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Modulation of the Specific Interaction of Cardiolipin with Cytochrome *c* by Zwitterionic Phospholipids in Binary Mixed Bilayers: A ^{2}H- and ^{31}P-NMR Study

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The interaction of cytochrome *c* with binary phospholipid mixtures was investigated by solid-state ^{2}H- and ^{31}P-NMR. To examine the effect of the interaction on the glycerol backbones, the glycerol moieties of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) were specifically deuterated. On the binding of cytochrome *c* to the binary mixed bilayers, no changes in the quadrupole splittings of each of the components were observed for the PC/PG, PE/CL and PE/PG liposomes. In contrast, the splittings of CL decreased on binding of protein to the PC/CL liposomes, although those of PC did not change at all. This showed that cytochrome *c* specifically interacts with CL in PC/CL bilayers, and penetrates into the lipid bilayer to some extent so as to perturb the dynamic structure of the glycerol backbone. This is distinctly different from the mode of interaction of cytochrome *c* with other binary mixed bilayers. In the ^{31}P-NMR spectra, line broadening and a decrease of the chemical shift anisotropy were observed on the binding of cytochrome *c* for all binary mixed bilayers that were examined. These changes were more significant for the PC/CL bilayers. Furthermore, the line broadening is more significant for PC than for CL in PC/CL bilayers. Therefore, it can be concluded that with the polar head groups, not only CL but also PC are involved in the interaction with cytochrome *c*.

Keywords: Bilayer, Cardiolipin (CL), Cytochrome *c*, ^{2}H-NMR spectra, ^{31}P-NMR spectra, Zwitterionic phospholipid

Introduction

Cytochrome *c* is a peripheral membrane protein, which is involved in the electron transport system that operates in the inner mitochondrial membrane. The lipid-protein interactions may have relevant functional effects on the efficiency of the electron transfer. For instance, cytochrome *c* binds to acidic phospholipid membranes (Waltham *et al.*, 1986; Pinheiro & Watts, 1994a). In particular, its binding to CL bilayers has been thoroughly investigated (Kruijff & Cullis, 1980; Demel, 1989). CL bilayers are also known to be required for optimal activity of cytochrome *c* oxidase (Robinson *et al.*, 1980). Although the reason for this requirement is still unclear, there is evidence that acidic phospholipids, especially CL, facilitate the binding of cytochrome *c* to cytochrome oxidase (Vik, 1981). The major lipids in the mitochondrial inner membrane are PC, PE, and CL (Awasthi & Chuang *et al.*, 1971; Krebs *et al.*, 1979). Since acidic phospholipids constitute only a small fraction of the total mitochondrial membrane lipids, it is important to examine the effect of other lipid components of the mitochondrial membrane on the apparently strong and specific interaction between acidic phospholipids and cytochrome *c*.

The molecular behavior of the cytochrome *c*-lipid bilayer complexes has been investigated by solid-state nuclear magnetic resonance (NMR). The effects of cytochrome *c* binding on the deuterium quadrupole splittings, and the relaxation times of PC and phosphatidylserine (PS) in the hydrocarbon chains and head groups, were investigated by Devaux *et al.* (Devaux *et al.*, 1986). Although the binding affected the NMR parameters from the polar head groups of PS, it did not change those from the hydrocarbon chains. Watts and his co-workers (Spooner & Watts, 1991a, b, 1992; Pinheiro & Watts, 1994a, b) researched a series of work on the interaction of cytochrome c with the polar head groups of phospholipids. It was suggested that a specific interaction between CL and cytochrome *c* induces reversible protein unfolding. No information has been provided so far on the effect of cytochrome *c* binding on the glycerol backbone, the interface between the hydrocarbon chains, and polar head groups. Furthermore, the nature of the binding of cytochrome *c* to mixed bilayers has not been studied systematically. To

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investigate these points, we selectively deuterated the glycerol moieties of PC, PE, CL, and PG. We then measured the ^{2}H- and ^{31}P-NMR spectra of PC/CL, PC/PG, PE/CL, and PE/PO bilayers in the absence and presence of bound cytochrome *c*. The specific interaction of cytochrome *c* with CL was also confirmed in this work. In addition, the specificity turned out to be modulated by the zwitter-ionic phospholipid in the binary mixtures.

Materials and Methods

Specific deuteration of the glycerol moieties of phospholipids Synthesis of perdeuterated glycerol ([$^{2}H5$]-glycerol) was performed as described elsewhere (Yoshikawa *et al.*, 1988). The deuterated glycerol was incorporated into an *E. coli* mutant that required glycerol (*E. coli* K-12 GRA) at 37°C. Phospholipids were extracted from the cells according to the reported method (Yoshikawa *et al.*, 1988). Purity was confirmed by silicic acid thin layer chromatography (TLC). The extent of deuteration was estimated from a ^{1}H-NMR spectrum of PE. It averaged about 70%.

Sufficient amounts of the deuterated PE and CL were obtained from the *E. coli* cells. PG was synthesized by transphosphatidylation of the purified PE using cabbage phospholipase D. PC was synthesized by methylation of the PE that was purified from the cells, which was described previously according to the modified method (Stockton *et al.*, 1974; Akutsu *et al.*, 1986). Specific deuteration of the glycerol backbone of PC was carried out by using specifically deuterated PE.

Purification of cytochronte *c* Prior to use, cytochrome *c* from a horse's heart (typeVI, Sigma Chemical Co.) was converted to the fully oxidized form by addition of excess $K_3Fe(CN)_6$. It was then purified by ion-exchange chromatography on Whitman CM-32, eluted with 0.5 M NaCl, 10 mM phosphate buffer at pH 7.0. Eluent, which contained the purified protein, was concentrated by ultrafiltration using Amicon YM-3 ultrafiltration membranes, then dialyzed extensively to remove the phosphate (Spooner & Watts, 1991a).

Preparation of NMR samples To prepare binary mixtures, zwitter-ionic and acidic phospholipids (4 : 1, w/w) were dissolved-in chloroform/methanol (1 : 1, v/v). A dry film was formed under rotary evaporation after the polyvalent metal ions were removed with a 0.5 M Na_2SO_4, 2.0 mM EDTA solution (pH 7.2). The film was then put under high vacuum for almost one day in order to remove all traces of the organic solvent. Multilamellar liposomes were prepared by dispersing the dry lipid film to a 5 mM Tris-HCI buffer (pH 7.4) in deuterium depleted water (less than 0.2 ppm, CEA) at 50°C. Typically, about 30 mg of the deuterated lipid was used for the ^{2}H-NMR measurement. The liposome-cytochrome *c* complexes were prepared by addition of a protein solution (5 mM Tris-HCI, pH 7.4) to the previously formed multilayer liposomes. The lipid-protein complexes were obtained by centrifugation at 4000 rpm (Tomy TS-7 rotor) and 4°C. The clear supernatant was then used for the protein analysis. The amount of binding protein was determined from the amount of remaining protein in the supernatant. The absorption spectra were measured with a SHIMADZU UV-2000 spectrophotometer. A sample preparation was carried out in a nitrogen atmosphere to prevent the oxidation of the lipid fatty acyl chains. The lipid/protein ratios in the mixtures were calculated with respect to the acidic phospholipid in the binary mixtures. The molar ratio of lipid/protein of the lipid-protein complexes that were used in the present study was in the range of 10-20 relative to the acidic phospholipid component.

NMR measurements ^{1}H-NMR spectra were obtained with a Bruker AM-400 spectrometer operation at 61.1 MHz that was equipped with a CP/MAS probe for a 5-mmϕ tube. The 90° pulse width was 4 μs. The quadrupole echo pulse sequence $(90_x\text{-}\tau_1\text{-}90_y\text{-}\tau_2)$ was employed with $\tau_1 = 30$ μs, $\tau_2 = 20$ μs, and 0.5 s of recycle time. ^{31}P-NMR spectra were recorded on a Chemagnetics CMX-400 spectrometer that operated at 161.15 MHz under proton decoupling. The 90° pulse width was 3.5 μs for ^{31}P. A chemical shift was shown in the spectrum relative to 85% phosphoric acid.

Results

^{2}H-NMR spectra and deuterium quadrupole splittings of phospholipid glycerol backbones The binding of cytochrome c to the binary mixed bilayers of PC and CL was examined. The molar ratio of CL to cytochrome *c* was 10 to 1. Figs. 1A, B, C, and D represent ^{2}H-NMR spectra of PC/CL*, PC/CL*-cytochrome *c*, PC*/CL, and PC*/CL-cytochrome *c* bilayers, respectively, at 35°C. The asterisk (*) stands for the phospholipid that was perdeuterated in the glycerol moieties. All of ^{2}H-NMR spectra in Fig. 1 exhibited superimposed powder patterns that are characteristic for nuclei with $I = 1$ under strong magnetic fields. The assignments for the highest

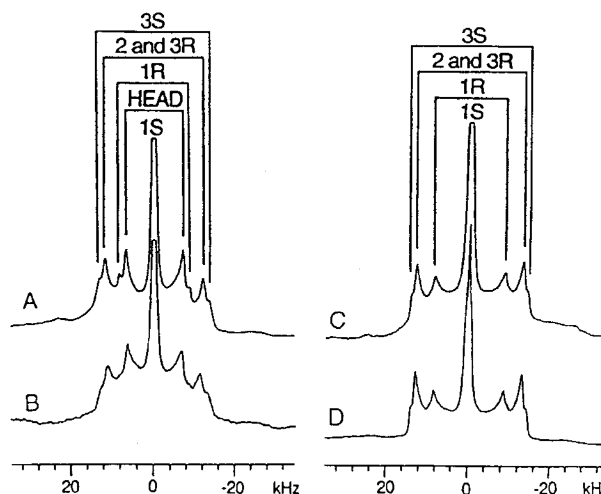

Fig. 1. ^{2}H-NMR spectra of the deuterated glycerol backbone of the binary mixed bilayers of PC and CL (4 : 1, w/w) without and with cytochrome *c* at 61.6 MHz and 35°C. (A) PC/CL* mixed bilayers, (B) PC/CL* mixed bilayers with cytochrome *c*, (C) PC*/CL mixed bilayers, (D) PC*/CL mixed bilayers with cytochrome assignments are given at the top of the spectra. HEAD denotes the head group of CL, and the others stand for the prochiral deuterons of the glycerol backbone.

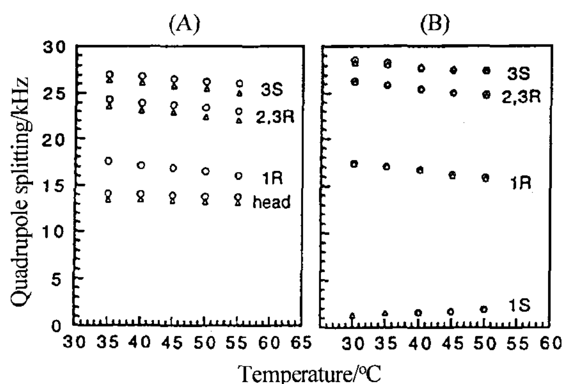

Fig. 2. Temperature dependence of the deuterium quadrupole splittings of the binary mixed bilayers of PC and CL (4 : 1,w/w) without (circle) and with cytochrome *c* (triangle). (A) PC/CL* liposomes, (B) PC*/CL liposomes. The asterisk (*) stands for the deuterated phospholipid.

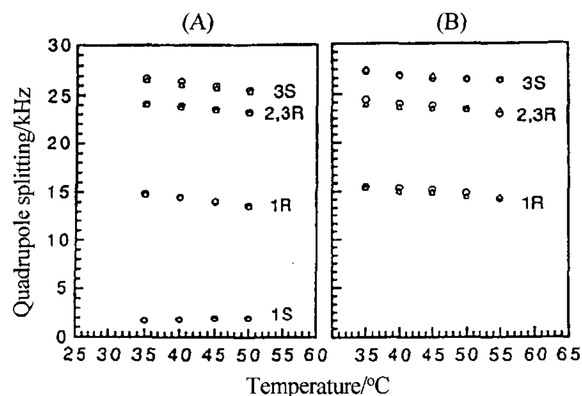

Fig. 3. Temperature dependent of the deuterium quadrupole splittings of the binary mixed bilayers of PC and PG (4 : 1, w.w) without (circle) and with cytochrome *c* (triangle). (A) PC/PG* liposomes, (B) PC*/PG liposomes. The asterisk (*) stands for the deuterated phospholipid.

peaks were given on the basis of the reported ones (Allegrini *et al.*, 1984; Strenk *et al.*, 1985; Yoshikawa *et al.*, 1988). For example, 1S stands for the pro-S deuteron at the C1 site of the glycerol backbone. A strong signal pair with the quadrupole splitting of about 14 kHz in Figs. 1A and B was attributed to the five deuterons of the head group of CL. The ^{2}H-NMR spectra of CL* in PC/CL* liposomes changed on the binding of cytochrome *c* (Figs. 1A and B). A reduction in the signal intensity and an increase of spectral linewidth was observed. In contrast, the ^{2}H-NMR spectrum of PC* in the mixed bilayers revealed no change on the incorporation of cytochrome *c* (Figs. lC and D). The quadrupole splittings of PC/CL liposomes with and without cytochrome *c* were plotted as a function of temperature in Fig. 2. Although the changes were insignificant, all of the quadrupole splittings of the glycerol deuterons of CL became smaller on binding of cytochrome *c* to the PC/CL* liposomes (Fig. 2A). However, the quadrupole splittings of the PC backbone did not change on the binding of cytochrome *c* (Fig. 2B). Therefore, it can be concluded that while the bound cytochrome *c* affects the dynamic structure of the glycerol backbone and head group of CL, it does not alter the dynamic structure of the glycerol backbone of PC. So far, the effect of the binding of cytochrome *c* has been undetected outside of the polar head groups of the lipid bilayers by solid-state NMR.

The ^{2}H-NMR spectra of the PC/PG*, PC*/PG liposomes in the absence and presence of bound cytochrome *c* were also examined (spectra not shown). The molar ratio of PG/ cytochrome *c* was about 20 in the liposome-cytochrome *c* complex. The observed quadrupole splittings were plotted as a function of temperature in Fig. 3. Both of the spectral line shapes and quadrupole splittings of the ^{2}H-NMR spectra of PC* and PG* were identical for the free and cytochrome c-bound liposomes. This suggests that the dynamic structures of the glycerol backbones of both components were unaffected by the binding of die protein to the PC/PG liposomes. Consequently, the mode of interaction of cytochrome *c* with CL should be different from that with PG.

The binary mixed bilayers of PE/CL and PE/PG gave results similar to that of the PC/PC bilayers. The molar ratios of acidic phospholipids to cytochrome *c* were similar to those of the PC/CL and PC/PG liposomes with cytochrome *c*, respectively. The temperature dependence of the quadrupole splittings for the PE/CL and PE/PG systems is illustrated in Figs. 4 and 5, respectively. The quadrupole splittings of all of the phospholipid components in the mixed bilayers were unaffected by the binding of cytochrome *c* to the membranes. These results showed that the mode of interaction of cytochrome *c* with CL is also different for the PC/CL and PE/ CL liposomes. Since the glycerol backbone structure was perturbed by cytochrome *c* only for CL in the PC/CL bilayers, the protein must interact most strongly with the PC/CL bilayers among the binary mixtures that were examined.

^{31}P-NMR spectra and phosphorus chemical shift anisotropy To monitor the polymorphic structure of the binary lipid samples, and the effect of intermolecular interactions on the polar head groups by the binding of cytochrome *c* , ^{31}P-NMR spectra were measured for the same samples that were used for the ^{2}H-NMR measurements. The ^{31}P-NMR spectra of the PC/CL* and PC/PG* liposomes with and without cytochrome *c* are shown in Fig. 6. Their powder patterns indicate that most of phospholipids are in liquid-crystalline bilayers in all of the samples. All spectra clearly consist of two superimposed, axially symmetric powder patterns with different chemical shift anisotropies. A similar superimposed spectrum was reported for the DOPC/CL bilayers (Spooner & Watta, 1992), POPC/*E.coli* PE bilayers (Ghosh, 1988) and DMPC/DMPG/DDAB bilayers (Marassi & Macdonald, 1991). DOPC, POPC, and DMPC stand for dioleoyl-, palmitoyloleoyl-, and dimyristoylphosphatidylcholines, respectively. DMPG and DDAB denote dirnyristoylphosphatidylglycerol and

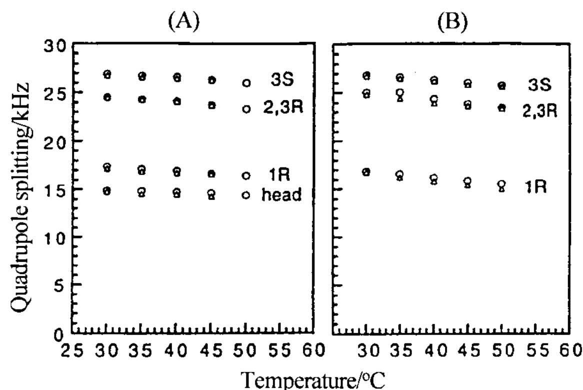

Fig. 4. Temperature dependent of the deuterium quadrupole splittings of the binary mixed bilayers of PE and CL (4 : 1, w.w) without (circle) and with cytochrome *c* (triangle). (A) PE/CL* liposomes, (B) PE*/CL liposomes. The asterisk (*) stands for the deuterated phospholipid.

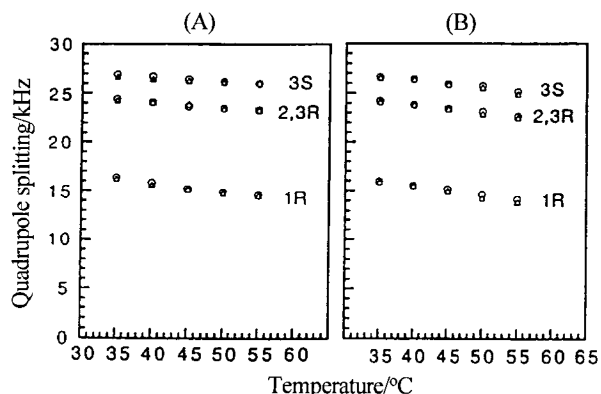

Fig. 5. Temperature dependence of the deuterium quadrupole splittings of the binary mixed bilayers of PC and PG (4 : 1,w/w) without (circle) and with cytochrome *c* (triangle). (A) PE/PG* liposomes, and (B) PE*/PG liposomes. The asterisk (*) stands for the deuterated phospholipid.

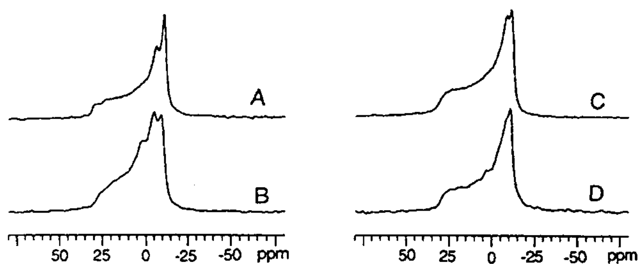

Fig. 6. Proton-decoupled ^{31}P-NMR spectra of binary mixed bilayers, including PC at 161.15 MHx and 35°C. (A) PC/CL liposomes, (B) PC/CL liposomes with cytochrome *c*, (C) PC/ PG liposomes and (D) PC/PG liposomes with cytochrome *c*.

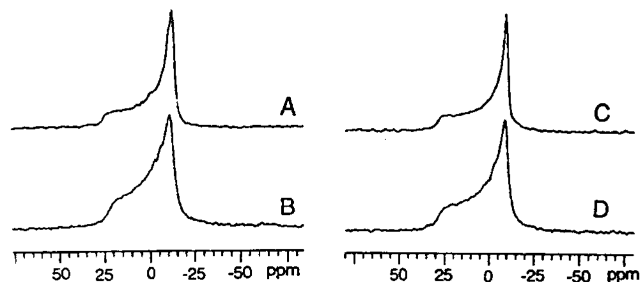

Fig. 7. Proton-decoupled ^{31}P-NMR spectra of binary mixed bilayers, including PE at 161.15 MHx and 35°C. (A) PE/CL liposomes, (B) PE/CL liposomes with cytochrome *c*, (C) PE/PG liposomes and (D) PE/PG liposomes with cytochrome *c*.

didodecyldimethyl-ammonium bromide, respectively. In every case, two axially symmetric powder patterns were ascribed to each phospholipid species. Therefore, the powder patterns with large and small chemical shift anisotropies can be ascribed to PC and CL (or PG), respectively, in Fig. 6. The apparent chemical shift anisotropy of the larger component of the PC/CL and PC/PG bilayers was about –44 ppm. For the PC bilayers, it was –46 ppm. This suggests that the conformation of the phosphate group of PC changed when it was mixed with acidic phospholipid. It is well known that the polar head group of PC changes its conformation in the presence of a membrane surface change (Akutsu & Seelig, 1981; MacDonald *et al.*, 1991). As can be seen in Figs. 6B and D, a small but distinct isotropic component at around 4 ppm appeared in the presence of cytochrome *c*. The appearance of the isotropic component was reported for the ^{31}P-NMR spectra of single acidic phospholipid bilayers (Kruijiff & Cullis, 1980; Waltham *et al.*, 1986; Pinheiro & Watts, 1994a) and mixed bilayers in the presence of bound cytochrome *c* (Pinheiro & Watts, 1994b).

Line broadening of the spectra and a decrease of the chemical shift anisotropy were observed on the binding of cytochrome *c*. It was even more significant for PC/CL bilayers (Figs. 6A and B). Furthermore, the peak height of the wider component of the powder patterns was considerably reduced in comparison with the narrow component in Fig. 6B. This suggests that the line broadening was more significant for PC than for CL. Therefore, it can be concluded that as far as the polar head groups are concerned, not only CL but also PC are involved in the interaction with cytochrome *c*. In the case of the PC/PG liposomes, two components showed a similar line broadening. The line broadening was also reported for CL and PC in the three-component mixture of PC/PE/CL on the binding of cytochrome *c* (Pinheiro & Watts, 1994b).

The ^{31}P-NMR spectra of the PE/PG* liposomes with and without cytochrome *c* at 35°C are presented in Fig. 7. In contrast to Fig. 6, the spectra of the binary mixed bilayers gave rise to a single powder pattern with an averaged chemical shift anisotropy. The presence of protein slightly decreased the average chemical shift anisotropy of the powder pattern and induced spectral line broadening. It should be noted that neither the isotropic signal nor the hexagonal II pattern appeared in these binary mixtures, even in the presence of bound cytochrome *c*. The apparent chemical shift anisotropy of the wider component of the mixed bilayers with and

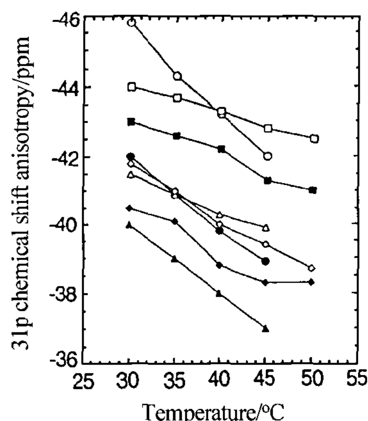

Fig. 8. Temperature dependence of apparent ^{31}P chemical shift anisotropy of the binary mixed bilayers without (open symbol) and with cytochrome *c* (filled symbol). Circle, PC/CL liposomes; Square, PC/PG; Triangle, PE/CL; Diamond, PE/PG.

without cytochrome *c* was plotted as a function of temperature in Fig. 8. On the binding of cytochrome *c*, the ^{31}P chemical shift anisotropy decreased for all of the mixed phospholipid bilayers that were examined by about 1-4 ppm. The largest decrease was observed for the PC/CL bilayers.

Discussion

The results of the ^{2}H-NMR experiment showed that cytochrome *c* specifically interacts with CL in the PC/CL bilayers and penetrates into the lipid bilayer to some extent, so as to perturb the dynamic structure of the glycerol backbone. This is distinctly different from the mode of interaction of cytochrome *c* with other binary mixed bilayers. Although cytochrome *c* was bound to the membrane, such as PC/PG, PE/CL, and PE/PG bilayers, it perturbed only the surface of the phospholipid bilayers. This is consistent with earlier reports (Pinheiro & Watts, 1992,1994b). However, direct evidence for the penetration of cytochrome *c* into the membrane on the basis of solid state NMR was obtained for the first time in this work. There is a report on the binding of cytochrome *c* to the PC/CL small unilamellar vesicles that were studied by liquid-state high resolution NMR (Brown & Wuthrich, 1977). It showed that the binding of the protein affects the relative intensity and spin-lattice relaxation time of the carbons (^{13}C) in the region from the polar head groups to the ester bonds of the fatty acids. However, the affected phospholipid species could not be identified. The affected region deduced in the reported study agrees with our results.

CL showed a specific interaction with cytochrome *c* in the PC/CL bilayers, but it did not in the PE/CL bilayers. This fact suggests that the specific interaction of cytochrome *c* with CL can be modulated by the co-existing phospholipid molecules. Since PE showed no strong interaction with cytochrome *c*, it cannot compete with CL in the interaction with the protein. Therefore, the lipid-lipid interactions should be responsible for the modulation of the specific interaction of cytochrome *c* with CL. A distinct difference between PC and PE is the ability to form hydrogen bonds. CL cannot form hydrogen bonds with PC, but it can with PE. Therefore, the CL-PE interaction should be much stronger than that of CL-PC. Such a strong interaction would prevent the penetration of cytochrome *c* into the membrane.

Deuterium quadrupole splittings of CL in the PC/CL bilayers decreased for all deuterons of the glycerol backbones and head groups. This suggests that the fluctuation of the molecule increased on the binding to cytochrome *c* in the bilayers. Some extent of the penetration of cytochrome *c* could induce a structural perturbation in the bilayers, which would result in more fluctuation on average. On the other hand, the quadrupole splittings of PC did not change at all, but the chemical shift anisotropy and the linewidth of the phosphorus powder pattern changed to a certain extent. This is in good agreement with the results of the ternary mixed bilayers of PC/PE/CL at a high temperature (Pinheiro & Watts, 1992,1994b). Although the PC molecule does not interact strongly with cytochrome *c*, it is involved in the interaction. Since there is no bound signal in the ^{2}H- and ^{31}P-NMR spectra, cytochrome *c* should change the binding sites among CL and PC at a rate faster than the NMR time scale, as suggested by Pinheiro and Watts. However, the major binding site, which induces the penetration of cytochrome *c*, should be the CL molecules.

It was shown that although CL is not essential for the activity of cytochrome oxidase, it can enhance the activity. One of proposed mechanisms to explain the observation was that CL would increase the association constant of the cytochrome oxidase with cytochrome *c*. The penetration of cytochrome *c* into lipid bilayers could facilitate the interaction with the cytochrome oxidase on mitochondrial inner membranes. This would result in an increase of the association constant. Therefore, the asymmetric distribution of phospholipids in the mitochondrial inner membrane is very interesting. In the inner membrane of the beef heart mitochondria, PC and PE are mainly located in the outer and inner leaflets of the inner membrane, respectively (Krebs, 1979). Since cytochrome *c* interacts with the outer leaflet of the membrane in the intermembrane space, CL may exert its specificity in the interaction with cytochrome *c* in the presence of dominant PC.

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