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Association of ferri- and ferro-cytochrome *c* with lipid multilayers: a ^{31}P solid-state NMR study

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The ^{31}P nuclear magnetic resonance anisotropies of dispersions of diacylphosphatidic acid and diacylphosphatidylserine were slightly increased in the presence of cytochrome *c*: no increase was observed with cardiolipin. However, the ^{31}P spin-lattice relaxation times (T_1) for all of these lipids were reduced markedly by the protein. As similar effects were observed with ferri-cytochrome *c* and with the reduced protein, which is diamagnetic, we suggest that the changes in T_1 reflect a reduction in the spectral density of fast motions for the lipid headgroups attendant on binding of protein, rather than paramagnetic relaxation of the phosphorus nuclear spin.

Cytochrome *c* is distinguished from other cytochrome components of the respiratory chain in being readily washed out from swollen mitochondria at high ionic strengths. Since phospholipids have long been considered as the major binding site of cytochrome *c*, this observation is consistent with it being an extrinsic membrane protein bound by a primarily electrostatic interaction to the phospholipid bilayer on the cytosolic side of the inner mitochondrial membrane [1]. It has been proposed [2–4] that association with lipids may allow the protein to diffuse within the plane of the membrane and thereby to transfer electrons during collisions on the membrane surface. Recently Mitchell and Moyle [5] have further proposed that the nature of the interaction between lipid and protein changes on oxidation of the protein, and that this change is accompanied by a release of protons that is in part responsible for establishing the transmembrane proton gradient that drives the mitochondrial ATPase.

Interactions of cytochrome *c*, mostly of ferricytochrome *c*, with lipid monolayers and bilayers have been widely studied (reviewed in Refs. 6–8) but concensus on the nature of the interaction has not been reached. Some experiments have been interpreted as showing partial penetration of the protein into bilayers (see, for example Refs. 9 and 10), whereas others have lead to a contrary conclusion [11–13]. Evidence also exists for differences in the modes of interaction of oxidized and reduced cytochrome *c* with lipids. From measurements of bilayer thickness, the effectiveness of sodium chloride in releasing the protein from lipids, and the absorption spectrum of the proteins, Letellier and Shechter [14] have concluded that ferri- and ferro-cytochrome *c* interact differently with cardiolipin, the former binding electrostatically and the latter partly hydrophobically.

Abbreviations: diacylPA, diacylphosphatidic acid; diacylPS, diacylphosphatidylserine; CSA, chemical shift anisotropy; FID, free induction decay.

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However, others have described ferri-cytochrome *c* as also binding to diacylphosphatidic acid (diacylPA) dispersions through both electrostatic and hydrophobic interactions [15].

We have used binding measurements and ^{31}P-NMR to explore further the interaction of oxidized and reduced cytochromes *c* with multilayer dispersions of three acidic lipids; cardiolipin, diacylPA, and diacylphosphatidylserine (diacylPS).

DiacylPA was prepared by digestion of egg diacylphosphatidylcholine with phospholipase D, and purified as previously described [16,17]. DiacylPS was prepared from bovine brain [18]. The sodium salt of bovine heart cardiolipin, and horse heart cytochrome *c* (Type III) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. The lipids were better than 95% pure as assessed by TLC of 50–100 μg in chloroform/methanol/ammonia (65 : 25 : 4, by vol.) and chloroform/methanol/water (65 : 25 : 5, by vol.). Cytochrome *c* was reduced in 5 mM sodium ascorbate.

Unilamellar lipid vesicles were formed using a 200 watt bath sonicator. Lipids were dried under vacuum for several hours, a few ml of buffer (0.1 M Tris-HCl, 1 mM EDTA, 2 mM sodium azide, pH 7.5 measured before and after the addition of protein) were added, and the contents sealed under nitrogen prior to sonication. The binding of proteins to these lipid vesicles was measured by separation of unbound protein from the lipid-protein complexes by centrifugation in a 0.5–50% (w/v) linear sucrose gradient at 100 000 × *g*. Free protein, which remained at the top of the gradient, was assayed colorimetrically [19]. The phosphorus contents of fractions from the gradients were measured by the method of Bartlett [21].

Dispersions were prepared by addition of 5–10 ml of the Tris-HCl buffer, or solutions of protein in buffer, to 50–150 mg dried lipid. Vortexing and repeated freeze-thaw cycles under nitrogen were used to mix the lipid and protein, and minor adjustments to the pH made when necessary. The protein-lipid complexes were sedimented by centrifugation at 85 000 × *g* for 8–16 h. The pellets were sealed under nitrogen in plastic Beam electronmicroscopy embedding capsules. ^{31}P-NMR spectra were recorded at 121.44 MHz on a Bruker CXP300 spectrometer using 90° free induction decay or ($90° \sim \tau \sim 180° \sim \tau \sim$ FID) pulse sequences, with a 90° pulse duration of 8–10 μs, a repetition time of 3 s, and 25 kHz (a proton 90° pulse length of 10 μs) of proton decoupling field. Chemical shifts were measured relative to phosphoric acid. Spin lattice relaxation times (T_1) were obtained by the inversion-recovery method with a waiting time of approximately 5 T_1 between pulses.

For comparison of peak intensities, pulses were separated by at least 4 T_1 to ensure full relaxation between pulses. The proton decoupling was gated to remove the ^{31}P (^{1}H) nuclear Overhauser effect [20]. The spectra were recorded under identical machine conditions with the whole sample contained within the receiver coil. Subsequently the samples were redispersed, with the addition of methanol, or of sodium dodecyl sulphate to a final concentration of 10% (w/v), to obtain an even dispersion, and the total lipid content measured by triplicate phosphate analyses [21]. Peak intensities were obtained by cutting and weighing photocopies of the spectra. Multiple measurements indicated a variety of less than 4% in intensity when this procedure was followed. Multiple standard determinations demonstrated that the resonance intensity observed was proportional to the amount of phospholipid. All spectra were obtained at 25 ± 2°C. The chemical shift anisotropies were determined by comparison with simulated spectra.

The binding of cytochrome *c* to a fixed weight of each of the three lipids is shown in Fig. 1. For diacylPA and diacylPS vesicles, the binding of the oxidized protein reached a plateau at 2 and 1.5 g protein/g lipid, respectively, corresponding to a minimum of 8–12 moles phospholipid/mole protein. Less ferro-cytochrome *c* was bound to both of these lipids, in accord with previous reports [22,23]. Cardiolipin bound substantially more ferri-cytochrome *c* than diacylPS and diacylPA, and showed a greater discrimination between the oxidized and reduced proteins. At saturation there were about four molecules of cardiolipin per ferri-cytochrome *c*, in agreement with the results of Azzi et al. [24]. 100 mM sodium chloride reduced the binding of both forms of the protein to cardiolipin by 20% (data not shown), a result that is consistent with a predominantly ionic interaction.

The slightly lower charge on the reduced pro-

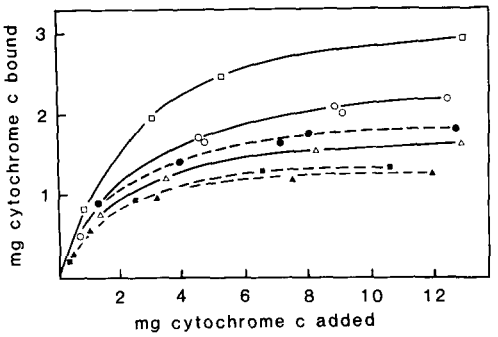

Fig. 1. Binding of ferri- (open symbols) and ferro- (filled symbols) cytochrome *c* to vesicles of diacylPA (○, ●), diacylPS (△, ▲), and cardiolipin (□, ■). The data are presented for 1 mg samples of lipid.

tein may in part be responsible for its lower affinity for lipids. It has also been proposed [14] that the difference may result from the adoption of different conformations by the two forms of the protein in the presence of lipids. In support of the latter explanation, more apocytochrome *c* than ferri-cytochrome *c* binds to lipids, even though the apoprotein has a lower net charge [9].

The ^{31}P chemical shift anisotropies of diacylPA and diacylPS, but not of cardiolipin, were increased by ferri- and ferro-cytochrome *c* (Table I). Comparable increases have been observed with several other basic proteins in dispersions with acidic lipids [25]. Fig. 2 shows typical spectra for diacylPA and cytochrome *c*/diacylPA dispersions prepared with reduced and oxidized protein: similar results were obtained using diacylPS and cardiolipin. The simplest, but not unique, explanation for the increased chemical shift anisotropy is that the protein increases the order of the lipid phosphate group. Some samples gave spectra containing, in addition to the lamellar component, a small isotropic peak, as observed also by De Kruijff and Cullis [26] who assigned this peak to a small population of inverted micelles within the bilayers. These authors also observed that addition of cytochrome *c* to preformed vesicles sometimes led to the formation of an hexagonal phase. In the current work all mixed dispersions were prepared by hydration of the lipid in the presence of protein, and none showed hexagonal phase components in the NMR spectra.

Addition of calcium ions to bilayers of negatively charged lipids causes a marked reduction in the apparent spectral intensity (Fig. 3). Calcium forms a complex with the phosphate groups in which the latter are greatly restricted in motion [27], causing the phosphorus chemical shift anisotropy to approach the rigid lattice values of 192 ppm (diacylPS), and 88 ppm (diacylPA) [27,28].

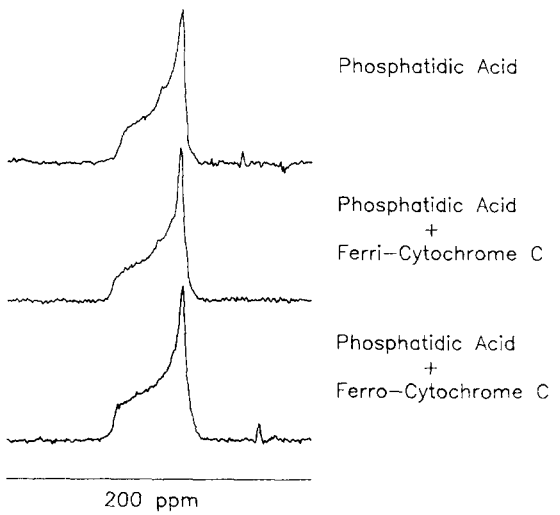

Fig. 2. ^{31}P-NMR spectra of dispersions of diacylPA in the absence and presence of oxidized and reduced cytochrome *c*. About 600 scans were accumulated at 298 K using 60–70 mg lipid. The spectra were plotted using 25 Hz line broadening. In the dispersions containing cytochrome *c* the protein/lipid weight ratio was 0.35 ± 0.05.

TABLE I

^{31}P CHEMICAL SHIFT ANISOTROPIES (CSA) FOR MULTILAMELLAR DISPERSIONS OF PHOSPHOLIPIDS WITH CYTOCHROME *c*

Values given are the mean and standard deviation from the number of measurements in parenthesis.

Sample	CSA (ppm)
DiacylPS	-47 ± 2 (5)
DiacylPS/ferri-cytochrome *c*	-51 ± 1 (5)
DiacylPS/ferro-cytochrome *c*	-51 ± 2 (5)
DiacylPA	-37 ± 2 (6)
DiacylPA/ferri-cytochrome *c*	-43 ± 3 (5)
DiacylPA/ferro-cytochrome *c*	-43 ± 2 (5)
Cardiolipin	-25 ± 1 (5)
Cardiolipin/ferri-cytochrome *c*	-25 ± 1 (6)
Cardiolipin/ferro-cytochrome *c*	-26 ± 1 (6)

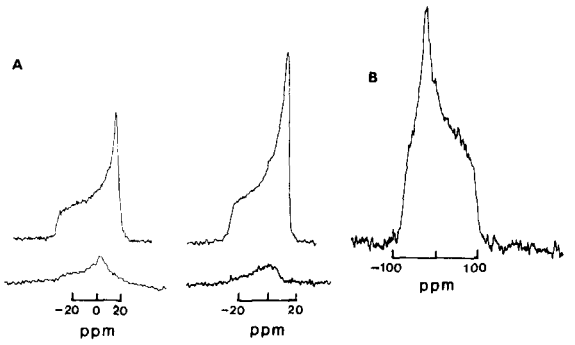

Fig. 3. (A) ^{31}P-NMR spectra of dispersions of diacylPS (left) and diacylPA (right) in the presence (bottom) and absence (top) of equimolar amounts of Ca^{2+}. Each set of spectra was accumulated using identical spectrometer settings and amounts of lipid. Chemical shifts are relative to the resonance positions of unilamellar vesicles of diacylPA at pH 7.5. The sweep width was 60 kHz. (B) ^{1}H-^{31}P cross-polarization spectrum of diacylPS with equimolar Ca^{2+} in 0.1 M Tris-HCl (EDTA-free) buffer at pH 7.5. This spectrum was recorded at 287 K using a contact time of 1 ms, and 4400 90° pulses with a repetition time of 2 s. A 9.5 μs ^{1}H pulse was used.

Cross-polarization from protons greatly enhanced the ^{31}P spectrum, revealing the rigid lattice powder pattern for an axially asymmetric shielding tensor typical of an immobilized phospholipid (Fig. 3B).

As basic proteins could also cause a loss of intensity as a result of severely broadening the spectrum of some lipid molecules, measurements of the proportion of phospholipid contributing to the NMR signal were attempted. Although a linear relationship was demonstrated between total lipid in protein-free samples and the integrated resonance intensities, occasional inconsistencies, which could not be ascribed to errors in the phosphate analyses, make us cautious in using this technique to measure small intensity losses. However, with several basic proteins, including lysozyme and myelin basic protein, we have seen no evidence of intensity losses in diacylPA or diacylPS dispersions. Similarly with diacylPS and cardiolipin dispersions containing ferro- or ferricytochrome *c* all of the phosphorus appears to contribute to the NMR spectrum. Dispersions of PA containing reduced or oxidized cytochrome sometimes exhibited intensity losses of 10–20%, exceeding the errors arising from peak integration and phosphate analysis. But some samples showed no such losses and even the greatest intensity loss was far below the loss of over 80% observed with dispersions of diacylPS and diacylPA containing equimolar amounts of Ca^{2+}. We therefore conclude that cytochrome *c* does not immobilize a significant number of lipid molecules to the extent that they are not detected when using the 90°-FID pulse sequence.

Both ferri- and ferro-cytochrome *c* reduced the spin-lattice relaxation times of the phosphorus atom in dispersions of diacylPS, diacylPA, and cardiolipin (Table II). No anisotropy in T_1 of the lamellar component was evident. Observation of a single T_1 and chemical shift anisotropy implies that lipid molecules move in and out of the sphere of influence of the protein molecules at a rate that is fast on the ^{31}P-NMR timescale (i.e. with a correlation rate $< 10^3$ s^{-1}). The T_1 values given in Table II are therefore ensemble averages, and the T_1 values of molecules bound to cytochrome *c* must be smaller than the observed values. T_1 always increased with increasing temperature.

Following the NMR experiments the oxidation state of the protein was examined by visible spectroscopy. The dispersions (10–80 mg lipid) were sonicated for a few minutes in a bath sonicator, with the samples under nitrogen in 3–5 ml of the Tris-HCl buffer, to form small vesicles and thus diminish the light scattering. After recording the spectrum from 600 nm to 380 nm, small aliquots of 0.25 M sodium ascorbate (at pH 7.6) and 0.3 mM phenazine methosulphate were added to give final concentrations of 4.5 mM and 1 μM, respectively, and the spectrum was recorded again after 0.25–3 h incubation at room temperature. The ferro-cytochrome *c*/lipid dispersions showed no change in A_{550}/A_{540} on addition of reducing agent, indicating that the protein had been fully reduced in the samples used for the NMR studies [29,30]. As the iron atom in the reduced protein has a d^6 low spin electrogenic configuration, and is therefore diamagnetic, the reductions in T_1 cannot be attributed to paramagnetic relaxation of the phosphorus nuclear spin: they are possibly a result of restrictions in headgroup motion that lower the spectral density of fast motions (on the 10^{-9} s timescale) of the phosphorus atoms. Such restrictions could be caused by binding of the monoester headgroup of diacylPA and the phosphodiesters of diacylPS and cardiolipin to the phosphate ion

TABLE II

^{31}P SPIN-LATTICE RELAXATION TIMES (T_1) FOR MULTILAMELLAR LIPID DISPERSIONS WITH CYTOCHROME *c*

Sample	T_1 [a] (ms)	Protein/lipid weight ratio	Lipid/protein mole ratio [b]
DiacylPS	1140 ± 180 (11) [c]		
DiacylPS/ferri-cytochrome *c*	693 ± 102 (3) [c]	≈ 0.92	≈ 17
DiacylPA	1150 ± 100 (2)		
DiacylPA/ferri-cytochrome *c*	650	0.25	63
	570	0.32	49
	545	0.61	26
DiacylPA/ferro-cytochrome *c*	560	0.28	56
	296	0.37	42
Cardiolipin	970 ± 60 (2)		
Cardiolipin/ferri-cytochrome *c*	465	0.55	18
	232	0.67	14
Cardiolipin/ferro-cytochrome *c*	556	0.47	20
	496	0.54	18

[a] Values given are the mean and standard deviation from the number of measurements in parenthesis.
[b] Assuming lipid molecular weights of 700, 800 and 1300 for diacylPA, diacylPS, and cardiolipin, respectively.
[c] From Ref. 24.

binding site of cytochrome *c* [31].

Our NMR studies have shown no profound effects of protein oxidation state on the mode of lipid binding, though the binding measurements indicate, as others have found, that more of the oxidized protein binds to vesicles formed from acidic lipids. Cytochrome *c* has little effect on the ^{31}P-NMR anisotropy of diacylPA or cardiolipin. Similarly, this protein has been reported to cause only slight changes in the ^{2}H spectra of dimyristoylPS labelled in the headgroup or acyl chain and co-dispersed with dimyristoylPC, and in the ^{31}P spectra of these dispersions or dispersions of pure diacylPS (Ref. 25 and unpublished work cited in Refs. 8 and 32). However, cytochrome *c* has been reported to alter the ^{31}P spectrum of cardiolipin-diacylphosphatidylethanolamine dispersions [27]. Several other basic proteins have also been shown to reduce the ^{31}P T_1 of diacylPA or diacylPS (Table II and Ref. 25), though their effects are smaller, even at higher protein/lipid ratios. This behaviour may be contrasted with that of calcium which has been reported to increase the T_1 of diacylPS from 0.5 ± 0.1 s to 16 ± 3 s [28].

Interactions at the lipid/water interface clearly have pronounced effects on the state of lipid, inducing partial (with proteins) or virtually complete (with calcium) immobilization of the polar segments of the lipids. In addition, protons and myelin basic protein [17], have been shown to induce changes in phase. The recent studies [33] of the binding of the pentapeptide, pentagastrin, and its analogues have further emphasized the subtlety of the interactions between water soluble peptides or proteins, and lipids.

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