widths were measured from the resonances at half-height.

RESULTS AND DISCUSSION

As PC and LPC are amphophilic surfactants having different hydrophilicities, molecular weights and fatty acid compositions, they may show different affinities for proteins. Thus, various molar ratios of PL/ovalbumin mixture were prepared to investigate the binding molar ratio of PC or LPC to ovalbumin. Figure 1 shows the changes of the molar ratio of binding PC or LPC to ovalbumin. When the incubation molar ratio of PLs/protein was under 40, the ratio of binding PLs increased with the ratio of PLs/protein in the incubation mixture. When the molar ratio of PLs/protein in the mixture was increased to 40, the amount of free PC and LPC was gradually increased. Maximum binding molar ratio of PC and LPC reached a plateau at about 60. LPC/ovalbumin and LPC/linoleic acid/ovalbumin complex formation was not influenced by pH, but PC/ovalbumin complex formation depended on pH, showing lower affinity in 20 mM NaOH. The result indicated that most PC or LPC bound to ovalbumin when the molar ratio of PLs/protein was under 40 in 20 mM HCl. From these observations, the preparation of the PL/protein complex and the emulsion composed of

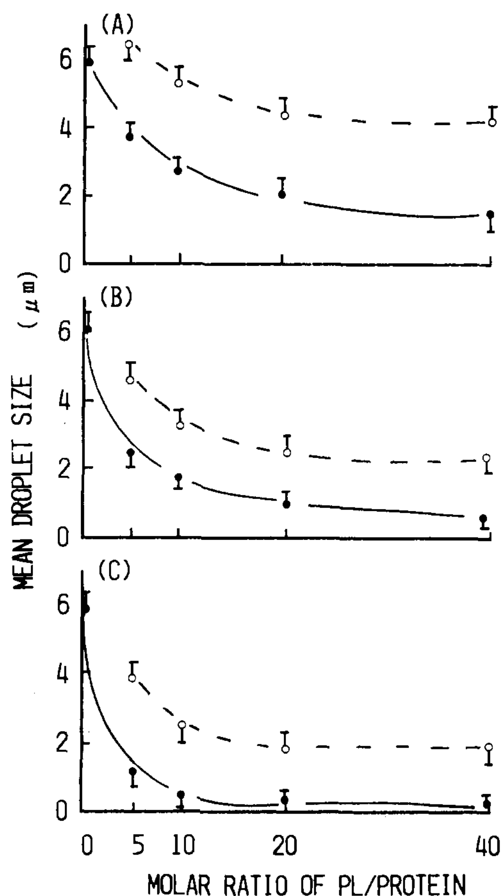

Figure 2. Changes of the mean droplet size of emulsion composed of PL and protein (—) or just PL (- - -) as the emulsifier. HCl (20 mM) and triolein were used as aqueous and oil phase, respectively. The ratio of oil/protein (w/w) was 3. (A) PC (O), PC/ovalbumin, (●); (B) LPC (O), LPC/ovalbumin (●); (C) LPC/linoleic acid (O), LPC/linoleic acid/ovalbumin (●).

them was accomplished in acidic conditions, and the molar ratio of PL/protein was controlled to be under 40. Figure 2 shows the changes of the mean droplet size of emulsion composed of triolein and aqueous dispersion of PL/protein complexes. Protein concentration was maintained at 45 mg/2 mL in 20 mM HCl. The mean droplet size of emulsion composed of just aqueous dispersion of ovalbumin and triolein was about 5.8 μm, and over 95% of the total volume of oil was formed into an emulsion with droplet size between 3.3 and 8.6 μm. As the molar ratio of PLs/protein increased, the mean droplet diameter of the emulsion became smaller. The LPC/ovalbumin complex formed smaller droplets compared to PC/ovalbumin complex. Moreover, the LPC/ovalbumin complex in the presence of linoleic acid formed smaller droplets in comparison with those formed in the absence of linoleic acid. These results indicated that interfacial adsorptivity of ovalbumin was promoted by interaction with PC or LPC and the formation of microemulsion was further promoted by linoleic acid.

The phosphorus signals of ^{31}P NMR are influenced by the motional properties of phosphate moieties in molecules (Gorenstein, 1982; Wu et al., 1984). The phosphorus signals of LPC and ovalbumin broadened after their adsorption on the interface. This result shows that the motions of the part of the ampholytic molecule in the aqueous dispersion are restricted by reconstitution on the interface (Chiba and Tada, 1990b; Mine et al., 1992). It was therefore expected that the phosphorus signal of PL and ovalbumin changed with the formation of the complex or its reconstitution at the interface. The aqueous dispersions and emulsions composed of PC/ovalbumin, LPC/ovalbumin complex, and LPC/ovalbumin complex in the presence of linoleic acid in 20 mM HCl were subjected to ^{31}P NMR analysis, and the line width of ^{31}P NMR spectra was measured (Figure 3; Table I). The molar ratio of PC and LPC to protein was 10. The complex of protein and PLs gave three separated phosphorus signals, and these signals were assigned to phosphoserine 68 and phosphoserine 344 of ovalbumin (Vogel and Bridger, 1982) and to the phosphate residues of PLs, by addition of PLs to ovalbumin solution, as shown in Figure 3. The line widths of ^{31}P NMR spectra of phosphoserine 68 and phosphoserine 344 of ovalbumin, SUV-PC, and LPC micelles in 20 mM HCl were 7, 5, 72, and 12 Hz, respectively. In the PC/ovalbumin complex, the line width of phosphorus signal of PC in aqueous dispersion became broader (130 Hz wide) with the formation of complex with ovalbumin. The line width of PC became again narrower (74 Hz wide) on emulsification. The increasing ratio of line widths of two phosphoserines of ovalbumin in PC/ovalbumin complexes was only 1.2–1.4-fold wider in emulsion than in aqueous dispersions. Moreover, the phosphorus signal of LPC in aqueous dispersion of LPC/ovalbumin complex and LPC/ovalbumin complex with linoleic acid was scarcely changed with the interaction of ovalbumin. The results indicate that the headgroup motion of LPC was scarcely restricted in spite of the interaction with protein. On the other hand, ^{31}P NMR spectra of the two phosphoserines of ovalbumin at the interface differed markedly between LPC/ovalbumin complex and LPC/linoleic acid/ovalbumin complex. In the presence of linoleic acid, the mean droplet size of emulsion became smaller (<0.5 μm) and over 95% of the total volume of oil was formed into a fine emulsion with droplet size between 0.09 and 0.8 μm. The line width of phosphoserine 68 of ovalbumin was broadened about 3-fold compared to that in aqueous dispersion. These results suggested that the specific restriction of the phosphoserine 68 moiety of ovalbumin was important to form fine emulsions with the interaction of LPC and linoleic acid. LPC and linoleic acid mutually changed the structure of ovalbumin to increase the interfacial adsorption of ovalbumin.

We investigated the effect of LPC and linoleic acid on a protein with poor emulsifying capacity to confirm promotion of the interfacial adsorptivity of protein by the interaction with LPC and FFA. In a previous paper (Mine et al., 1992), we reported that the motions of the two phosphoserines of *p*-ovalbumin, formed by cleaving the N-terminal residues (1–22) of ovalbumin by pepsin digestion, were little restricted by emulsification and this correlated with lower interfacial adsorptivity, ^{31}P NMR spectra, and the line width of LPC/*p*-ovalbumin complex in the presence of linoleic acid in aqueous dispersion and emulsion (Figure 4; Table I). The molar ratio of LPC to protein was 10. The shape of ^{31}P NMR spectra of the complex in the emulsion was very similar to that of the LPC/ovalbumin complex in the presence of linoleic acid, and the mean droplet diameter of emulsion was 0.6 μm. The motion of phosphoserine 68 of *p*-ovalbumin was much restricted by emulsification with the interaction of LPC and linoleic acid as well as the LPC/ovalbumin complex in the presence of linoleic acid.

As mentioned above, linoleic acid promoted the interfacial adsorptivity of the LPC/protein complex. The surface activity of lysophospholipid was influenced by the degree of unsaturation and chain length of free fatty acid which coexisted with lysophospholipid (Fujita and Suzuki, 1990). We investigated the kind of free fatty acid

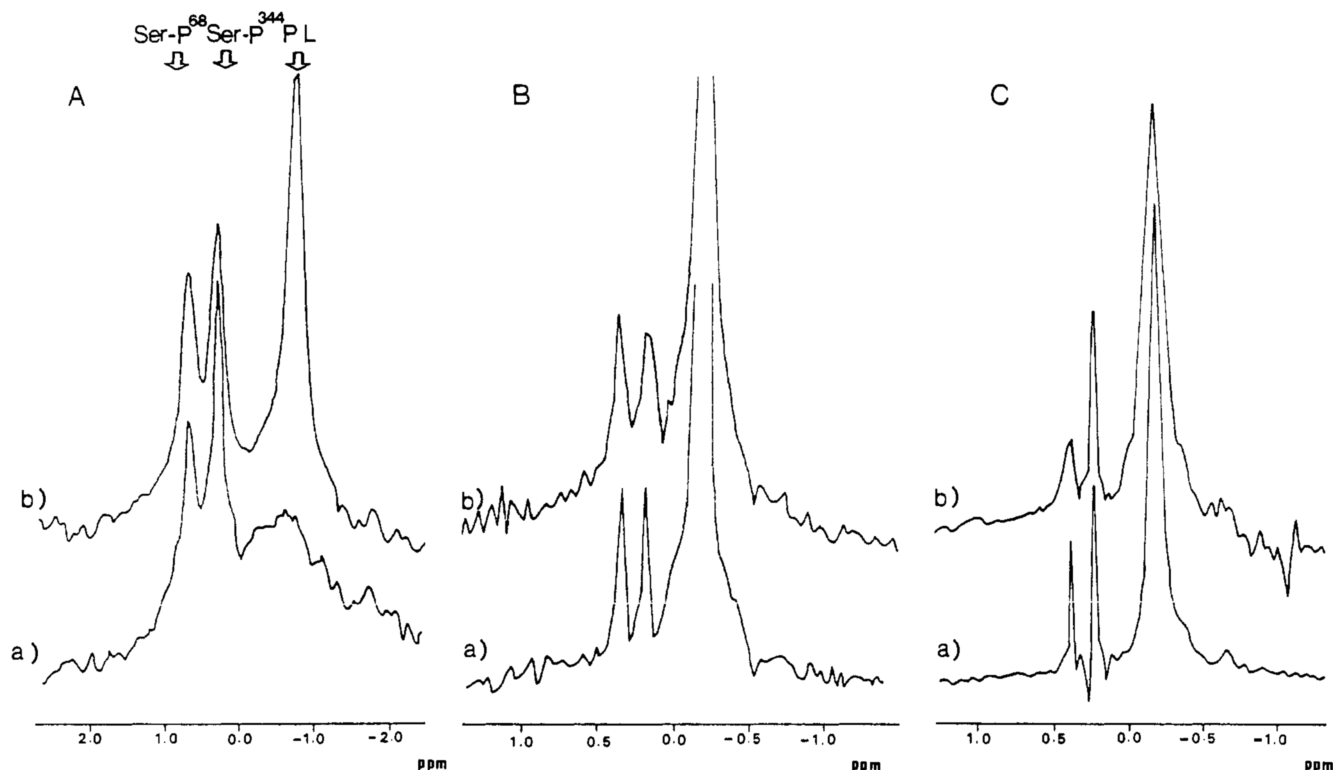

Figure 3. ^{31}P NMR spectra of complexes in an aqueous dispersion (a) and emulsion (b). (A) PC/ovalbumin (10:1, mol/mol); (B) LPC/ovalbumin (10:1, mol/mol); (C) LPC/linoleic acid/ovalbumin (10:10:1, mol/mol). The emulsions were prepared with triolein and the complex of PL and protein dispersed in 20 mM HCl. The ratio of oil/protein (w/w) was 3, respectively. A total of 12 000 acquisitions were made, and additional line broadening was 0.1 Hz. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm.

Table I. Phosphorus Line Widths of Complexes in Aqueous Dispersion and Emulsion and Mean Diameter of the Dispersion and Emulsion[a]

		aqueous dispersion, Hz (mean diam, μm)	emulsion, Hz (mean diam, μm)
PC/OA[b]	PC	130.0 (0.065)	49.2 (2.3)
	OA Ser-P^{344}	22.1	28.0
	Ser-P^{68}	24.3	34.2
LPC/OA	LPC	12.3 (0.020)	26.9 (1.6)
	OA Ser-P^{344}	8.8	14.0
	Ser-P^{68}	9.2	11.9
LPC/C_{18-2}[c]/OA	LPC	15.0 (0.021)	29.9 (0.5)
	OA Ser-P^{344}	7.1	8.9
	Ser-P^{68}	7.0	>20.0[e]
LPC/C_{18-2}/p-OA[d]	LPC	14.6 (0.018)	35.2 (0.6)
	POA Ser-P^{344}	9.5	14.5
	Ser-P^{68}	12.8	>20.0[e]

[a] Emulsions were prepared with the complex in 20 mM HCl and triolein at 25 °C. The ratio of oil/protein (w/w) was 3, respectively. [b] Ovalbumin. [c] Linoleic acid. [d] p-Ovalbumin. [e] The line widths were measured manually because of lower peak height.

on the interfacial adsorptivity of the LPC/protein complex. Figure 5 shows the changes of mean droplet size under the influence of emulsifying temperature and the kind of, or unsaturation degree, of free fatty acid which coexisted with LPC. The mean droplet size of emulsion was influenced little by saturated fatty acid. On the other hand, the mean droplet size decreased as the unsaturation degree of free fatty acid fell. Smaller droplets were obtained by heating during emulsification. The size distribution of LPC/linoleic acid bilayer vesicles was 50–200 nm. We also observed that the bilayer vesicles were destroyed by the interaction with protein and the size

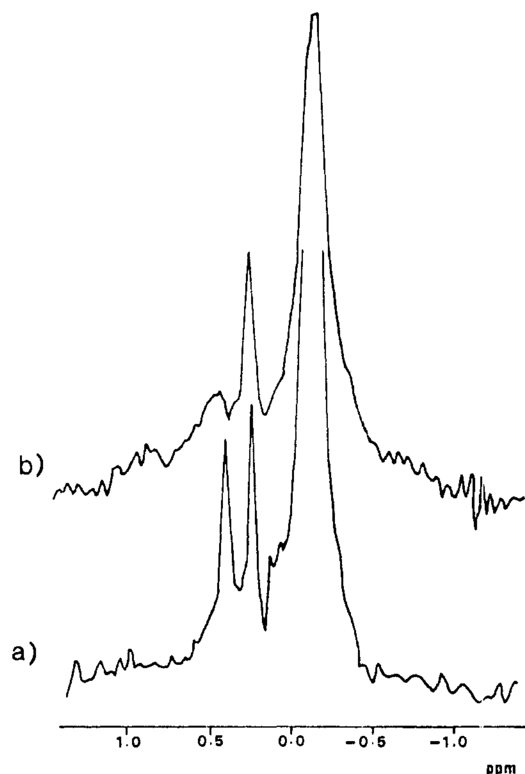

Figure 4. ^{31}P NMR spectra of complex in an aqueous dispersion (a) and emulsion (b). Complex was composed of LPC/linoleic acid/p-ovalbumin (10:10:1, mol/mol). The condition of emulsification was the same as in Figure 3. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm.

distribution of LPC/linoleic acid/ovalbumin complex was 10–20 nm, as measured with laser light scattering photometer.

In conclusion, the interfacial adsorptivity of ovalbumin

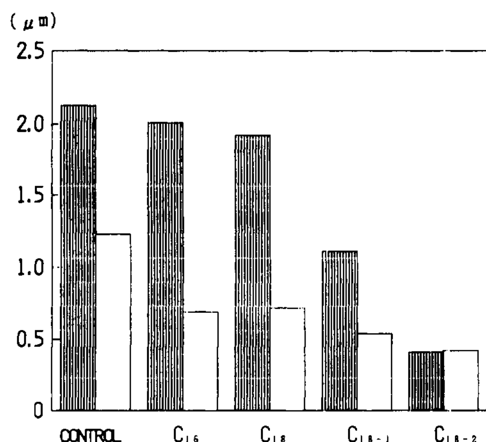

Figure 5. Mean droplet size of the emulsions composed of complex and triolein. The complex was composed of LPC/free fatty acid/ovalbumin (10:10:1, mol/mol) in 20 mM HCl. The ratio of oil/protein (w/w) was 3. The emulsion were prepared at 25 (shaded bars) or 80 °C (open bars): control; LPC/ovalbumin (10:1, mol/mol), C_{16-0}, palmitic acid; C_{18-0}, stearic acid; C_{18-1}, oleic acid; C_{18-2}, linoleic acid.

was promoted by interacting with PLs, and the formation of the microemulsion was further promoted by the interaction with LPC and FFAs. It was found that the phosphoserine 68 moiety of ovalbumin was specifically restricted at the interface when the complex formed microemulsion by ^{31}P NMR. Moreover, the bilayer vesicles composed of LPC and FFA were destroyed by the formation of complex with ovalbumin; that is, higher interfacial adsorptivity resulted from a complex of LPC, FFA, and the protein. It is suggested that the LPC/FFA/protein complexes are mutually interacted and show highly interfacial adsorptivity. As the interfacial adsorptivities of complexes were markedly influenced by the kind of FFA and the emulsifying temperature, it is suggested that the dynamic state of complexes correlate with their interfacial adsorptivity. It is necessary to further investigate the dynamic state of LPC/FFA/ovalbumin complex at the interface and the role of FFA on the promotion of interfacial adsorption of protein including the conformational changes of protein.

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