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Thermotropic Phase Behavior of Monoglyceride-Dicetylphosphate Dispersions and Interactions with Proteins: A ^{2}H and ^{31}P NMR Study

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ABSTRACT The phase behavior of a 1-[$^{2}H_{35}$]-stearoyl-*rac*-glycerol ([$^{2}H_{35}$]-MSG)/dicetylphosphate (DCP) mixture and its interaction with β-lactoglobulin and lysozyme were studied by ^{2}H and ^{31}P nuclear magnetic resonance (NMR). The behavior of the lipids was monitored by using deuterium-labeled [$^{2}H_{35}$]-MSG as a selective probe for ^{2}H NMR and DCP for ^{31}P NMR. Both ^{2}H and ^{31}P NMR spectra exhibit characteristic features representative of different phases. In the lamellar phases, ^{31}P NMR spectra of DCP are different from the spectra of natural phospholipids, which is attributable to differences in the intramolecular motions and the orientation of the shielding tensor of DCP compared with phospholipids. The presence of the negatively charged amphiphile DCP has a large effect on the phase behavior of [$^{2}H_{35}$]-MSG. At low temperature, the presence of DCP inhibits crystallization of the gel phase into the coagel. Upon increasing the temperature, the gel phase of [$^{2}H_{35}$]-MSG transforms in the liquid-crystalline lamellar phase. In the presence of DCP, the gel phase directly transforms into an isotropic phase. The negatively charged β-lactoglobulin and the positively charged lysozyme completely neutralize the destabilizing effect of DCP on the monoglyceride liquid-crystalline phase and they even stabilize this phase. Without DCP the proteins do not seem to interact with the monoglyceride. These results suggest that interaction is facilitated by electrostatic interactions between the negatively charged DCP and positively charged residues in the proteins. In addition, the nonbilayer-forming DCP creates insertion sites for proteins in the bilayer.

INTRODUCTION

Monoglycerides are amphiphatic neutral lipid molecules in which a hydrophobic fatty acid is attached at the *sn*-1(3) position of a hydrophilic glycerol backbone via an ester bond. Despite their relatively simple chemical structure, monoglycerides can form various phases found in membrane phospholipid/water systems, namely the coagel or lamellar crystalline (L_c) phase, the lamellar gel (L_β) phase, the lamellar liquid-crystalline (L_α) phase, bicontinuous cubic phases of different symmetry, the inverted hexagonal (H_{II}) phase, and the inverted micellar (L_2) phase. The lipid organization in monoglyceride-water systems is strongly dependent on the chemical structure of the monoglyceride and on environmental conditions such as temperature, water, and salt content.

The ability of monoglyceride/water systems to form different structures offers many interesting opportunities for studies on membrane structure and function, as well as for industrial applications. Being a natural emulsifier, monoglycerides are widely used in the food industry (Krog, 1990). During fat digestion the enzymatic hydrolysis of triglycerides leads to accumulation of a large amount of monoglycerides, which may result in formation of a bicontinuous cubic phase. Such a cubic phase could be a suitable matrix for interfacial enzymatic processes and for transport of lipid molecules between the fat droplets and mixed micelles of monoglycerides/fatty acids and bile acids (Lindblom and Rilfors, 1989). The monoglyceride cubic phase has also been successfully used as a matrix for crystallization of membrane proteins (Landau and Rosenbusch, 1996; Rummel et al., 1998; Kolbe et al., 2000).

In fat digestion and in industrial applications, monoglycerides are always present as mixtures with other lipids and proteins. The same is the case for crystallization of membrane proteins from the cubic phase of monooleoylglycerol. This phase is enriched by detergents, which are used for the solubilization of hydrophobic proteins. Potentially, additives introduced into monoglycerides can significantly change the properties of the system. For example, it was hypothesized that in the cubic phase of monoolein, protein crystals grow at the locally formed L_α phase, which is stabilized by detergents (Ai and Caffrey, 2000). For these reasons, mixed monoglyceride systems are of utmost importance for both biochemical research and industrial application. Although the polymorphic behavior of monoglyceride-water systems has been studied for many years (Lutton, 1965; Krog and Larson, 1968; Larsson and Quinn, 1994; Briggs and Caffrey, 1994; Qiu and Caffrey, 2000) little knowledge is as yet available on the properties of mixed monoglyceride systems. This applies in particular to systems containing proteins. Proteins are present in all systems mentioned above, and, therefore, the interaction between monoglycerides and proteins may play an important role in structural organization and function of these systems.

In our previous papers (Leenhouts et al., 1997; Boots et al., 1999, 2001) we reported on the interaction of β-lactoglobulin and lysozyme with monoglyceride monolayers and

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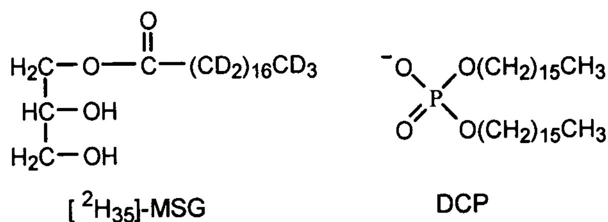

FIGURE 1 [$^2H_{35}$]-Monostearoyl-*rac*-glycerol ([$^2H_{35}$]-MSG) and DCP.

vesicles. In this paper, we present results on the interaction of β-lactoglobulin and lysozyme with a monostearoylglycerol/dicetylphosphate two-component mixture in different phase states. The negatively charged dicetylphosphate (DCP) (Fig. 1) was included because we observed previously (Leenhouts et al., 1997; Boots et al., 1999, 2001) that low concentrations of DCP facilitate insertion of proteins into monoglyceride monolayers. Lysozyme and β-lactoglobulin have a similar size, 14.3-kDa versus 18.3-kDa, and number of positively charged amino acids, 17 versus 18. However, lysozyme contains far less negatively charged amino acids, 9 versus 26, giving it an isoelectric point of 9.3 compared with 5.2 for β-lactoglobulin. Therefore, lysozyme is positively charged and β-lactoglobulin is negatively charged at neutral pH. The questions, which we are addressing in this study, are 1) how the presence of DCP can affect the lipid organization and phase properties of the MSG/water system, and 2) how the negatively charged β-lactoglobulin and the positively charged lysozyme interact with this monoglyceride system.

The lipid organization and phase behavior were studied by means of solid-state ^{2}H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Deuterium-labeled 1-[$^2H_{35}$]-stearoyl-*rac*-glycerol ([$^2H_{35}$]-MSG) (Fig. 1) with a fully deuterated acyl chain was used as a selective probe for ^{2}H NMR. The behavior of DCP was followed by ^{31}P NMR. In this approach, the behavior of individual molecular components can be monitored in the same sample, which is important for monoglyceride/water systems known to form long-living metastable phases.

MATERIALS AND METHODS

Materials

Bovine β-lactoglobulin (a mixture of genetic variants A and B) and DCP were obtained from Sigma Chemical Company (St. Louis, MO). Lysozyme was obtained from Boehringer (Mannheim, Germany). Fully deuterated stearic acid-D_{35}, 13-[$^2H_2$]-palmitic acid, and deuterium-depleted water were obtained from Cambridge Isotope Laboratories (Cambridge, MA). 1,2–Dipalmitoyl-*sn*-glycerophosphocholine was obtained from Avanti Polar Lipids (Alabaster, AL). 1-[$^2H_{35}$]-Monostearoyl-*rac*-glycerol with a fully deuterated acyl chain was synthesized according to the procedure described previously (Chupin et al., 2001).

Sample preparation

Samples were prepared by mixing known amounts of [$^2H_{35}$]-MSG and DCP stock solutions in $CHCl_3$/MeOH (3:1). The solvents were evaporated and then residual solvent was removed under high vacuum for at least 4 h. The lipids were subsequently hydrated by adding buffer and heating the samples at 65°C for at least 5 min and cooling to room temperature. After three cycles of heating and cooling, the samples were used for NMR experiments. The samples consisted of 50–70 μmol lipid (10% by weight) hydrated in buffer. Samples containing proteins were obtained by hydration of a dry lipid powder with a solution of β-lactoglobulin or lysozyme in buffer without or with 100 mM sodium chloride. A molar lipid-to-protein ratio of 100 was used to achieve saturation in protein binding (Boots et al., 1999, 2001). All buffers were prepared with deuterium-depleted water: 20mM Tris, pH 7, with or without 100 mM NaCl. Potassium palmitate sample was prepared according to Davis and Jeffrey (1977) and contained 10% (mol) of DCP and 10% (mol) of 13-[$^2H_2$]-palmitate.

NMR measurements

All NMR spectra were recorded on a Bruker MSL 300 spectrometer (Bruker Karlsruhe, Germany). Using a high-power 7.5-mm broadband probe, 46.1 MHz ^{2}H NMR spectra were obtained, as described previously (Chupin et al., 2001). A quadrupolar echo technique (Davis et al., 1976) with a 3-μs π/2-pulse and a 40-μs τ delay was used. 121.1 MHz ^{31}P NMR spectra were recorded using a high-resolution 10 mm broadband probe. A Cyclops sequence with broadband, gated proton decoupling was used. The recycling delay was 1 s and the π/4-pulse width was 8 μs. Typically, 15 000 free induction decays for the L_β phase and ~1000 for the L_α and L_2 phases were accumulated. In the L_c phase, ^{31}P NMR spectra were obtained using a 5-mm probe. A spin echo technique with a 6-μs π/2-pulse, a 30-μs τ delay, and 8-s recycling delay was used. An exponential multiplication with a line-broadening factor of 300 Hz for the L_c and L_β phases, and 30 Hz for the L_α and L_2 phases was used before performing the Fourier transformation. All ^{2}H NMR spectra were symmetrized. Chemical shifts in ^{31}P NMR spectra were measured relative to the isotropic signal.

Theory and application of ^{2}H NMR (Davis, 1983; Seelig and Macdonald, 1987; Smith, 1989) and ^{31}P NMR (Seelig, 1978; Smith and Ekiel, 1984) spectroscopy in lipid systems is described in the literature. ^{31}P NMR spectra of DCP in the L_α phase were simulated using the Lorentzian broadening.

RESULTS

The phase behavior and lipid organization of a [$^2H_{35}$]-MSG:DCP mixture and its interaction with β-lactoglobulin and lysozyme were investigated in excess water by using ^{2}H and ^{31}P NMR. The [$^2H_{35}$]-MSG:DCP mixture in a molar ratio of 9 to 1 was chosen based on our previous observations that even low concentrations of DCP facilitate interaction of β-lactoglobulin and lysozyme with monoglycerides (Leenhouts et al., 1997; Boots et al., 1999, 2001). We previously demonstrated that ^{2}H NMR spectroscopy on deuterated acyl chain monoglycerides is a convenient technique to monitor phase transitions in monoglyceride-water systems (Chupin et al., 2001) because all different phases give rise to characteristic features in the ^{2}H NMR spectra. Similarly, ^{31}P NMR should allow for following the behavior of DCP. However, the line shape of ^{31}P NMR spectra of DCP in different phases is not known. Therefore, we first characterized DCP in different phases by ^{31}P NMR.

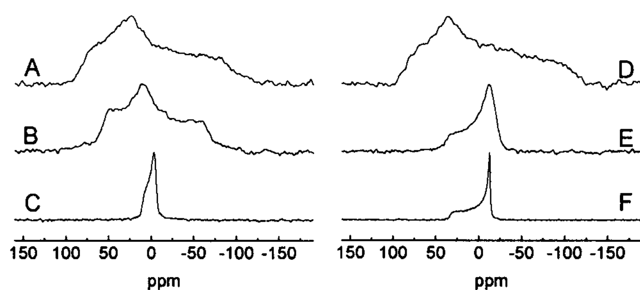

FIGURE 2 ^{31}P NMR spectra of DCP and dipalmitoylphosphatidylcholine (DPPC) in different phases. (*A*) DCP in the L_c phase of potassium palmitate at 20°C (dry powder). (*B*) DCP in the L_β phase of potassium palmitate at 25°C. (*C*) DCP in the L_α phase of potassium palmitate at 50°C. (*D*) DPPC in the L_c phase at 20°C (dry powder). (*E*) DPPC in the L_β phase at 25°C. (*F*) DPPC in the L_α phase at 50°C.

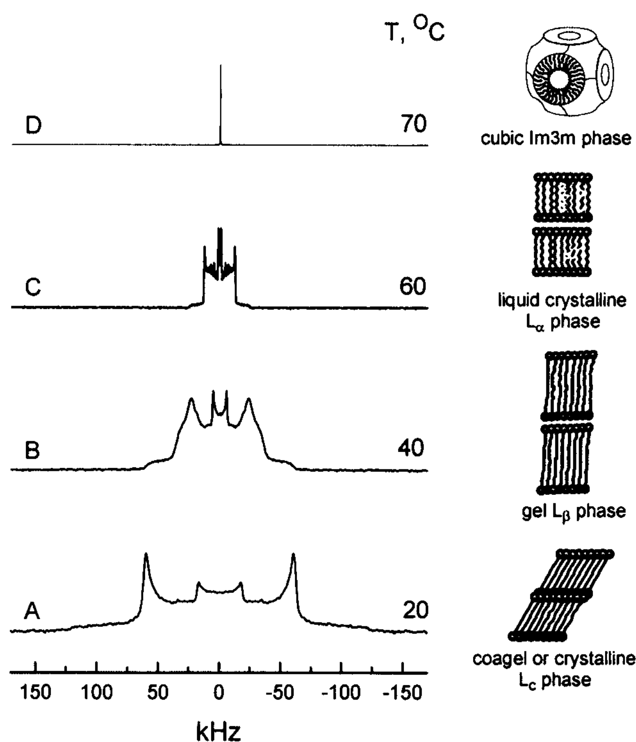

FIGURE 3 ^{2}H NMR spectra (*left panel*) representing the different phases of [$^2H_{35}$]-MSG, as schematically depicted (*right panel*). (*A*) L_c phase; (*B*) L_β phase; (*C*) L_α phase; and (*D*) Cubic phase.

^{31}P NMR spectra of DCP in different phases

Although ^{31}P NMR spectra of DCP in different phases have never been published, it is easy to predict the line shape for DCP in L_2 phases as well as in the L_c phase. In cubic or other isotropic phases, the fast isotropic motion of lipid molecules will result in a narrow symmetric signal in NMR spectra independent of the structure of these molecules (Lindblom and Rilfors, 1989). With respect to the coagel, it was shown that ^{31}P NMR spectra of crystalline phosphates exhibit a broad powder type pattern with three components of the chemical shielding tensor. Principal tensor values of $\sim\sigma_{11} = 80$, $\sigma_{22} = 20$, and $\sigma_{33} = -110$ are almost identical for different diester phosphates, which is characteristic for immobilized phosphate groups (Seelig, 1978). Indeed, the ^{31}P NMR spectrum of DCP in the crystalline bilayer exhibits similar features (Fig. 2 *A*) as the crystalline powder of phospholipids (Fig. 2 *D*).

However, the line shape of ^{31}P NMR spectra of DCP or structurally related compounds in gel and liquid-crystalline bilayers is not known. As DCP may not form such phases on its own, the experimental spectra of DCP in the L_β and L_α phases were obtained by using potassium palmitate as a bilayer matrix (Davis and Jeffrey, 1977) that is transparent for ^{31}P NMR. The phase state of the matrix was first checked by ^{2}H NMR using [13-$^2H_2$]-palmitate as a probe. ^{2}H NMR demonstrated that the presence of a small amount of DCP does not affect the bilayer organization of potassium palmitate (not shown). ^{31}P NMR spectra of a DCP: potassium palmitate mixture recorded below and above the L_β-to-L_α phase transition are shown (Fig. 2, *B* and *C*) and compared with the spectra of dipalmitoylphosphatidylcholine in the L_β and L_α phase (Fig. 2, *E* and *F*).

^{31}P NMR spectra of DCP both in the L_β and L_α phases significantly differ from the spectra of phospholipids in the corresponding phases. In the L_β phase, the ^{31}P NMR spectrum of DCP exhibits a line shape, which is characteristic of a partially immobilized phosphate group (Fig. 2 *B*). The spectrum of DCP in the L_β phase clearly has a nonaxial symmetry indicating that the phosphate group of DCP does not undergo fast rotation. Three components of the chemical shielding tensor are resolved with values of $\sim\sigma_{11} = 51$, $\sigma_{22} = 10$, and $\sigma_{33} = -62$ ppm at 25°C. These values are much smaller than those obtained for the L_c phase, indicating that some motions occur in the L_β phase. In contrast to DCP, ^{31}P NMR spectra of phospholipids exhibit a line shape close to an axial symmetric pattern with parallel and perpendicular components (Fig. 2 *E*).

In the L_α phase, the ^{31}P NMR spectrum of DCP has a high-field peak and low-field shoulder (Fig. 2 *C*) similar to phospholipids in liquid-crystalline bilayers (Fig. 2 *F*). However, the chemical shift anisotropy (CSA) of the DCP signal is ~15 ppm, which is much smaller than the values of ~40 ppm for most phospholipids.

The difference in line shape and CSA of DCP as compared with phospholipids can be explained by the chemical structure of DCP as will be discussed below.

Effect of DCP on the phase behavior of [$^2H_{35}$]-MSG

In our previous paper we reported on ^{2}H NMR spectra of [$^2H_{35}$]-MSG in different phases (Chupin et al., 2001). As a control, these spectra are shown in Fig. 3, *A–D*. A pictorial

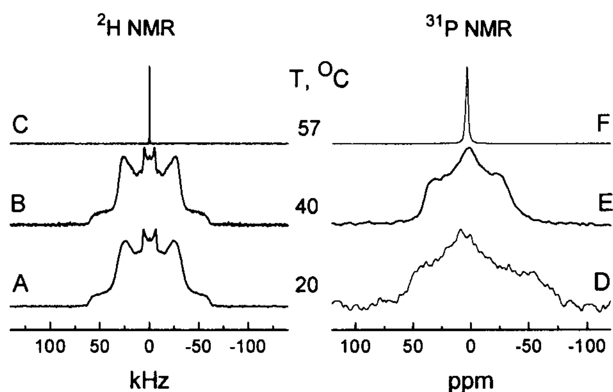

FIGURE 4 ^{2}H NMR (*A–C*) and ^{31}P NMR (*D–F*) spectra of [$^2H_{35}$]-MSG:DCP, 9:1 (mol/mol) at different temperatures, as indicated in the figure.

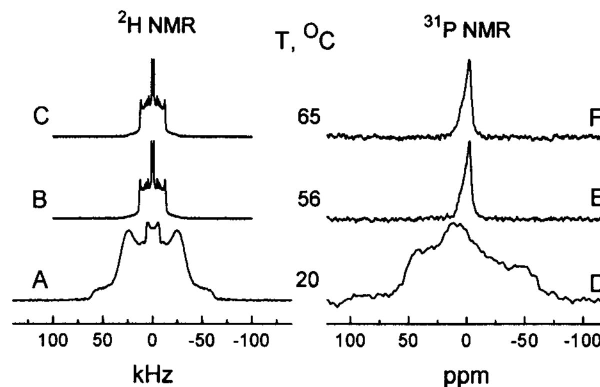

FIGURE 5 ^{2}H NMR (*A–C*) and ^{31}P NMR (*D–F*) spectra of [$^2H_{35}$]-MSG:DCP, 9:1 (mol/mol) in the presence of β-lactoglobulin at different temperatures. Lipid:protein ratio, 100:1 (mol/mol); pH 7; 0 mM NaCl.

description of the different monoglyceride phases is shown on the right panel. In the L_c phase of [$^2H_{35}$]-MSG at 20°C, the spectrum is characteristic for immobilized acyl chains and consists of two well defined powder patterns, one for the methylene groups with a quadrupolar splitting ($\Delta\nu_Q$)of 120 kHz and another for the methyl groups with a splitting of 35 kHz (Fig. 3 *A*). In the L_β phase at 40°C, the spectrum of [$^2H_{35}$]-MSG again shows two resolved signals, but now with reduced $\Delta\nu_Q$ of ~50 kHz and 11 kHz, indicating partial motional averaging (Fig. 3 *B*). The L_β phase of [$^2H_{35}$]-MSG is metastable and gradually transforms into the coagel during several hours at 20°C (not shown). The ^{2}H NMR spectrum in the L_α phase of [$^2H_{35}$]-MSG consists of a number of resolved resonances from different labeled sites along the acyl chain (Fig. 3 *C*). In the cubic phase (the cartoon shows the Im3m phase proposed for MSG (Lindblom et al., 1979)), the ^{2}H NMR spectrum of [$^2H_{35}$]-MSG shows a narrow signal originating from isotropically moving lipid molecules (Fig. 3 *D*).

The presence of DCP significantly changes the phase behavior of the monoglyceride. This is shown by the ^{2}H NMR and ^{31}P NMR spectra of [$^2H_{35}$]-MSG:DCP, 9:1 (mol/mol), at different temperatures (Fig. 4, *A–F*). Once heated above the Kraft point, the [$^2H_{35}$]-MSG:DCP mixture forms a long-living L_β phase even at low temperatures, as can be concluded from the ^{2}H NMR spectrum with $\Delta\nu_Q$ of ~50 and 11 kHz at 20°C consistent with the presence of a L_β phase (Fig. 4 *A*). The ^{31}P NMR spectrum of this mixture (Fig. 4 *D*) shows a line shape with reduced anisotropy, which is a characteristic of the partially immobolized phosphate group of DCP in the L_β phase (cf. Fig. 2 *B*). In contrast to pure [$^2H_{35}$]-MSG dispersions, the L_β phase of MSG:DCP is stable during months at room temperature and transforms into the coagel very slowly (not shown). Upon heating the L_β phase of [$^2H_{35}$]-MSG:DCP, the $\Delta\nu_Q$ in the ^{2}H NMR spectra and the total anisotropy in the ^{31}P NMR spectra are reduced (Fig. 4, *B* and *E*), but no L_α phase is formed. Instead, upon further heating, the L_β phase of [$^2H_{35}$]-MSG:DCP transforms directly into an L_2 phase as indicated by the narrow symmetric peaks in the ^{2}H and ^{31}P NMR spectra (Fig. 4, *C* and *F*). The L_β-to-L_2 phase transition in [$^2H_{35}$]-MSG:DCP was found at 56°C for both lipid molecules (not shown), which is approximately the same as the L_β-to-L_α phase transition temperature of 55°C of pure [$^2H_{35}$]-MSG indicating that no significant phase separation takes place. This was also confirmed by DSC data (not shown).

Interaction of β-lactoglobulin and lysozyme with [$^2H_{35}$]-MSG:DCP

To investigate the possible effects of β-lactoglobulin and lysozyme, the phase behavior of MSG:DCP mixtures was investigated in the presence of these proteins. At pH 7, the lipid bilayer surface is negatively charged because of the presence of DCP whereas lysozyme has a positive net charge and β-lactoglobulin is negatively charged. Buffers with low and high ionic strength were used to modulate the electrostatic interactions between protein and lipid surface. NMR spectra of the lipid-protein mixtures were recorded in the temperature range from 20°C until the temperature of denaturation of β-lactoglobulin and lysozyme, 70° and 65°C, respectively (Ericsson et al., 1983; Iametti et al., 1996).

Fig. 5 shows the ^{2}H and ^{31}P NMR spectra of a [$^2H_{35}$]-MSG:DCP mixture which was hydrated with a solution of β-lactoglobulin. At low temperature, both ^{2}H and ^{31}P NMR spectra (Fig. 5, *A* and *D*) exhibit a line shape which is representative of the L_β phase of the pure lipid system (cf. Fig. 4, *A* and *D*). The L_β phase of the [$^2H_{35}$]-MSG:DCP:β-lactoglobulin mixture slowly transforms into the coagel during months at 20°C with approximately the same rate as a [$^2H_{35}$]-MSG:DCP mixture (data not shown). Apparently, the presence of protein does not affect the stability of the L_β phase. Upon heating, the L_β

phase of the [$^2H_{35}$]-MSG:DCP:β-lactoglobulin mixture transforms into the L_α phase (Fig. 5, *B* and *E*). Strikingly, the presence of β-lactoglobulin thus completely neutralizes the destabilizing effect of DCP on the L_α phase of [$^2H_{35}$]-MSG (cf. Fig. 4, *B* and *E* and Fig. 5, *B* and *E*), indicating that the protein interacts with [$^2H_{35}$]-MSG: DCP bilayers. The protein even stabilizes the L_α phase of [$^2H_{35}$]-MSG:DCP, which is now more stable as compared with the pure [$^2H_{35}$]-MSG. No isotropic signal was observed up to a temperature of 70°C at low ionic strength, whereas the pure [$^2H_{35}$]-MSG transforms into the cubic phase at 67°C (Chupin et al., 2001). At high ionic strength, this effect is decreased and the L_α phase of [$^2H_{35}$]-MSG:DCP transforms into the L_2 phase at a temperature of 65°C, indicating that electrostatic interactions are involved. Control experiments demonstrated that β-lactoglobulin does not significantly affect the phase behavior of pure [$^2H_{35}$]-MSG (not shown), indicating that the presence of DCP is of importance for the interaction of this negatively charged protein with the negatively charged lipid surface.

The positively charged lysozyme has a similar effect on the phase behavior of [$^2H_{35}$]-MSG:DCP mixtures as β-lactoglobulin (not shown). Lysozyme stabilizes the L_α phase at the expense of the L_2 phase up to 65°C, the temperature of its denaturation. The presence of lysozyme does not accelerate transformation of the metastable gel into the stable coagel, again similar to the effect of β-lactoglobulin. In the presence of either protein, the transition into the L_α phase occurs at the same temperature for both [$^2H_{35}$]-MSG and DCP (not shown), indicating that there is no phase separation of lipids involved.

Interestingly, the line shape of the ^{2}H NMR spectra of [$^2H_{35}$]-MSG in the L_α phase (Fig. 6 *A*) is clearly different from that of the mixture of [$^2H_{35}$]-MSG:DCP:protein (Fig. 6 *B*). In the presence of protein, the spectrum exhibits lower resolution and decreased intensity of the signal with the largest $\Delta\nu_Q$. This signal is a superposition of the unresolved resonances of the acyl chain methylenes located near the monoglyceride headgroup. In the presence of protein the order in this region is decreased, indicative of lipid-protein interactions. There are two possible explanations of this effect. The first is that insertion of protein molecules into the bilayer decreases the resolution of the spectrum (Fig. 6 *B*). This effect could be attributable to the destruction of the uniform orientation of the acyl chains and/or slowing down of the acyl chain motion, which reduces the deuteron T_2 relaxation time. The second is that the resolution in the spectrum of [$^2H_{35}$]-MSG is higher because of the magnetic orientation of the lipid bilayers (Chupin et al., 2001) and the proteins destroy this orientation in [$^2H_{35}$]-MSG:DCP mixture. Taking our data obtained on MSG:DCP monolayers (Boots et al., 1999, 2001) into account, the first explanation seems more likely.

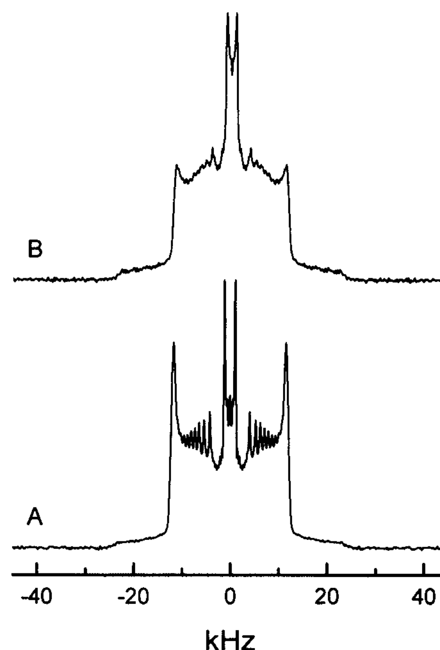

FIGURE 6 ^{2}H NMR spectra of [$^2H_{35}$]-MSG (*A*) and [$^2H_{35}$]-MSG:DCP, 9:1 (mol/mol) in the presence of lysozyme (*B*) in the L_α phase at 60°C. Lipid:protein ratio, 100:1 (mol/mol); pH 7; 0 mM NaCl.

DISCUSSION

^{31}P NMR spectra of DCP in different lamellar phases

The line shape of ^{31}P NMR spectra is determined by the values of the principal elements of the shielding tensor, its orientation, and averaging because of molecular and intramolecular motions. The principal elements of the shielding tensor of different phosphodiesters are almost identical (Seelig, 1978). Their values are of $\sigma_{11} = 80$, $\sigma_{22} = 20$, and $\sigma_{33} = -110$ ppm. The orientation of the chemical shielding tensor with respect to the molecular frame is known for barium diethylphosphate (Herzfeld et al., 1978), which is structurally related to DCP. The σ_{11} element is approximately perpendicular to the O(3)-P-O(4) angle and the σ_{22} element approximately bisects the O(3)-P-O(4) angle, where O(3) and O(4) are nonesterified oxygen atoms. The problem then is how to relate the orientation of the chemical shielding tensor with respect to the molecular frame of DCP in a bilayer. Fortunately, a DCP molecule has a symmetric shape. Both alkyl chains attached to the phosphate group are chemically equivalent, as are the nonesterified oxygen atoms. Therefore, it is reasonable that both chains are embedded into the bilayer in the same way, whereas both charged nonesterified oxygens are exposed into the water phase again in the same way. In this case, the direction of the σ_{22} element coincides with the bilayer normal and the elements σ_{11} and σ_{33} are parallel to the bilayer plane (Fig. 7 *A*).

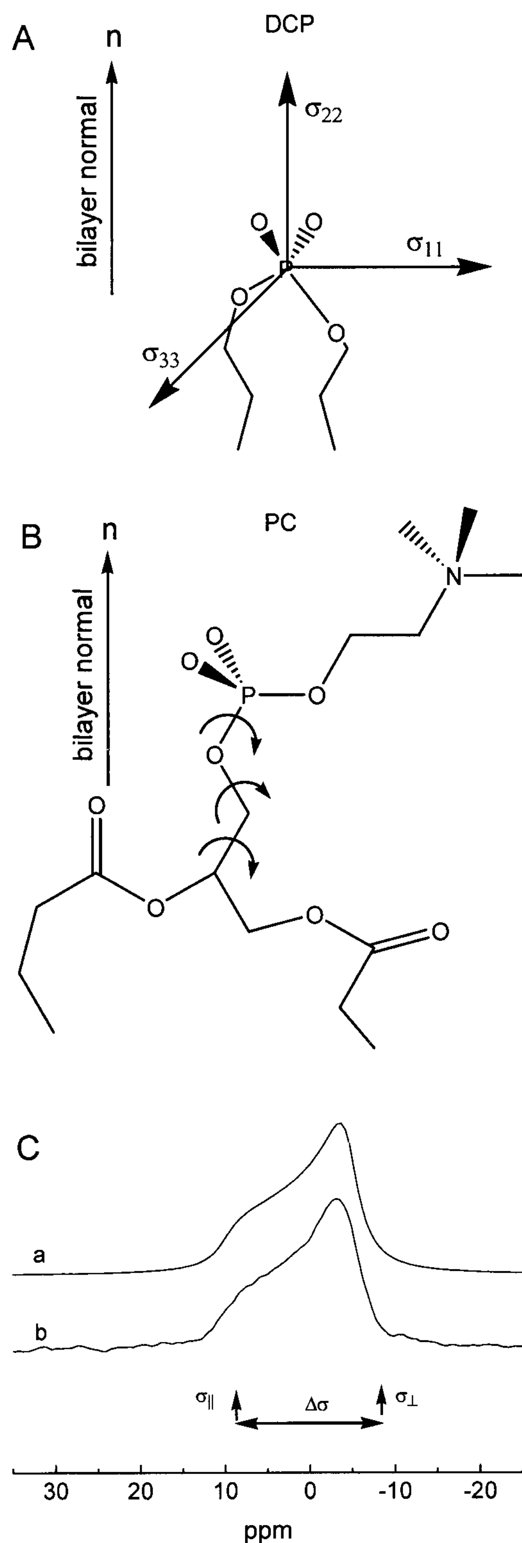

FIGURE 7 Different orientation of the diester phosphate group of DCP (*A*) and phosphatidylcholine (*B*) in a bilayer with respect to the bilayer normal, *n*. The orientation of the shielding tensor is shown for DCP (*A*). Arrows indicate possible intramolecular rotations of the phosphatidylcholine headgroup, which can reduce the CSA (*B*). Simulated (*a*) and experimental (*b*) ^{31}P NMR spectra are shown for DCP in the L_α lamellar phase (*C*). The simulated spectrum was obtained by using $\Delta\sigma = 15$ ppm and 200-Hz line broadening.

In the L_c phase, the ^{31}P NMR spectrum of DCP exhibits a line shape with three components of the shielding tensor similar to other crystalline diester phosphates (Fig. 2 *A*). Qualitatively, we observed the same line shape in the L_β phase of DCP (Fig. 2 *B*). However, the total CSA of DCP is significantly reduced in the L_β phase compared with the L_c phase, as indicated by the values of $\sigma_{11} = 51$, $\sigma_{22} = 10$, and $\sigma_{33} = -61$ ppm. This means that the phosphate group of DCP in the L_β phase is not completely immobilized and that some motions average the CSA compared with the crystalline phases. However, the phosphate group is not experiencing fast rotation on the NMR time scale, as indicated by the spectrum of a nonaxial symmetry (Fig. 2 *B*).

The line shape of the spectrum of DCP in the L_β phase (Fig. 2 *B*) is significantly different from that of phospholipids (Fig. 2 *E*). When DCP is embedded into the bilayer, any intramolecular rotations of the phosphate group are restricted because it is attached to the two hydrophobic alkyl chains. In contrast to DCP, rotation of the phosphate group of phospholipids is not hindered and can contribute to the reduction of the CSA.

In liquid-crystalline bilayers, lipid molecules undergo fast rotation around the bilayer normal. For the case of fast rotation about a fixed axis, the original tensor is replaced by an effective tensor, which is axially symmetric and has the elements:

$$\sigma_\parallel = (\sin^2\beta\,\cos^2\alpha)\sigma_{11} + (\sin^2\beta\,\sin^2\alpha)\sigma_{22} + (\cos^2\beta)\sigma_{33} \quad (1)$$

$$\sigma_\perp = \tfrac{1}{2}[\sigma_{11} + \sigma_{22} + \sigma_{33}) - \sigma_\parallel] \quad (2)$$

where $\sigma_\parallel$ and $\sigma_\perp$ are the components parallel and perpendicular to the rotation axis and the angles α and β are Euler angles between the rotation axis and the principal angles of the shielding tensor (Mehring et al., 1971; Seelig, 1978).

In the case of DCP, we proposed that the direction of the element σ_{22} coincides with the bilayer normal. Therefore, fast molecular rotation will completely average the elements σ_{11} and σ_{33} and not affect the element σ_{22}, resulting in equations:

$$\sigma_\parallel = \sigma_{22} \quad (3)$$

$$\sigma_\perp = \tfrac{1}{2}(\sigma_{11} + \sigma_{33}) \quad (4)$$

$$\Delta\sigma = \sigma_\parallel - \sigma_\perp \quad (5)$$

The anisotropy $\Delta\sigma$ which is observed depends now only on the values of σ_{ii}. In the L_α phase, the intramolecular rotations of the DCP phosphate group are restricted for the same reason as in the L_β phase. Therefore, the CSA of DCP in the L_α phase can be calculated assuming that fast molecular rotation is the main contribution in the additional averaging of the CSA compared with the L_β phase. For this, the values of the partially averaged shielding tensor in the L_β phase of DCP were used to calculate $\Delta\sigma$ of DCP in the L_α phase

(Eqs. 3–5). The calculated value of 15 ppm is in a good agreement with the experimental data. The simulated and experimental spectra of DCP in the L_α phase are shown in Fig. 7 *C*.

^{31}P NMR spectra of DCP in the L_α phase exhibit a significantly smaller anisotropy compared with phospholipids, although the motional freedom is more restricted in the case of DCP. The reason for this is in the different orientation of the shielding tensor of DCP and phospholipids with respect to the lipid bilayer. Polar groups of phospholipids are aligned essentially parallel (within 30°) to the plane of the bilayer (Scherer and Seelig, 1989), whereas both hydrocarbon chains attached to the phosphate group of DCP are embedded in the bilayer perpendicular to the bilayer surface (Fig. 7, *A* and *B*).

DCP stabilizes the L_β phase and destabilizes the L_α lamellar phase in monostearoylglycerol

An interesting feature of monoglyceride/water systems is the ability to form a great variety of different phases. In contrast to phospholipids, phase transformations of monoglycerides occur in a relatively narrow temperature range. Qualitatively, the phase behavior of monoglycerides can be easily understood by relating the packing properties of lipid molecules to their shape (Israelachvili et al., 1977). The headgroup of monoglycerides consists of a glycerol moiety. Because of their low hydration capacity (Morley and Tiddy, 1993), the size of the headgroup is almost the same in different phases except in the coagel, in which water is not present. The dynamically averaged volume of the acyl chain increases upon heating because of chain rotation and *trans*/*gauche* isomerization along the chain inducing the phase transitions. When lipid molecules have a cylindrical shape, they are packed in a bilayer structure. Upon heating, the acyl chain volume increases and molecules now have more of a cone-like shape with the headgroup at the pointed end. The self-packing of such cone-like molecules can lead to the formation of highly curved cubic phases or inverted phases with a negative curvature.

The phase behavior of the MSG:DCP mixture can likewise be related to the molecular shape of these lipids. Fig. 8 illustrates the difference in the molecular shape of MSG and DCP. The molecule of DCP consists of a relatively small headgroup with two attached alkyl chains. Therefore, the dynamically averaged molecular shape of DCP will be cone-like. Being placed into a flat bilayer will increase the propensity of lipids to be organized into inverted structures with a negative curvature (de Kruijff, 1997a, b). Indeed, when DCP is present the [^{2}H$_{35}$]-MSG:DCP mixture does not form a "fluid" liquid-crystalline bilayer, but directly transforms from the L_β into the L_2 phase instead.

Another effect of DCP on the phase behavior of [^{2}H$_{35}$]-MSG is the stabilization of the metastable L_β phase. This can be understood considering the way the L_c phase is

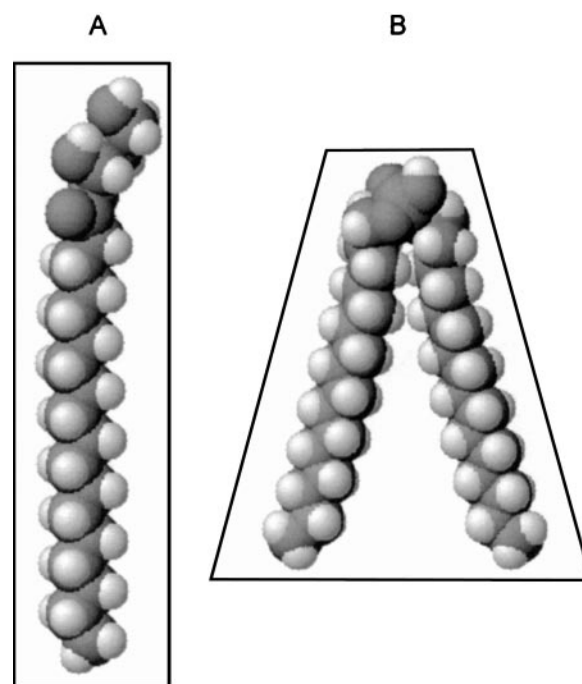

FIGURE 8 Molecular models of MSG (*A*) and DCP (*B*) in the extended conformation. The dynamically averaged molecular shape is illustrated by the surrounding lines. Light spheres represent hydrogen atoms. Dark spheres represent carbon, oxygen, and phosphorus atoms.

thought to form. Crystallization of the L_β phase into the coagel occurs via expelling of water and formation of hydrogen bonds between monoglyceride molecules in adjacent bilayers. The presence of the negatively charged DCP will inhibit the bilayers to come in a close proximity, thereby preventing crystallization of the monoglyceride bilayers.

Water-soluble proteins stabilize the L_α lamellar phase in [^{2}H$_{35}$]-MSG:DCP mixture

The water-soluble β-lactoglobulin and lysozyme change the phase behavior of the monoglyceride dispersion when DCP is present, demonstrating that DCP is responsible for lipid-protein interactions in this system. Although incorporation of DCP in [^{2}H$_{35}$]-MSG destabilizes the L_α lamellar phase, in the presence of the proteins, this effect is completely eliminated. Because β-lactoglobulin and lysozyme do not affect the phase behavior of pure [^{2}H$_{35}$]-MSG, this suggests that electrostatic interactions between the proteins and the negatively charged DCP are involved. Also, it was found that a high ionic strength decreases the stabilizing effect of these proteins on the L_α phase of [^{2}H$_{35}$]-MSG:DCP. However, the total charge of protein does not seem to be important for interaction of the protein with the negatively charged lipid-water interface, as β-lactoglobulin has a negative net charge and lysozyme has a positive net charge. This implies that electrostatic interactions must take place

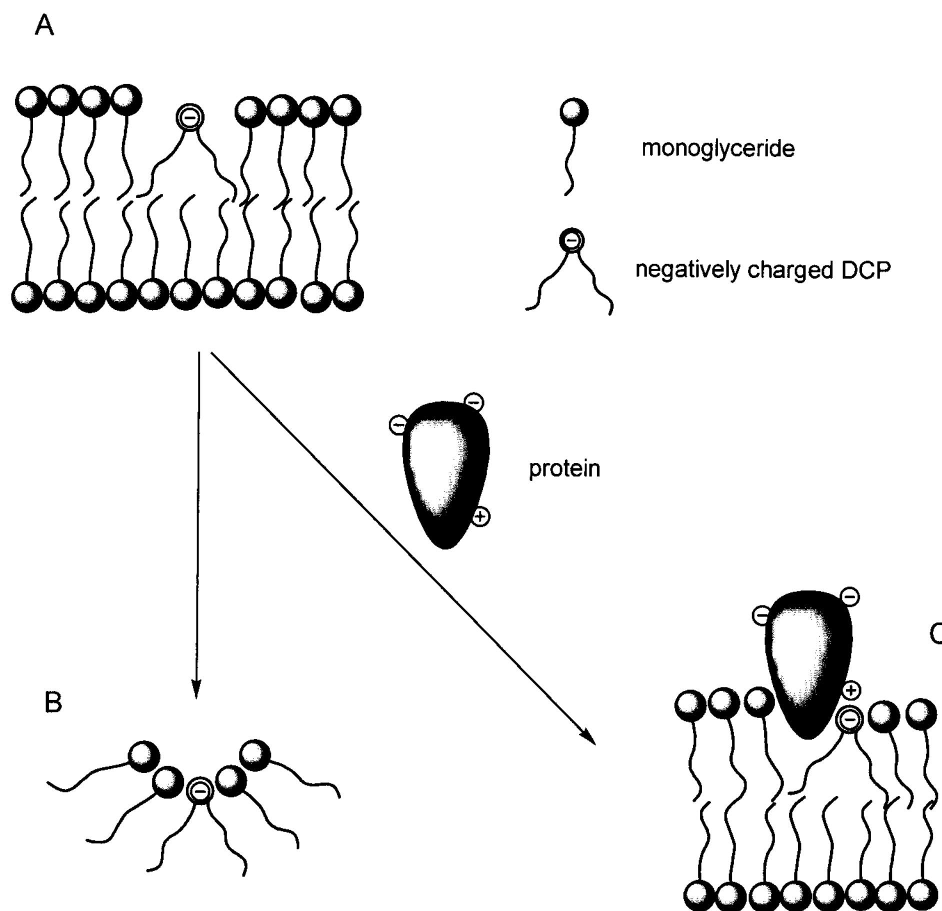

FIGURE 9 Protein insertion into the MSG:DCP bilayer. Being placed into a bilayer, a nonbilayer-forming DCP creates a space at the interface (*A*). In the absence of proteins, the flat liquid crystalline bilayer enriched with DCP transforms into the inverted phase with negative curvature (*B*). When protein is inserted into the bilayer the volume of the headgroup region is increased, effectively neutralizing the tendency of DCP to be organized in inverted structures (*C*).

on the level of the positively charged amino acid residues of the proteins. In contrast, in the presence of these proteins, the L_α phase of the [$^2H_{35}$]-MSG:DCP mixture is observed up to higher temperatures than in pure [$^2H_{35}$]-MSG. This can not be explained by only electrostatic interactions, because the simple neutralization of the electrical charge of DCP would instead rather destabilize the L_α phase. The mechanism and consequences of lipid-protein interactions in [$^2H_{35}$]-MSG:DCP can be understood if one takes into account both electrostatic interactions and the special effect of nonbilayer-forming lipids on liquid-crystalline bilayers (de Kruijff, 1997a, b; Van den Brink-van der Laan et al., 2001). This is illustrated in Fig. 9.

According to this model, both β-lactoglobulin and lysozyme interact with the [$^2H_{35}$]-MSG:DCP interface. Although lysozyme and β-lactoglobulin differ in the sign of the net charge, they have almost the same number of positively charged residues, which can electrostatically interact with the negatively charged DCP. In addition, the relatively small headgroup of DCP creates a space at the interface, thereby facilitating protein insertion (Fig. 9 *A*). This is in agreement with our observation that the presence of DCP or dioctadecylphosphate facilitates insertion of β-lactoglobulin and lysozyme into monoglyceride monolayers (Boots et al., 1999, 2001). In line with this model is also the observation that insertion of proteins may destroy the acyl chain packing order of [$^2H_{35}$]-MSG at the lipid/water interface, as indicated by the line shape changes in ^{2}H NMR spectra (Fig. 6). In the absence of proteins, the flat bilayer enriched with a nonbilayer-forming lipid DCP (Fig. 9 *A*) transforms into the inverted phase (Fig. 9 *B*). However, when protein is inserted into the bilayer the volume of the headgroup region is increased neutralizing the tendency of DCP to be organized in inverted structures (Fig. 9 *C*).

At low temperature the presence of β-lactoglobulin and lysozyme does not accelerate transformation of the thermodynamically metastable L_β phase of nonsonicated [$^2H_{35}$]-MSG:DCP dispersions into the coagel. This is in contrast to the results obtained for sonicated dispersions (Boots et al., 1999, 2001), in which we showed that β-lactoglobulin and

lysozyme induce fast crystallization of small sonicated [$^2H_{35}$]-MSG:DCP L_β phase vesicles into the coagel at 20°C. A likely explanation for this apparent discrepancy is that the lipid molecules are less tightly packed at the interface of the outer monolayer of highly curved sonicated vesicles compared with the flat, extended bilayers of nonsonicated dispersions (Huang and Mason, 1978). This may facilitate interaction of proteins with the outer monolayer of sonicated vesicles. Indeed, β-lactoglobulin and lysozyme readily interact with sonicated vesicles of [$^2H_{35}$]-MSG:DCP at 20°C, inducing disruption of the lipid bilayer (Boots et al., 1999). The following rearrangement of the lipid molecules results in the formation of the coagel, which is thermodynamically stable under these conditions. In addition, nonsonicated dispersions, which consist of randomly oriented extended bilayers, are much more viscous. Stacking of these bilayers into the L_c phase can only proceed after mechanical disruption of the bilayers, which slows down the rate of crystallization. In line with this explanation is the observation that shearing of the monoglyceride gel accelerates the coagel formation (Cassin et al., 1998).

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