Resolution of Individual Lipids in Mixed Phospholipid Membranes and Specific Lipid–Cytochrome *c* Interactions by Magic-Angle Spinning Solid-State Phosphorus-31 NMR†

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ABSTRACT: A model of the inner mitochondrial membrane was constructed with dioleoylphosphatidylcholine (PC), dioleoylphosphatidylethanolamine (PE), and cardiolipin (CL) at a PC:PE:CL molar ratio of 2:2:1, and the interaction of the peripheral membrane protein cytochrome *c* with this mixed membrane has been investigated by static and magic-angle spinning (MAS) solid-state ^{31}P NMR. The static ^{31}P NMR spectrum of the three-component membrane is a typical broad powder pattern for phospholipids in a bilayer structure, and is a result of three overlapping spectra of each individual phospholipid component in the mixed membrane, with an average effective chemical shift anisotropy of approximately 41 ppm. Using magic-angle spinning NMR methods, three resolved resonances are observed in the narrowed MAS ^{31}P NMR spectrum, each of which has been assigned to each lipid component in the mixed membrane. This allows the investigation of individual phospholipid–protein interactions in multicomponent lipid bilayers. The interaction of cytochrome *c* with each lipid in a model mitochondrial membrane could now be evaluated. Phosphorus-31 spin–lattice (T_1) relaxation times for each lipid phosphate were measured as a function of temperature, in the absence and presence of bound cytochrome *c*. T_1 was not affected for any lipid upon binding of cytochrome *c* over the temperature range analyzed. However, averaging of the phosphorus-31 chemical shift anisotropy for the cardiolipin component in mixed PC/PE/CL bilayers at lower temperatures ceases to be axially symmetric on binding of cytochrome *c*, while for PC and PE components the axial symmetry is retained over the temperature interval studied here. The results suggest that the strongest interaction of cytochrome *c* in this model mitochondrial membrane occurs with the cardiolipin component, but less so with the other components. Some lipid lateral-phase separation in the mixed CL/PE/PC bilayer seems to be induced on binding of cytochrome *c*, in which PE is segregated into isotropic structures on the phosphorus-31 NMR time scale.

Detailed molecular information about the nature and specificity of the interaction of peripheral proteins with membranes is not widely available. Peripheral membrane proteins interact primarily with the membrane surface, in an association with phospholipid headgroups at the interface between the hydrophobic interior of the membrane and the aqueous environment. Such interactions are originally driven by electrostatic forces between charged amino acid residues on the protein surface and charged lipid headgroups at the membrane surface. The binding of a peripheral protein to a lipid membrane may, however, be followed by other modes of lipid–protein interactions, such as hydrophobic interactions involving partial penetration of the protein into the hydrophobic core of the bilayer.

Cytochrome *c* has been known to bind strongly to anionic phospholipid membranes (Kimelberg *et al.*, 1970; Rietveld *et al.*, 1983; Waltham *et al.*, 1986), in particular to cardiolipin bilayers (Brown & Wüthrich, 1977; de Kruijff & Cullis, 1980; Demel *et al.*, 1989). The binding of cytochrome *c* to anionic electrode surfaces (Hildebrandt *et al.*, 1989a,b) and anionic phospholipid bilayers (Hildebrandt *et al.*, 1990a; Heimburg *et al.*, 1991) has been shown by surface-enhanced resonance Raman spectroscopy to induce conformational changes in the protein, whereby the sixth coordination ligand (Met-80) to the heme iron is disrupted and an intermediate high-spin state of the iron in the protein is induced. Those conformational changes in the protein can be detected on ^{31}P nuclear magnetic resonance (NMR)[1] studies of the lipid phosphate in phospholipid–cytochrome *c* complexes, whereby a strong paramagnetic enhancement of phosphorus-31 spin–lattice relaxation on binding of cytochrome *c* has been observed (Spooner & Watts, 1991; Pinheiro & Watts, 1994). A dynamic conformational equilibrium is established between a low-spin six-coordinated heme iron and a high-spin five-coordinated heme iron in cytochrome *c*, which is controlled by the electrostatic interaction between the positively charged lysine residues in the protein surface and the negatively charged phospholipid headgroups in the membrane surface.

Cytochrome *c* acts in the mitochondrial electron-transfer chain as an intermediate between the b–c_1 complex and cytochrome *c* oxidase. It is probable that the lipid-induced conformational changes in cytochrome *c*, described above, may have relevant functional implications on the efficiency of electron transfer. The intervention of a membrane-bound intermediate of cytochrome *c* in this process is plausible; however, this has been a matter of controversy (Gupte & Hackenbrock, 1988a,b; Hildebrandt *et al.*, 1990b; Heimburg

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[1] Abbreviations: CL, cardiolipin; cyt *c*, cytochrome *c*; DOPC or PC, dioleoylphosphatidylcholine; DOPE or PE, dioleoylphosphatidylethanolamine; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time.

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et al., 1991). The major lipid components of the inner mitochondrial membrane are as follows: phosphatidylcholine (PC), 40%; phosphatidylethanolamine (PE), 39%; cardiolipin (CL), 17%; and phosphatidylinositol (PI), 2% (weight percentages of the total weight of lipids) (Daum, 1985). In the present study, we have constructed a mitochondrial model membrane containing the major phospholipid components PC, PE, and CL, in the molar ratio 2:2:1, respectively, and by using magic-angle spinning (MAS) phosphorus-31 NMR, the interaction of cytochrome *c* with the individual lipids in the mixed membrane has been examined. The temperature profiles of phosphorus-31 spin–lattice (T_1) relaxation times of the lipid headgroup phosphates are analyzed in the absence and presence of bound cytochrome *c*. Through the spinning sideband analysis, the phosphorus-31 chemical shift anisotropy parameters for the individual phospholipids in the model mitochondrial membrane could be evaluated, and the specific effects upon binding of cytochrome *c* were investigated.

MATERIALS AND METHODS

Cardiolipin (CL), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylethanolamine (DOPE) were purchased from Sigma Chemical Co., St. Louis, MO, and used without further purification. Cytochrome *c* from horse heart, type VI (Sigma Chemical Co.), which comes contaminated with a variety of deaminated forms of the protein, was purified by ion-exchange chromatography on Whatman CM-32, and eluted with 65 mM phosphate buffer, pH 7.0 (Brautigan *et al.*, 1978). The eluent containing the purified protein was concentrated by ultrafiltration using Amicon YM-5 ultrafiltration membranes, followed by extensive dialysis against cold distilled water (4 °C) to remove phosphate. Aqueous concentrations of cytochrome *c* were measured spectrophotometrically using a molar absorptivity of 2.95×10^4 M^{-1} cm^{-1} at 550 nm and pH 7.0 for the protein reduced with sodium dithionite (Margoliash & Walasek, 1967).

Sample Preparation. A ternary mixture of the phospholipids CL, DOPC, and DOPE in the molar ratio 1:2:2, respectively, was prepared from their stock solutions in chloroform/methanol or chloroform/ethanol, 1:1, v/v, and a dry film was formed under rotatory evaporation, which was then left under high vacuum for a minimum of 8 h to remove all traces of organic solvent. Multilamellar liposomes were prepared by hand-shaking the preformed lipid film with excess 20 mM cacodylate buffer at pH 7.0, containing 100 mM NaCl and 0.5 mM EDTA. Lipid–cytochrome *c* complexes were prepared by addition of protein in buffer solution to the previously formed phospholipid bilayers, followed by three cycles of freeze–thawing. The lipid–protein complexes were separated by ultracentrifugation at 75000*g* for 1 h at 4 °C, and the clear supernatant was removed for protein analysis. The amount of protein in the pellets was determined from the amount of remaining protein in the supernatant as determined spectrophotometrically. From the aqueous protein concentrations in the supernatant, it was estimated that less than 5% of the protein content in the pelleted lipid–protein complexes was in an unbound state. For all sample preparations, the buffer was previously deoxygenated with nitrogen gas, and all steps were carried out under a nitrogen atmosphere to prevent oxidation of the unsaturated lipid acyl chains. Lipid:protein ratios in the complexes were calculated with respect to the cardiolipin fraction in the three-component lipid bilayers of CL, DOPC, and DOPE in the molar ratio 1:2:2, respectively. Lipid–protein complexes used in the present study were in the lipid:protein mole ratio range of (15–20):1 relative to the cardiolipin component.

Phosphorus-31 NMR Spectroscopy. Proton-decoupled ^{31}P NMR spectra were recorded at 161.98 MHz on a Bruker MSL 400 spectrometer using a Hahn echo pulse sequence. Static and magic-angle spinning (MAS) spectra were obtained by using a 5-μs $\pi/2$ pulse width and application of a 10-G proton decoupling field during acquisition. MAS experiments were performed on Bruker double-bearing probe heads for 7-mm sample rotors. In general, no special procedures were required to enable MAS of the hydrated lipid or lipid–protein complexes, other than to seal the samples under nitrogen atmosphere with a Teflon insert top and nonvented rotor caps. Low-temperature MAS experiments were achieved by cooling the bearing gas while the drive gas was maintained at room temperature. The equipment for cooling the nitrogen gas is described elsewhere (Allen *et al.*, 1991). Phosphorus-31 spin–lattice (T_1) relaxation times were measured with the inversion–recovery pulse sequence π–τ–$\pi/2$, with proton decoupling, and with a recycle time of typically 5 s, which is at least $5 \times T_1$.

Spinning Sideband Analysis. Spinning sideband analysis of the individual resonances in the ^{31}P MAS NMR spectra of the mixed phospholipid bilayers containing CL, DOPC, and DOPE, with and without cytochrome *c*, was performed by the Herzfeld–Berger method (Herzfeld & Berger, 1980), using the fitting package developed by de Groot *et al.* (1991). By this method, the phosphorus-31 chemical shift anisotropy parameters σ'_{11}, σ'_{22}, and σ'_{33} as well as the asymmetry parameter η were calculated for the individual lipid components in the mixed bilayers.

RESULTS

^{31}P MAS NMR Spectra. A typical phosphorus-31 powder pattern spectrum of phospholipids in bilayer structure, resulting from an axially symmetric ^{31}P chemical shift tensor, was obtained for the three-component bilayer system containing cardiolipin, DOPC, and DOPE, in the molar ratio 1:2:2, respectively, as illustrated in the *top spectrum* of Figure 1. This spectral line shape containing an intense high-field edge ($\sigma_\perp$) and a shallow low-field shoulder ($\sigma_\parallel$) is a result of the averaging of the phosphorus chemical shift anisotropy by fast rotation about the bilayer normal (parallel to the long molecular axis) of the entire phospholipid molecule, and additional headgroup wobbling motion (Smith & Ekiel, 1984). The separation of the edges in a ^{31}P NMR spectrum, *i.e.*, the breadth between the lower intense low-field shoulder ($\sigma_\parallel$) and the more intense high-field peak ($\sigma_\perp$), defines the residual chemical shift anisotropy. The *top spectrum* of Figure 1 is the result of three overlapping powder pattern spectra for each of the individual phospholipid components in the mixed membrane, with an averaged effective chemical shift anisotropy of approximately 41 ppm.

Spinning at the magic angle (54.7°) at rates (ω_r) smaller than the chemical shift anisotropy, the powder pattern is broken into a high-resolution spectrum composed of a central band flanked on both sides by spinning sidebands spaced by multiples of ω_r. The identification of the central band can be achieved by varying the spinning rate, as illustrated in Figure 1. When the spinning rate is increased by $\Delta\omega$, all the sidebands move apart from the central band by the same amount $\Delta\omega$, while the central band remains at the same frequency, and simultaneously the sideband intensity is moved into the central band. An advantage of the spinning NMR experiment is that the individual resonances for each phospholipid component in the mixed bilayer can now be observed, as the central band contains the resolved isotropic shifts for each lipid phosphate

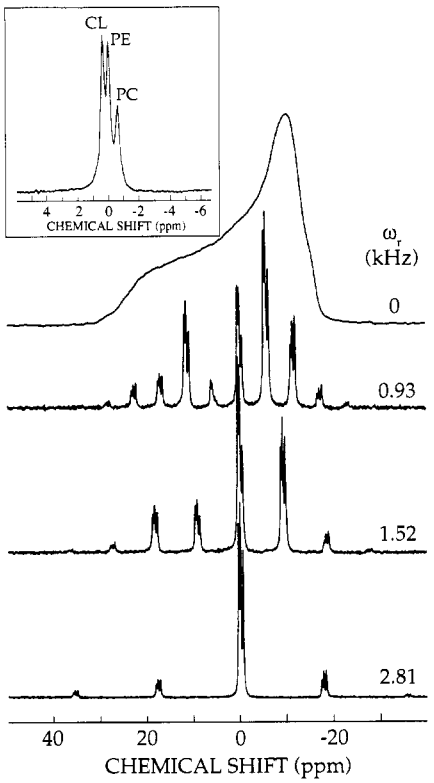

FIGURE 1: 161.98-MHz proton-decoupled ^{31}P NMR spectra of a mixed three-component phospholipid membrane, containing cardiolipin (CL), dioleoylphosphatidylcholine (PC), and dioleoylphosphatidylethanolamine (PE) in the molar ratio 1:2:2, respectively. *Top spectrum*, static axially symmetric powder pattern; *lower spectra*, illustration of the spinning sideband profile in the corresponding MAS spectra at various spinning rates (ω_r) for identification of the central band. *Insert*: central band of the spectrum at 1.52 kHz showing the assignment of the individual resonances (*see text* for full description).

in the mixed bilayer (*insert* of Figure 1). The identification of the isotropic shifts for each phospholipid in the MAS ^{31}P NMR spectrum was made in comparison with parallel experiments on two-component systems of CL/DOPC and CL/DOPE (spectra not shown), and the resulting assignments are the following: CL, +0.395 ppm; DOPE, +0.018 ppm; DOPC, −0.584 ppm relative to 85% H_3PO_4 at 25 °C. No variations in the chemical shifts with temperature or on binding of cytochrome *c* were observed.

In Figure 2, the effect on the lineshape of the static ^{31}P NMR spectra on addition of cytochrome *c* to the mixed lipid membrane can be observed. Some differences are readily detected between the protein-free and protein-containing bilayers. The presence of cytochrome *c* is accompanied by the appearance of an additional isotropic component at around 2 ppm, a decrease in the average chemical shift anisotropy of the powder pattern from 41 to 35 ppm, and a reduction in the intensity of the low-field shoulder. The corresponding MAS ^{31}P NMR spectra at 1.5 kHz are also shown in Figure 2 (spectra b and d). A noticeable reduction in the signal intensities and loss of spectral resolution for the bilayers containing cytochrome *c* (Figure 2d) when compared with the protein-free bilayers (Figure 2b) can be readily seen. Isotropic spectral components have been observed in static ^{31}P NMR spectra of cardiolipin–cytochrome *c* complexes (de Kruijff & Cullis, 1980; Rietveld *et al.*, 1983; Spooner *et al.*, 1993), and with other anionic phospholipid–cytochrome *c* complexes (Pinheiro & Watts, 1994). The interpretation of such isotropic components is still unclear. In some studies, these isotropic components have been attributed to vesicular or micellar structures in oron the bilayer, possibly associated with

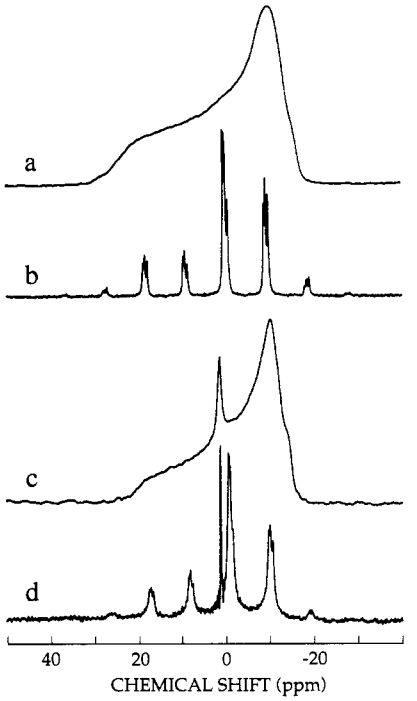

FIGURE 2: 161.98-MHz proton-decoupled ^{31}P NMR spectra of a mixed three-component phospholipid membrane containing cardiolipin, DOPC, and DOPE in the molar ratio 1:2:2, respectively. Static powder pattern spectra (*a*, *c*) obtained with the samples at the magic angle; high-resolution magic-angle spinning spectra at a spinning rate of 1.5 kHz (*b*, *d*) for protein-free bilayers (*a*, *b*); cytochrome *c*-containing bilayers in the molar lipid to protein ratio of 20:1, relative to the cardiolipin fraction (*c*, *d*).

precursors of hexagonal H_{II}-phase formation (de Kruijff & Cullis, 1980; Farren & Cullis, 1980). Others have attributed them to formation of smaller diameter (≤ 500 nm) vesicles induced upon protein binding, or a result of motional averaging, without involvement of nonbilayer structures (Spooner & Watts, 1991; Spooner *et al.*, 1993; Pinheiro & Watts, 1994).

Spin–Lattice Relaxation. Phosphorus-31 spin–lattice (T_1) relaxation times for each phospholipid component, CL, DOPE, and DOPC, in the mixed membrane were evaluated by performing inversion–recovery experiments on spinning samples at various temperatures, for the mixed bilayers alone, and with bound cytochrome *c*. The isotropic regions of sequences of spectra typically obtained during T_1 measurements on protein-free and protein-containing bilayers are shown in Figure 3. Here, it can be observed that the spin–lattice relaxation for all three lipid components in the presence of cytochrome *c* (Figure 3B) is indistinguishable from that observed in the protein-free bilayers (Figure 3A). However, the "extra" isotropic component at around 2 ppm clearly has a longer T_1 relaxation time. This contrasts with previous observations by static ^{31}P NMR measurements on single-component phospholipid bilayers, including cardiolipin (Spooner & Watts, 1991), phosphatidylglycerol, and phosphatidylserine (Pinheiro & Watts, 1994), where the isotropic component had a shorter spin–lattice relaxation time compared with the broad spectral bilayer component, and on binding of cytochrome *c*, a marked enhancement in the phosphorus T_1 relaxation was observed for the bilayer component. The temperature variation of the phosphorus spin–lattice relaxation times for all the spectral components observed in mixed CL/PC/PE bilayers, with and without cytochrome *c*, is shown in Figure 4. A T_1 minimum can be estimated between 20 and 30 °C for the individual lipid components (Figure 4A–C),

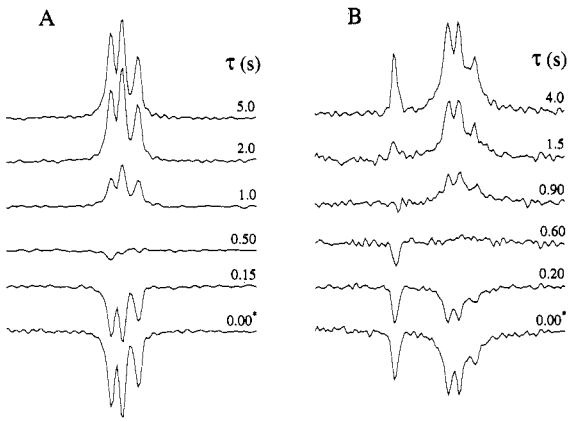

FIGURE 3: Isotropic regions of some ^{31}P MAS NMR spectra from inversion–recovery experiments used for measuring ^{31}P spin–lattice (T_1) relaxation times in CL/PC/PE (1:2:2) bilayers alone (A) and with bound cytochrome *c* (B) showing the delay times (τ) between π and $\pi/2$ pulses. The lipid:protein mole ratio was 20:1 with respect to the cardiolipin fraction. Spectra were recorded at 0 °C; spinning rates, $\omega_r \cong 1.5$ kHz; recycle times, 5 s; number of acquisitions, 4 (A) and 16 (B). (Asterisk) $\tau = 50$ μs.

being somehow better defined in the protein-containing bilayers for all three lipid phosphates than in the protein-free bilayers. The T_1 relaxation time of the "extra" isotropic spectral component from bilayers containing cytochrome *c* (see Figures 2d or 3B) has a different temperature dependence (Figure 4D) from that observed for all the other isotropic components (Figure 4A–C). The spin–lattice relaxation time of the extra component increases rapidly from −10 °C up to 0 °C, and decreases gradually above 0 °C.

The observation of a ^{31}P T_1 minimum in phospholipid membranes requires a fortunate combination of a few conditions, including a relatively high magnetic field strength, a low lipid-phase transition temperature to allow sufficient slowing of the motion to occur at lower temperatures without formation of the gel phase, and additional headgroup–headgroup interactions (Seelig *et al.*, 1981). This explains the scarce examples reported in the literature of T_1 minima in biological samples (Seelig *et al.*, 1981; Tamm & Seelig, 1983; Ghosh, 1988; Spooner & Watts, 1991; Pinheiro & Watts, 1994). The importance of the T_1 minimum rests on the fact that is not necessary to know the exact relaxation mechanism(s) in order to evaluate the correlation time (τ_c) for the molecular motion at the temperature of the minimum. The spin–lattice (T_1) relaxation time is related to τ_c according to eq 1 (Abragam, 1961), where ω_0 is the Larmor frequency. If

$$1/T_1 \propto \tau_c/(1 + \omega_0^2\tau_c^2) \quad (1)$$

the value of the molecular correlation time τ_c is such that $\omega_0\tau_c \cong 1$, then T_1 is most efficient; *i.e.*, the T_1 relaxation time is minimized. If the molecular motions are slower or faster than the Larmor frequency, the T_1 relaxation time becomes longer. As the molecular motion depends on the temperature, the variation of T_1 with temperature can exhibit a minimum if at a certain temperature the condition $\omega_0\tau_c \cong 1$ is satisfied. At this point, the relation $\tau_c \sim 1/\omega_0$ holds for all types of relaxation mechanisms. At the resonance frequency used here, a correlation time for the lipid headgroup phosphates in the mixed membranes is found at around 1 ns for the temperature of the minimum. As expected, this is a typical value for phospholipid headgroup motion in hydrated bilayers, which has been reported for bilayers of DOPC at around 0 °C (Seelig *et al.*, 1981), for POPC at around 15 °C (Tamm & Seelig, 1984), for cardiolipin at around 25 °C (Spooner & Watts, 1991), and for DOPG, DOPS, and diacyl-PI at around 15, 18, and 37 °C, respectively (Pinheiro & Watts, 1994).

The magnitude of the minimum for each lipid component in the mixed membrane containing cytochrome *c* appears to be different from those reported for the corresponding single-lipid bilayers. In the mixed CL/PC/PE bilayers of this study, the minimum for PE occurs at around 30 °C with a T_1 value of 670 ± 13 ms (Figure 4A); for PC, the minimum lies between 20 and 35 °C with a T_1 of 610 ± 20 ms (Figure 4B); for the CL component, it can be define at around 30 °C, with a T_1 value of 635 ± 18 ms (Figure 4C). The minimum for cardiolipin in bilayers alone, measured at 161.98 MHz, occurs at around 25 °C and is approximately 1 s (Spooner & Watts, 1991). For DOPC in bilayers alone, the minimum occurs at around 0 °C with a T_1 value of 1 s, measured at 121.4 MHz (Seelig *et al.*, 1981). For both PC and CL components, a decrease in the magnitude of the T_1 minimum is observed in the mixed bilayer PC/PE/CL when compared with the single-lipid bilayers. For PE in bilayers alone, no T_1 minimum data are available, since the stability for this lipid in a bilayer conformation is temperature-dependent (see below).

Chemical Shift Anisotropy. In a MAS NMR experiment, high resolution is achieved at the expense of the information contained in the chemical shift anisotropy. However, by choosing intermediate spinning speeds and with a suitable analysis of the spinning sideband intensities, the chemical shift anisotropy parameters can be obtained (Herzfeld & Berger, 1980). Spinning sideband analysis was performed for ^{31}P MAS NMR spectra of mixed CL/PE/PC bilayers with and without cytochrome *c*. The principal values of the chemical shift tensors σ_{11}, σ_{22}, and σ_{33} are arranged in the conventional assignment so that

$$|\sigma_{33} - \sigma_i| \geq |\sigma_{11} - \sigma_i| \geq |\sigma_{22} - \sigma_i| \quad (2)$$

where σ_i is the isotropic shift, and the anisotropy parameter η is defined as

$$\eta = (\sigma_{22} - \sigma_{11})/(\sigma_{33} - \sigma_i) \quad (3)$$

The principal elements of the shielding tensor of various phospholipids have been obtained from powder pattern spectra, and are all very similar with values of $\sigma_{11} = -80$, $\sigma_{22} = -20$, and $\sigma_{33} = +110$ ppm (Seelig, 1978). However, no information about the orientation of the chemical shift tensor in the molecular frame of the phosphate group can be obtained from powder-type spectra. Since phospholipid crystals of sufficient size for ^{31}P NMR measurements are not available, the orientation has been determined in crystals of the model compounds phosphoethanolamine and barium diethylphosphate (Kohler & Klein, 1976; Herzfeld *et al.*, 1978), and it is assumed that the orientation of the chemical shielding tensor of phospholipids in bilayers is the same as that observed in the model compounds. On average, the ^{31}P chemical shift tensor of a phospholipid molecule in the bilayer has its principal component σ_{11} oriented approximately perpendicular to the bilayer plane, *i.e.*, parallel to the long molecular axis, while σ_{22} and σ_{33} will be approximately coplanar to the bilayer surface. Due to the rotation about the long molecular axis (on average, normal to the bilayer surface) and additional wobbling motions of the phospholipid headgroup in a bilayer structure, σ_{11} is averaged to an effective value σ'_{11}, and σ_{22} and σ_{33} become degenerate in the bilayer plane to effective values $\sigma'_{22} = \sigma'_{33}$ (Smith & Ekiel, 1984). σ'_{11} is commonly referred to as $\sigma_\parallel$, the component parallel to the bilayer director, and $\sigma'_{22} = \sigma'_{33}$ are referred to as $\sigma_\perp$, the component

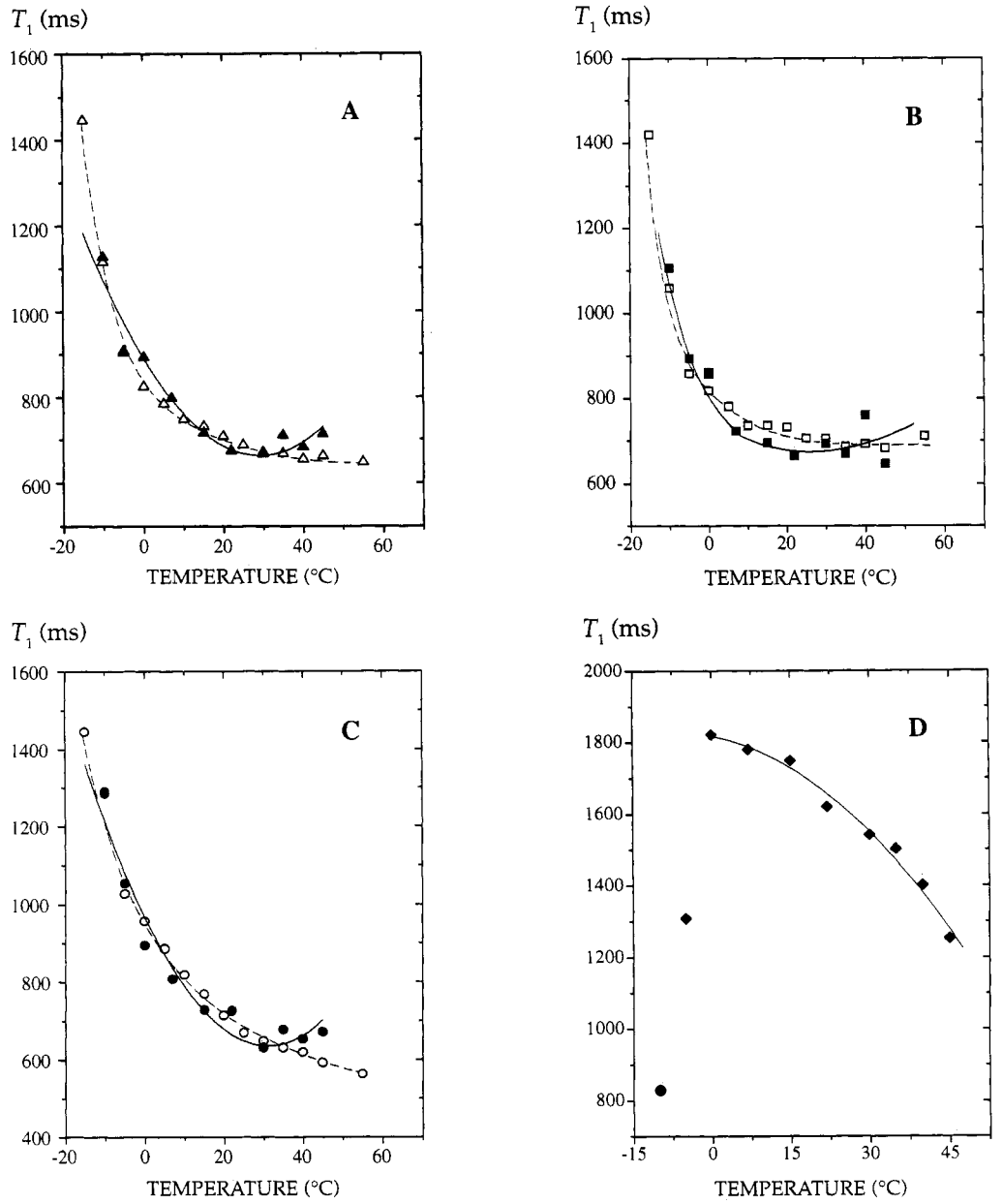

FIGURE 4: Temperature variation of the phosphorus-31 spin–lattice (T_1) relaxation time at 161.98 MHz of (A) dioleoylphosphatidylethanolamine (PE), (B) dioleoylphosphatidylcholine (PC), (C) cardiolipin (CL), and (D) an "extra" isotropic component in the model three-component mitochondrial membrane composed of PE, PC, and CL in the molar ratio 2:2:1, respectively. *Open symbols* and *dashed line* for protein-free bilayers; *filled symbols* and *straight line* for protein-containing bilayers at a lipid:cytochrome *c* molar ratio of 20:1, relative to the cardiolipin fraction.

perpendicular to the bilayer director. We will use here the nomenclature σ'_{11}, σ'_{22}, and σ'_{33} for the motionally averaged principal elements of the ^{31}P chemical shift tensor, direct outputs from the spinning sideband analysis, since in some cases σ'_{22} becomes unequal to σ'_{33} (see below).

Typical examples of the spectral simulations involved in spinning sideband analysis of the experimental MAS ^{31}P NMR spectra are shown in Figure 5 for the mixed lipid bilayers with (Figure 5B) and without cytochrome *c* (Figure 5A), where the calculated spectra for the individual phospholipid components in the mixed membrane, the total calculated spectrum, and the difference spectrum are all shown. Tables 1 and 2 summarize the chemical shift anisotropy parameters for the bilayers with and without cytochrome *c* for two temperatures. Over the temperature range from −10 to 20 °C, all lipid phosphates in the mixed bilayers without cytochrome *c* show the relations $\sigma'_{22} = \sigma'_{33}$ and $\eta = 0$, characteristic of an axially symmetric chemical shift tensor, as expected for phospholipids in a bilayer conformation (Herzfeld & Berger, 1980). On binding of cytochrome *c*, the elements σ'_{22} and σ'_{33} for the cardiolipin component become unequal for temperatures below or equal to −5 °C, indicating that the ^{31}P chemical shift tensor for this lipid has lost its axial symmetry. This is revealed in the value $\eta = 0.24$ for the CL component (Table 2), which shows appreciable deviation from axial symmetry. At 0 °C and higher temperatures, no significant differences are detected between σ'_{22} and σ'_{33} ($\eta = 0$) for any of the lipid phosphates.

The relative spectral intensities (RI) determined over all the spinning sidebands for each phospholipid component in protein-free bilayers closely reflect their respective molar proportions of CL:PC:PE = 1:2:2 (Table 1 and 2). Note that cardiolipin contains two phosphate groups per molecule, while both PC and PE have just one phosphate per lipid molecule. For the protein-containing bilayers, while the relative spectral intensities between CL and PC reflect their expected chemical proportionality, a reduction in the PE intensity is observed

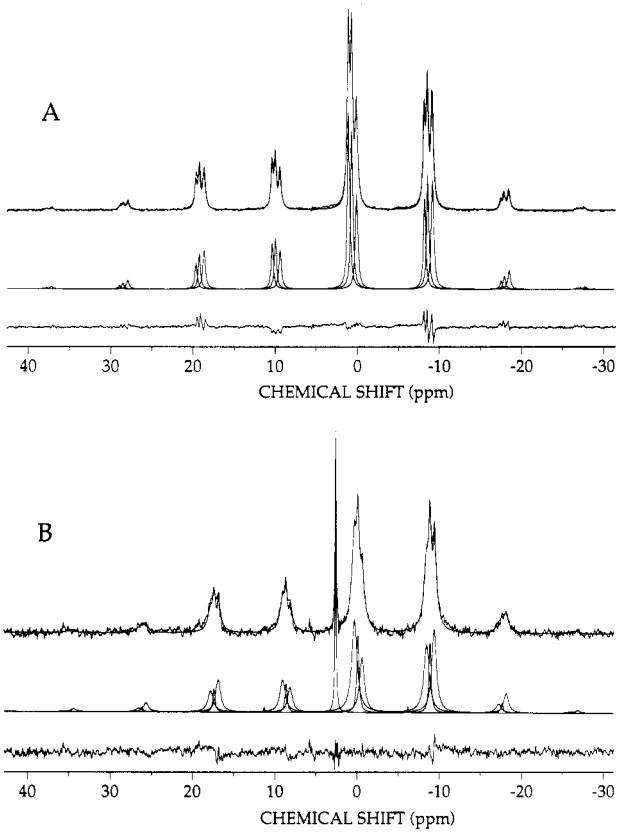

FIGURE 5: Typical examples of the spinning sideband analysis for ^{31}P MAS NMR spectra of mixed membranes containing cardiolipin (CL), PC, and PE in the molar ratio 1:2:2, respectively. (A) Protein-free bilayers: spinning rate, ω_r = 1.502 kHz; temperature, 20 °C. (B) Cytochrome *c*-containing bilayers at a lipid:protein mole ratio of 20:1 with respect to the cardiolipin component: ω_r = 1.416 kHz; temperature, −10 °C. *Top*: experimental spectrum and fit (*smooth line*). *Middle*: calculated spectra for the individual phospholipid components in the model mitochondrial membrane. *Bottom*: difference spectrum.

Table 1: ^{31}P NMR Isotropic Chemical Shifts[a] (σ_i), Relative ^{31}P NMR Intensities (RI), Motionally Averaged Principal Elements of the Chemical Shift Tensor (σ'_{ij}), Half-Height Line Width ($\Delta\nu_{1/2}$), and Anisotropy Parameter (η) for Individual Phospholipid Components in Mixed Bilayers of CL+PE+PC in the Mole Ratio 1:2:2 with and without Cytochrome *c* at 20 °C

component	RI[b] (%)	σ_i (ppm)	σ'_{11} (ppm)	σ'_{22} (ppm)	σ'_{33} (ppm)	$\Delta\nu_{1/2}$ (Hz)	η
CL+PE+PC (1:2:2)							
CL	33.7	0.395	21.8	−9.3	−9.3	53.8	0.00
PE	34.4	0.018	23.4	−10.6	−10.8	51.5	0.01
PC	31.9	−0.584	26.9	−13.2	−13.4	59.2	0.01
CL+PE+PC (1:2:2) with[c] Cytochrome *c*							
CL	34.5	0.395	19.9	−9.7	−10.1	113.3	0.02
PE	15.3	0.018	20.2	−10.2	−11.2	46.2	0.04
PC	33.7	−0.584	23.0	−12.6	−13.0	146.5	0.02
extra	16.5	2.323	7.9	−1.0	−1.2	28.3	0.04

[a] All chemical shifts are relative to external 85% H_3PO_4. [b] Integrated ^{31}P NMR intensities over all the MAS NMR spectra. [c] Lipid:protein mole ratio of 20:1 with respect to the cardiolipin fraction.

(Tables 1 and 2). However, this discrepancy is lost if the intensity of the "extra" isotropic component is taken into account, suggesting that an appreciable fraction of PE contributes to the "extra" component. Unsaturated phosphatidylethanolamines have a strong tendency to form hexagonal H_{II} phases (Cullis & de Kruijff, 1978). However, when in mixtures with other phospholipids, such as phosphatidylserine, cardiolipin (de Kruijff & Cullis, 1980), or phosphatidylcholines (Cullis & de Kruijff, 1979), PE is

Table 2: ^{31}P NMR Isotropic Chemical Shifts[a] (σ_i), Relative ^{31}P NMR Intensities (RI), Motionally Averaged Principal Elements of the Chemical Shift Tensor (σ'_{ii}), Half-Height Line Width ($\Delta\nu_{1/2}$), and Anisotropy Parameter (η) for Individual Phospholipid Components in Mixed Bilayers of CL+PE+PC in the Mole Ratio 1:2:2 with and without Cytochrome *c* at −10 °C

component	RI[b] (%)	σ_i (ppm)	σ'_{11} (ppm)	σ'_{22} (ppm)	σ'_{33} (ppm)	$\Delta\nu_{1/2}$ (Hz)	η
CL+PE+PC (1:2:2)							
CL	33.3	0.395	20.1	−9.5	−9.5	92.5	0.00
PE	34.5	0.018	21.8	−11.0	−11.0	51.3	0.00
PC	32.2	−0.584	26.5	−14.2	−14.2	59.1	0.00
CL+PE+PC (1:2:2) with[c] Cytochrome *c*							
CL	35.4	0.395	21.9	−7.9	−13.1	122.2	0.24
PE	20.8	0.018	23.6	−12.0	−12.0	52.8	0.00
PC	33.9	−0.584	27.9	−14.9	−14.9	101.1	0.00
extra	9.9	2.323	8.3	−0.2	−0.4	26.1	0.04

[a] All chemical shifts are relative to external 85% H_3PO_4. [b] Integrated ^{31}P NMR intensities over all the MAS NMR spectra. [c] Lipid:protein mole ratio of 20:1 with respect to the cardiolipin fraction.

stabilized in a bilayer conformation. The extent of this stabilization is dependent on the relative proportions of the lipids in the mixture, on the temperature, and on the external ligands. In particular, ^{31}P NMR studies have shown that on increasing the concentration of PC in mixtures with PE, the hexagonal H_{II} phase of PE alone (0 mol % PC) is completely converted into a bilayer structure at 50 mol % PC (Cullis & de Kruijff, 1979). For intermediate PC concentrations of 15 and 30 mol %, ^{31}P NMR spectra show a single isotropic spectrum and an overlapped isotropic component on a bilayer-type spectrum, respectively. Furthermore, addition of cations, polylysine, or cytochrome *c* results in destabilization of the bilayer conformation of PE in lipid mixtures, and formation of isotropic and hexagonal phases, as revealed by ^{31}P NMR (de Kruijff & Cullis, 1980; Cullis & Verkleij, 1979). In the pesent study, the bilayer conformation of CL/PC/PE mixtures in the molar ratio 1:2:2, respectively, was preserved over the entire temperature range from −10 to 55 °C. However, on addition of cytochrome *c*, an "extra" isotropic spectral component overlapping the bilayer-type spectrum is observed (Figure 2c), with an intensity similar to the reduction of PE in the bilayer conformation (Tables 1 and 2), suggesting that some segregation of PE arises on binding of cytochrome *c* to the three-component CL/PC/PE bilayers. This is also supported by the effects induced by cytochrome *c* on the half-height spectral line widths ($\Delta\nu_{1/2}$), also listed in Tables 1 and 2 for each ^{31}P resonance from the three lipid phosphates. While Figure 2d has suggested that the binding of cytochrome *c* to the mixed model mitochondrial membrane introduces a substantial line broadening, apparently in all lipid resonances, the spinning sideband analysis revealed that at any temperature the line broadening (evaluated by $\Delta\nu_{1/2}$) arises only in two of the spectral components, those being the CL and PC components in the mixed membrane (Tables 1 and 2). In contrast, the PE spectral component did not show any line broadening at any temperature on binding of cytochrome *c*.

DISCUSSION

The binding of cytochrome *c* to anionic phospholipid bilayers has been shown to induce some destabilization of the protein structure. On complexes with single-component phospholipid bilayers of cardiolipin (Spooner & Watts, 1991), phosphatidylserine (DOPS), or phosphatidylglycerol (DOPG) (Pinheiro & Watts, 1994), structural perturbations in cytochrome *c* have been detected via a paramagnetic enhancement of the

^{31}P spin–lattice relaxation of the lipid phosphorus in lipid–protein complexes. This is a manifestation of the formation of a high-spin state of the heme iron in cytochrome *c* induced upon binding to anionic lipid surfaces. The electrostatic interaction between the charged lysine residues surrounding the heme crevice and the negatively charged headgroups at the bilayer surface promotes the disruption of the sixth coordination to the heme iron (Met-80). This induces the formation of a five-coordinated high-spin heme iron in the protein. High-spin ferricytochrome *c* has been proposed as a labile intermediate formed during complexation with small anionic ligands (Sutin & Jandell, 1972). Surface-enhanced resonance Raman spectroscopy has shown that the binding of cytochrome *c* to anionic electrode surfaces (Hildebrandt *et al.*, 1989a,b) and anionic phospholipid bilayers (Hildebrandt *et al.*, 1990a; Heimburg *et al.*, 1991) induces the disruption of Met-80 to the heme iron and a high-spin form is detected, whereby a dynamic conformational equilibrium is established between a low-spin six-coordinated heme iron and a high-spin five-coordinated heme iron in cytochrome *c*. This process seems to be controlled by the electrostatic interaction between the positively charged lysine residues in the protein surface and the negatively charged phospholipid headgroups in a membrane surface.

In contrast to the ^{31}P spin–lattice relaxation effects observed for single-component phospholipid bilayers, including cardiolipin bilayers, no significant relaxation enhancements were observed on the ^{31}P spin–lattice relaxation for any of the phospholipid components in the mitochondrial model membrane studied here (Figure 4A–C). Compared to the single-lipid component cardiolipin bilayers, the three-component (PC, PE, CL) mitochondrial model membrane has a reduced bilayer surface charge density. The negatively charged cardiolipin headgroups are now diluted among the zwitterionic headgroups of phosphatidylcholine and phosphatidylethanolamine. This dilution of charge density at the bilayer surface reduces the electrostatic interaction between the protein and the negative charges in the membrane, which seems to inhibit the changes in the protein conformation that lead to the high-spin intermediate form of cytochrome *c*, such as observed on binding to single-lipid negatively charged bilayer surfaces of cardiolipin (Spooner & Watts, 1991), DOPS, or DOPG membranes (Pinheiro & Watts, 1994).

In previous studies, it was observed that the paramagnetic enhancement in the ^{31}P spin–lattice relaxation of the lipid headgroup in cytochrome *c*–phospholipid complexes increases with temperature (Spooner & Watts, 1991; Pinheiro & Watts, 1994). This suggested a progressive opening of the heme crevice, making the heme iron gradually more accessible to the interaction with the phosphate in the lipid headgroup. Recalling basic NMR concepts, the dipolar interaction (D) between the ^{31}P nuclear spin with the electron spin in the heme iron decays rapidly with the distance between the two interacting spins ($D \propto 1/r^3$). Furthermore, the dipolar interaction between two spins depends on the *magnitude* and *orientation* of their magnetic moments, and also on the *orientation* of the vector describing their relative positions. The distance r is initially determined by the electrostatic interaction between the negatively charged bilayer surface and the positive residues in the protein surface. As the temperature increases, the phosphate has greater accessibility to the heme iron due to the opening of the heme crevice, and the closest approach between the heme iron and the lipid phosphate group is ultimately determined by the steric properties of the phospholipid headgroup. This was supported by the absence of an enhancement in the ^{31}P spin–lattice relaxation for phosphatidylinositol–cytochrome *c* complexes (Pinheiro & Watts, 1994), in which the large headgroup moiety could not be accommodated closely enough in the heme crevice within the limits of maximal aperture before thermal denaturation of the protein takes place. In the present study, the lower surface charge density of the mixed CL/PE/PC bilayers, when compared with that of a single lipid negatively charged membrane, may result in a less compact binding state for the lipid–cytochrome *c* complex, *i.e.*, a larger r value between the two interacting spins. Thus, over the whole temperature interval analyzed here, the phosphate group does not come close enough to the heme iron to sense its paramagnetic effect.

Alternative arguments involving different orientations of cytochrome *c* between complexes with mixed- and single-component lipid bilayers are equally possible to explain the absence of interaction between the lipid phosphorus and the protein heme iron in mixed CL/PE/PC bilayers, when compared to that observed for protein complexes with single-lipid bilayers. Indeed, despite the almost spherical shape of cytochrome *c*, the electric potential field around the protein in asymmetric. A dipole electric moment of 325 D has been measured for ferric cytochrome *c* (Koppenol & Margoliash, 1982), which arises mainly due to the inhomogeneous distribution of the positive charges (mostly lysine residues) on the protein surface (Dickerson *et al.*, 1971). It has been proposed that the existence of this dipole moment causes cytochrome *c* to reorient itself in the electric field of its redox partners (Hildebrandt *et al.*, 1990b), or at the membrane surface (Vik *et al.*, 1988). The absence of paramagnetic enhancement of the lipid phosphorus T_1 relaxation for any of the lipid components in the mixed mitochondrial membranes studied here may also suggest that the protein could be bound in a different orientation than that in single-component anionic lipid bilayers, for which noticeable effects on the ^{31}P T_1 relaxation were detected (Spooner & Watts, 1991; Pinheiro & Watts, 1994). It is possible that in complexes with purely negative phospholipid bilayers, such as cardiolipin, DOPG, or DOPS, cytochrome *c* is bound through the front lysine patch surrounding the exposed heme edge, which permits the interaction between the lipid phosphates and the heme iron, therefore inducing a ^{31}P T_1 relaxation enhancement. In contrast, with the mixed bilayers of reduced surface charge density (negatively charged CL diluted among the zwitterionic PC and PE headgroups), the binding may occur through a different group of lysines, which no longer results in an efficient dipolar interaction between the ^{31}P nuclear spin and the transient electron spin in the heme iron. At present, we cannot exclude either of these possibilities, involving either *distance* or *orientation* arguments for the nonaccessibility of the heme iron to interaction with the lipid phosphates. Nonetheless, if changing the electric field at the membrane surface can lead to binding of cytochrome *c* in a different orientation, this could constitute a relevant mechanism for the interaction of cytochrome *c* in the inner mitochondrial membrane, in which reorientation of the protein may be modulated by fluctuating electric fields at the membrane surface prior to complexation with its redox partners. These fluctuating membrane surface electric fields could be generated by inhomogeneous lateral lipid distribution induced by the embedded integral proteins in natural membranes.

While the ^{31}P relaxation effects are directly associated with the immediate environment of the heme iron and its accessibility to the lipid phosphate, measurements of thermal denaturation of cytochrome *c* in lipid complexes have revealed

that the protein destabilization involves a more extensive structural perturbation of the entire protein molecule, being interpreted as a loosening of the overall protein tertiary structure (Pinheiro & Watts, 1994). Differential scanning calorimetry of the thermal denaturation of cytochrome *c* bound to the mixed bilayer CL/PC/PE (1:2:2) (data not shown) revealed a very broad endotherm (ΔT = 33 °C) with a peak temperature (T_p) of the transition decreased by about 11 °C, when compared with the value for the protein in buffer solution. However, the decrease in the thermodynamic parameters, T_p and ΔH^{cal}, is smaller than those observed for the thermal denaturation of cytochrome *c* complexes with single anionic phospholipid bilayers (Pinheiro & Watts, 1994), suggesting that destabilization of the protein structure occurs upon binding to the model mitochondrial membrane, but to a smaller extent than when in the single-component lipid bilayers. In a related ^{31}P NMR study of cytochrome *c* complexes with two-component lipid bilayers containing cardiolipin and DOPC (Spooner & Watts, 1992), no enhancement of the ^{31}P spin–lattice relaxation for the cardiolipin component was reported, in contrast with the results observed for the complexes of cytochrome *c* with single-component lipid bilayers (Spooner & Watts, 1991; Pinheiro & Watts, 1994). However, some perturbation of the cardiolipin component by the protein in mixed DOPC/CL bilayers may occur, in agreement with suggestions that possible phase separation of cardiolipin in mixed lipid membranes is induced upon binding of cytochrome *c*, as shown by spin-label ESR (Birrel & Griffith, 1976) and ^{1}H NMR studies (Brown & Wüthrich, 1977). Based solely on the relative intensities of the isotropic resonances in the central band of ^{31}P MAS NMR spectra of DOPC/CL bilayers with and without cytochrome *c*, a reduction in the chemical shift anisotropy of the cardiolipin component in this binary bilayer was predicted, indicating some selective influence of cytochrome *c* on CL. However, it was recognized that to support this interpretation a detailed spinning sideband analysis was lacking.

In the present study, we have carried out a complete analysis of the rotational sidebands for the individual phospholipid components in the mixed CL/PC/PE bilayers with and without cytochrome *c* at various temperatures. The analysis provides important insight into the interaction of cytochrome *c* with the individual lipid components in the mixed CL/PC/PE bilayers through (i) evaluation of the chemical shift anisotropy parameters for each lipid in the mixed membrane, (ii) individual line-broadening effects, and (iii) a quantitative assessment of the relative spectral intensities. In the mixed bilayers without protein, the chemical shielding tensor for all of the lipid components is axially symmetric, as expected for phospholipids in a bilayer conformation, and that was preserved at all temperatures. On binding of cytochrome *c*, the chemical shift tensor for the cardiolipin fraction ceases to be axially symmetric at low temperatures (–10 and –5 °C), while for the other lipids (PC and PE) the axial symmetry is retained through all temperature intervals studied here. Changes in the ^{31}P CSA of phospholipids in membranes seem to reflect changes in the wobbling motions that tilt the lipid headgroup, while the line broadening is caused by a slowing down of the molecular reorientation about the bilayer normal (Rajan *et al.*, 1981). The effects observed here on spectral line broadening (Tables 1 and 2) suggest that for both CL and PC fractions the reorientation of the entire lipid molecule about the long axis (normal to bilayer surface) is reduced on binding of cytochrome *c*. In contrast, only the chemical shift anisotropy for cardiolipin is affected upon protein binding. This selective influence of cytochrome *c* on CL suggests that the expected stronger electrostatic interaction between this negatively charged lipid headgroup (z = –2, from two ionized phosphate groups) and the positive lysine residues at the protein surface is probably affecting the tilting motions of the headgroup segment. However, this selective interaction seems to vanish with the increase of temperature, suggesting that at higher temperatures (>–5 °C) cytochrome *c* may be in fast exchange between PC and CL binding sites, and therefore only an averaged line broadening is detected. For lower temperatures (≤–5 °C), the protein–lipid exchange rate becomes slower, and differences between the two protein-bound headgroup moieties can be detected. The results indicate that the axial symmetry averaging of the ^{31}P chemical shift tensor for the cardiolipin headgroup phosphates is perturbed, while the PC phosphate is not affected, preserving its axial symmetry. This suggest a stronger interaction of cytochrome *c* with cardiolipin in the mixed model mitochondrial membrane containing PC, PE, and CL.

^{31}P NMR of mixed phospholipid bilayers containing phosphatidylethanolamines has shown the presence of a ^{31}P spectral isotropic component dependent on the temperature, the lipid proportion in the mixed membrane, and the external ligands (Cullis & de Kruijff, 1978, 1979). Quantitative analysis of the relative intensities (RI) in the spectra of each component for the mixed bilayers with and without cytochrome *c* (Tables 1 and 2) provides a strong indication that the protein can induce some phase separation of the various lipids in this multicomponent bilayer. The PE bilayer intensity is reduced from 34.4% in the absence of protein to 15.3% with protein, with the new isotropic component containing the remaining 16.5% of PE signal intensity. Phosphatidylethanolamine, which by itself would adopt a hexagonal H_{II} phase (Cullis & de Kruijff, 1978), has been "forced" into a bilayer conformation due to the intermolecular interactions with CL and PC. On addition of cytochrome *c*, this stabilizing factor is disrupted due to the involvement of those lipids, in particular cardioipin, in the interaction with the protein. PE molecules may be excluded from the heterogeneous mixture with CL and PC into intermediate structures, which give rise to an isotropic signal in the ^{31}P NMR spectrum. Such isotropic spectral components have been attributed to vesicular or micellar structures in or on the bilayer, and could be associated with precursors of hexagonal H_{II}-phase formation (Farren & Cullis, 1980). However, no evidence for the presence of hexagonal phases could be detected here in the spinning sideband patterns. The isotropic component superimposed on the broad bilayer spectrum (Figure 2c) is averaged out in a single central isotropic line in the corresponding MAS spectrum (Figure 2d), and its line broadening is not affected on binding of cytochrome *c*. Furthermore, the long spin–lattice relaxation times for the extra isotropic component are in good agreement with reported T_1 values for PE in PC/PE mixed vesicles (Yeagle *et al.*, 1976), where ^{31}P T_1 relaxation times are reported for various phospholipids in vesicles. While the majority of lipid types show a T_1 on the order of 1 s, the phosphorus in PE has a distinctly larger value of 2.2 s, attributed to strong intermolecular interactions. The results obtained here, either from the spinning sideband analysis or from the spin–lattice relaxation behavior, provide strong evidence for lipid lateral-phase separation in the mixed CL/PE/PC bilayers induced upon the binding of cytochrome *c*. In this process, PE seems to be segregated into isotropic structures, as monitored by the ^{31}P NMR time scale. It is also clear that protein interaction does occur with cardiolipin,

as revealed by the effects on the chemical shielding tensor at low temperatures. However, the results at higher temperatures suggest that the interaction is averaged by protein diffusion at the membrane surface and is thus translated to the PC component in the bilayer, possibly only to the extent of affecting the rate of its long-axis rotation and not sufficient to affect the headgroup conformation, since no changes in the anisotropy of the PC chemical shielding tensor are induced on protein binding. The difference in protein binding to mixed- rather than single-component charged bilayers is suggested to be due either to a different orientation of the protein on the two types of bilayer or to differences in the distance of the protein from the bilayer, either of which may have significance for the function of cytochrome *c* in vivo, where the heme in the protein is required to present itself in a specific way to the redox partners (integral membrane proteins) for efficient electron acceptance and donation.

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