Interaction of Cytochrome *c* with Mixed Dimyristoylphosphatidylcholine–Dimyristoylphosphatidylserine Bilayers: A Deuterium Nuclear Magnetic Resonance Study

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ABSTRACT: Deuterium nuclear magnetic resonance (^{2}H NMR) was used to study the interaction of cytochrome *c* (from horse heart) with bilayers of mixed dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS). Three types of labeled lipids were used: chain-perdeuterated phosphatidylcholine (DMPC-d_{54}), chain-perdeuterated phosphatidylserine (DMPS-d_{54}), and phosphatidylserine labeled at the α-position of the head group (DMPS-d_2). Liposomes containing equimolar mixtures of DMPC and DMPS were found to bind cytochrome *c* with a maximum ratio of about 1 mg of cytochrome *c* per 1 mg of DMPS. The ^{2}H NMR spectra of equimolar mixtures of DMPC-d_{54}–DMPS and DMPC–DMPS-d_{54} were examined with and without cytochrome *c*. No change of the NMR spectra of either DMPC or DMPS could be detected after protein addition, for temperatures both above and below the phospholipid phase transition region. On the other hand, in the liquid-crystalline state, the transverse relaxation time, T_{2e}, was reduced by 30–40% after protein addition. Measurements of the spin–lattice relaxation time, T_1, showed, under all circumstances, multiple components. For simplicity, we have examined the shape of the relaxation curves at short and long times. Addition of protein increased by 2-fold the value of the slow T_1 component of DMPS-d_{54} but not that of DMPC-d_{54}. Partially relaxed spectroscopy allowed us to assign this slow component (at least in part) to the methyl group and C^2H_2 groups near the methyl end of the chains, i.e., far from the binding sites of the extrinsic protein. The short-time behavior of the relaxation curves was not affected substantially by protein addition. The results for the head-group-labeled lipids contrasted with the above results. DMPS-d_2 in a 1 to 1 mixture of DMPC and DMPS gave two quadrupolar splittings of 3.3 and 10.0 kHz at 307 K. Protein binding left the smaller splitting unaffected but increased the larger splitting by 15%. Similar effects were observed if the mole ratio of DMPC to DMPS was 1:1, 5:1, or 10:1, but the change in the larger splitting decreased as the mole ratio of DMPC to DMPS increased. Upon protein addition, neither T_{2e} nor T_1 was affected substantially. These results suggest that cytochrome *c* binding to DMPC–DMPS mixtures does not result in a profound reorganization of the bilayer structure; only a small change in the average conformation of the DMPS-d_2 head group is observed. No evidence of protein-induced lateral phase segregation of DMPC and DMPS was found.

In the investigation of phospholipids at the boundary of intrinsic proteins, the technique of deuterium nuclear magnetic resonance (^{2}H NMR)[1] has been successful in showing the rapid exchange of those phospholipids in and out of direct contact with the protein (on the time scale of 10^{-5} s). The limited perturbation of order parameters of the lipid acyl chains caused by the proximity to intrinsic proteins has been emphasized in most articles concerned with the interpretation of ^{2}H NMR measurements in these systems. [for reviews, see Seelig & Seelig (1980), Jacobs & Oldfield (1981), Devaux (1983), and Bloom & Smith (1985)]. To date, very few ^{2}H NMR investigations have been performed on the interactions of charged lipids with membrane proteins, most experiments having involved zwitterionic phosphatidylcholine (PC) molecules. Exceptions are the studies by Seelig and collaborators of phosphatidylserine (PS) and phosphatidylglycerol (PG) in *Escherichia coli* membranes (Gally et al., 1979, 1980; Borle & Seelig, 1983) and reconstituted systems containing cytochrome *c* oxidase (Tamm & Seelig, 1983). These results show that the proteins have little or no effect on the ^{2}H NMR spectrum of the phospholipids. Since extrinsic proteins bind selectively to membranes containing charged lipids, they appear more promising candidates in the search for protein-induced perturbations of lipid configurations. Such interactions are important since the presence of charged lipids or charged intrinsic proteins is essential for the binding and functional activity of extrinsic proteins.

Extensive work has been carried out using phosphorus (^{31}P) and carbon (^{13}C) NMR to determine how lipid structures are influenced by extrinsic proteins such as cytochrome *c*, myelin basic protein, or β-hydroxybutyrate dehydrogenase [see the review by Devaux & Seigneuret (1985)]. To date, only one article has reported on the use of ^{2}H NMR to study this type of interaction. Sixl et al. (1984) have investigated the influence of myelin basic protein on the deuterium quadrupolar splittings

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; TLC, thin-layer chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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at specific positions of dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylcholine (DMPC) in mixed lipid bilayers. The authors reported a selective decrease of the quadrupolar splitting of head-group-labeled DMPG upon binding of the extrinsic protein which they interpreted as an illustration of specificity in lipid–protein interactions.

This article presents the results of the investigation of the interaction of cytochrome *c* with mixed lipid bilayers containing zwitterionic DMPC and charged DMPS. The behavior of both lipids can be studied independently by labeling either molecule; both perdeuterated acyl chains (DMPC-d_{54} and DMPS-d_{54}) and specifically deuterated head groups (DMPS-d_2) were used. In addition, some experiments were carried out at different mole ratios of DMPC to DMPS; namely, 1:1, 5:1, and 10:1. In all cases, spectra and relaxation times were obtained in the liquid-crystalline (L_α) and gel (P_β) regions of the phase diagram.

Cytochrome *c* in the inner mitochondrial membrane interacts mainly with cytochrome *c* oxidase, cardiolipin, and phosphatidylinositols. Phosphatidylserine is only a minor constituent lipid of mitochondria (Colbeau et al., 1971), and thus, the present investigation is designed as a model system for the interaction of extrinsic proteins with a charged lipid interface. The knowledge of the three-dimensional structure of cytochrome *c* (Dickerson et al., 1971) and the availability of large quantities of protein make it a reasonable choice as a representative extrinsic protein.

Materials and Methods

Lipid Synthesis. Perdeuterated (98 atom %) myristic acid was purchased from MSD Isotopes. The synthesis of DMPC-d_{54} was carried out according to Warner and Benson 1977). DMPS-d_{54} was obtained by enzymatic modification of the head group according to the method of Comfurius and Zwaal (1977). The labeled phospholipids were purified on a carboxymethyl (CM 52) cellulose column. The sodium salt of phosphatidylserine was made by washing the lipids with sodium citrate (Trevelyan, 1966).

Unlabeled DMPC was purchased from Sigma, and unlabeled DMPS was obtained from Avanti.

Synthesis of 3,3-Dideuterated Dimyristoylphosphatidylserine ([3-$^2H_2$]DMPS). Racemic *N*-acetyl-3,3-dideuterioserine was prepared according to King (1947) from 99% labeled paraformaldehyde (CEA France) and diethyl acetamidomalonate (Aldrich): after purification on Dowex 50 (Baker et al., 1953), the overall yield was 52% (not including a by-product fraction of *N*-acetylserine mixed with *N*-acetylglycine). Resolution of this derivative with acylase 1 (Sigma), by the procedure of Akabori et al. (1959), afforded 3,3-dideuterio-L-serine: yield 70%; $[\alpha]_D^{25}$ −8.0° (*c* 1.5; H_2O); deuterium content checked by NMR. Our method avoided the intermediate synthesis of racemic dideuterioserine, as described by Browning and Seelig (1979), leading us to a better overall yield of labeled L-serine.

Synthesis of 3,3-dideuteriated dimyristoylphosphatidylserine was achieved according to Browning and Seelig (1979, 1980). The phthalimidomethyl ester of the *N-tert*-butoxycarbonyl derivative obtained (45%) from 3,3-dideuterio-L-serine was coupled with dimyrsitoylphosphatidic acid (Lucerna), using the 2,4,6-triisopropylbenzenesulfonyl chloride technique, yielding, after purification by chromatography on silica gel, the protected phospholipid (55%). Removal of the protective groups by means of sodium thiophenolate and trifluoroacetic acid afforded, after purification, [3-$^2H_2$]DMPS (77%; single spot TLC on silica gel, R_f = 0.29; elution with $CHCl_3$–CH_3OH–H_2O (65:25:4)].

Binding Experiments. One milligram of DMPS plus variable amounts of DMPC (from 1 to 10 mg), solubilized in chloroform–methanol, was dried in a glass tube by pumping several hours with a vacuum pump. Horse heart cytochrome *c* (type VI from Sigma) was solubilized in 200 μL of a buffer composed of 50 mM Hepes, 40 mM NaCl, and 1 mM EDTA at pH 7.5. The concentration of protein in the buffer varied from 2 to 80 mg/mL. The solubilized proteins were added to the dried lipids, and the tube was shaken for 10 min at 35 °C. Then the tube was dipped in liquid nitrogen a few minutes and then thawed and vortexed. This operation (unless otherwise mentioned) was repeated 3 times. The lipids were afterwards centrifuged 90 min at 18 000 rpm at 4 °C. The amount of bound protein was determined from the amount of protein remaining in the supernatant as determined by optical absorption at 410 and 550 nm. (The absorption spectra correspond to the oxidized form of cytochrome *c*.) In some experiments, it was shown by phosphate determination that no lipids remained in the supernatant. Also in some experiments the amount of bound protein was double checked by resuspending the pellet in a Bligh and Dyer (1959) mixture [chloroform–methanol–water (1:8.1:1)] and then determining the amount of heme in the organic solvent. Control experiments with only DMPC, i.e., without DMPS, were also carried out to determine the amount of protein remaining bound in the uncharged liposomes.

Samples for NMR Experiments. Two procedures were employed to prepare the samples used for NMR. The first procedure was identical with that described for the binding assays of cytochrome *c* to DMPC–DMPS mixtures except that the scale was multiplied by 10 or 20 in order to have 10–40 mg of labeled lipids. A minimum of 50 wt % of buffer made with ^2H-depleted water (Aldrich) was added to the pellet. We found this precaution essential in order to obtain reproducible results. The lipid–protein complexes were transferred to glass NMR tubes (0.4–2.0 mL) as a frozen mixture obtained by cooling the sample in liquid nitrogen. The amount of bound protein was determined as described above by the optical absorption of the supernatant.

In the second procedure, the proteins were directly added in the volume of buffer used for the NMR experiments; thus, no attempt was made to separate the bound and free proteins. However, the samples were freeze–thawed 3 times before use in order to ensure better mixing. This procedure was utilized only with a very large excess of proteins in order that the lipid interface be saturated with proteins.

Calorimetry. Calorimetric traces were obtained by using a Microcal MC-1 differential scanning calorimeter with a scan rate of 30 °C/h.

NMR Methods. The ^2H NMR results were acquired at 35.4 MHz on a ^2H NMR spectrometer constructed in the electronics workshop of the UBC Physics Department. Digitization of the NMR signal was done with a Nicolet 2090 digital oscilloscope while signal averaging and basic data analysis were carried out on two Intel 8080A based microcomputers. More sophisticated data analysis such as de-Paking was carried out on The University of British Columbia Amdahl V8 computer which was serially connected to one of the Intel microcomputers. Temperature control of the sample was achieved with an oven regulated by a Bruker B-ST 100/700 temperature controller.

For the ^2H NMR spectra displayed in the figures and used for moment calculations, typically 100 000–150 000 transients were accumulated with a dwell time of 2, 5, or 10 μs and a repetition time of from 150 ms to 3 s. Quadupolar echo

radio-frequency pulse sequences (Davis et al., 1976) were employed with pulse lengths from 3 to 8 μs and pulse separations of 50–100 μs. The longer pulse lengths and dwell times and shorter repetition times were used for the head-group-labeled samples which had spectra with relatively narrow splittings and a short T_1. In all cases, the distortions of the spectra due to the finite pulse lengths (Bloom et al., 1980) were calculated and found to be negligible, and the pulse separations used were always less than one-quarter of T_{2e}. Spectra were obtained by Fourier transformation from the echo peak. When the accumulated quadrupolar echo signal did not have a digitized point exactly at the echo peak, the free induction decay was shifted by some fraction of a dwell time using an orthogonal polynomial interpolation routine (Davis, 1983) to ensure that the Fourier transform would start precisely on top of the quadrupolar echo. The spectral moments, M_1 and M_2, and the fractional mean squared deviation in the quadrupole splittings, $\Delta_2 = (M_2/1.35M_1^2)^{-1}$, were calculated by integration of the ^{2}H NMR spectra.

When investigating lipid–protein interactions by NMR, it is very important to ensure that the experiment can detect the type of signal which could arise from protein-bound lipids. Therefore, in some experiments, conditions were optimized to look for low-intensity, slowly relaxing, and very broad lines in the ^{2}H NMR spectra. In particular, spectra were accumulated with a 500-ns dwell time, repetition times of up to 3 s, and short high-power radio-frequency pulses (3 μs).

The quadrupolar echo decay rate, T_{2e}, was measured by plotting the intensity of the echo peak, $A(2\tau)$, as a function of 2τ, where τ is the separation between the pulses. For exponential relaxation

$$A(2\tau) = A(0)\exp(-2\tau/T_{2e}) \quad (1)$$

Spin–lattice relaxation times, T_1, were measured by an inversion–recovery technique where a nominal 180° pulse was applied a time, τ, before the quadrupolar echo pulse sequence. For exponential relaxation, the echo peak intensity follows the relation

$$A(\tau) = A(\infty) - [A(\infty) - A(0)]e^{-\tau/T_1} \quad (2)$$

A perfect 180° pulse would give $A(0) = -A(\infty)$, but the analysis of the experimental data for the head-group-labeled lipids was made with the more general eq 2. The ^{2}H NMR relaxation of the chain-perdeuterated phospholipids did not satisfy eq 2, and the data were treated differently as described under Results.

Results

Binding Studies of Cytochrome c. Separate binding studies were performed in order to determine how much protein is effectively interacting with the phospholipids under the experimental conditions required for ^{2}H NMR measurements. The protocol for these experiments, which is described under Materials and Methods, was the same as that used for preparations of NMR samples. Figure 1A shows the results of experiments in which the binding of cytochrome *c* with an equimolar mixture of DMPC and DMPS was measured as a function of cytochrome *c* added. A plateau was obtained which corresponded to a maximum binding of approximately 1 mg of cytochrome *c* bound to 1 mg of DMPS. In terms of mole ratio, this corresponds to 1 molecule of cytochrome *c* to 18 molecules of DMPS and 18 molecules of DMPC. Figure 1A also demonstrates that when pure DMPC was used, the amount of protein in the pellet was negligible. This proves that cytochrome *c* is not simply trapped within the interior of the liposomes but that it effectively binds to the negatively charged mixed lipid interface.

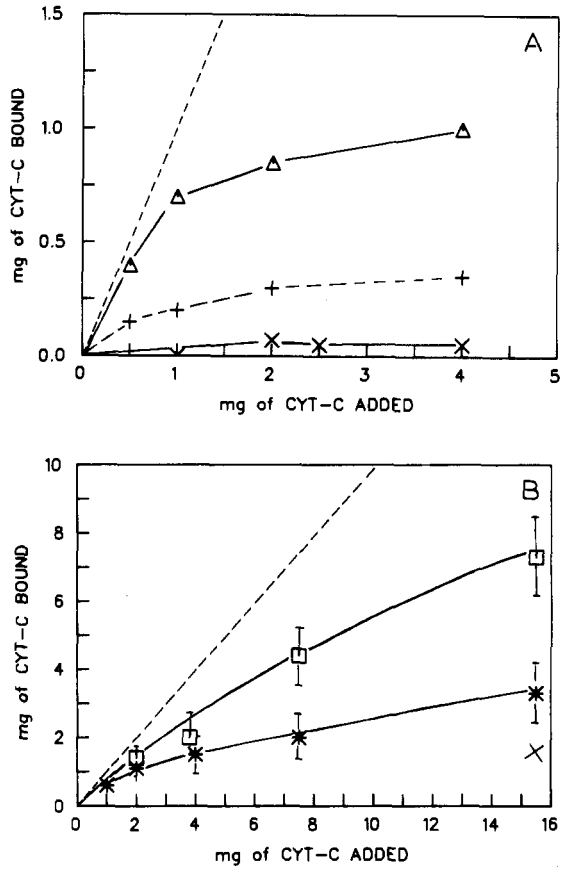

FIGURE 1: (A) Binding of cytochrome *c* to various liposomes as a function of the amount of protein added to the dispersions. (△) DMPC–DMPS (1 mg:1 mg) with a centrifugation volume of 0.2 mL; (+) DMPC–DMPS (1 mg:1 mg) with a centrifugation volume of 8.0 mL; (×) DMPC (2 mg) with a centrifugation volume of either 0.2 or 8.0 mL. (B) Binding of cytochrome *c* to liposomes of DMPC–DMPS as a function of the amount of protein added to the dispersions for different mole ratios of DMPC to DMPs (weight of DMPS constant at 1 mg): (□) 10:1 DMPC–DMPS; (*) 5:1 DMPC–DMPS; (×) 1:1 DMPC–DMPS.

It is important to bind the proteins using a reproducible procedure since the final extent of binding is strongly dependent on two factors: the volume of centrifugation and the number of freeze–thaw cycles used. When the centrifugation volume was increased from 0.2 to 8.0 mL, the total amount of bound protein was decreased by a factor of about 4. If no freeze–thawing was done, the total amount of bound protein was decreased 3-fold. It was observed that cycling more than 3 times did not result in additional binding.

Analysis of the binding curves in Figure 1B shows that if DMPS is further diluted with DMPC, the number of DMPS molecules required to bind one cytochrome *c* molecule can be considerably reduced. This indicates that one of the limitations of protein binding is a steric effect rather than an electrostatic requirement for a specific number of negative charges. Figure 1B shows the amount of cytochrome *c* bound as a function of incubated protein weight for 1 mg of DMPS in samples of ratios of 5:1 and 10:1 DMPC:DMPS, respectively. With the 10:1 mixture, it was not possible to reach protein saturation. Experiments on pure DMPC membranes demonstrate that the amount of protein bound by the zwitterionic lipid alone is negligible compared with that bound to the charged membrane composed of both DMPC and DMPS. Under the conditions of maximum protein binding, it was possible to estimate the number of charged lipid molecules interacting with each cytochrome *c* molecule. This is obviously a function of dilution

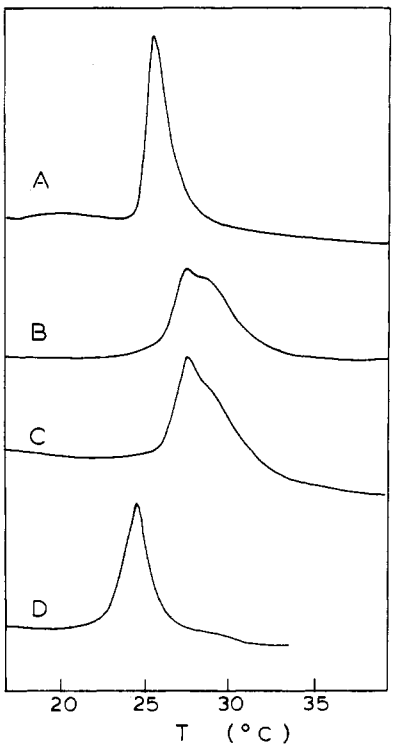

FIGURE 2: Differential scanning calorimetric traces for approximately 2 mg of lipids resuspended in 700 μL of buffer containing 20 mM Hepes at pH 7.5, 50 mM NaCl, and 1 mM EDTA. Plots are shown for increasing temperature at a scan rate of 30 K/h. (A) 3:1 DMPC–DMPS; (B) 1:1 DMPC–DMPS; (C) 1:1 DMPC–DMPS with bound cytochrome *c*; (D) 1:1 DMPC–DMPS-d_{54}.

of DMPS with DMPC: rough estimates suggest 18, 6, and 3 for 1:1, 5:1, and 10:1 mixtures, respectively.

Calorimetric Studies. Differential scanning calorimetry was performed on several mole ratio mixtures of DMPC and DMPS both with and without protein; these results are presented in Figure 2. Comparison of traces 2B and 2D shows that when chain-perdeuterated lipids were used, the liquid-crystalline to gel phase transition was shifted to lower temperatures.

^{31}P NMR. Spectra were obtained for the 1:1 DMPC–DMPS mixture with and without cytochrome *c*. There was no change in the magnitude or sign of the phosphorus chemical sheilding anisotropy and thus no evidence for the existence of nonbilayer structures. Therefore, it can be concluded that binding of this extrinsic protein conserves the symmetry of the membrane.

Chain-Perdeuterated Phospholipids: 1:1 DMPC:DMPS Molar Ratio. When the lipid–protein interaction was studied by using labeled acyl chains on both lipid species, it was considered desirable to conduct experiments on all possible permutations: DMPC-d_{54}:DMPS, DMPC-d_{54}:DMPS:cytochrome *c*, DMPC:DMPS-d_{54}, and DMPC:DMPS-d_{54}:cytochrome *c*. While experiments were performed at different protein concentrations, the results presented in this section correspond either to no protein or to the highest protein to lipid ratio possible.

The ^2H NMR spectra in the liquid-crystalline phase (310 K) of chain-perdeuterated DMPC and DMPS are compared in the absence and presence of bound protein in Figure 3. In both instances, no influence of the protein on the acyl chains of the lipid can be detected from the NMR line shape. The absence of variation in the quadrupolar splitting profile is more obvious in the corresponding oriented spectra shown in Figure 4. This is obtained by de-Paking of the powder spectrum.

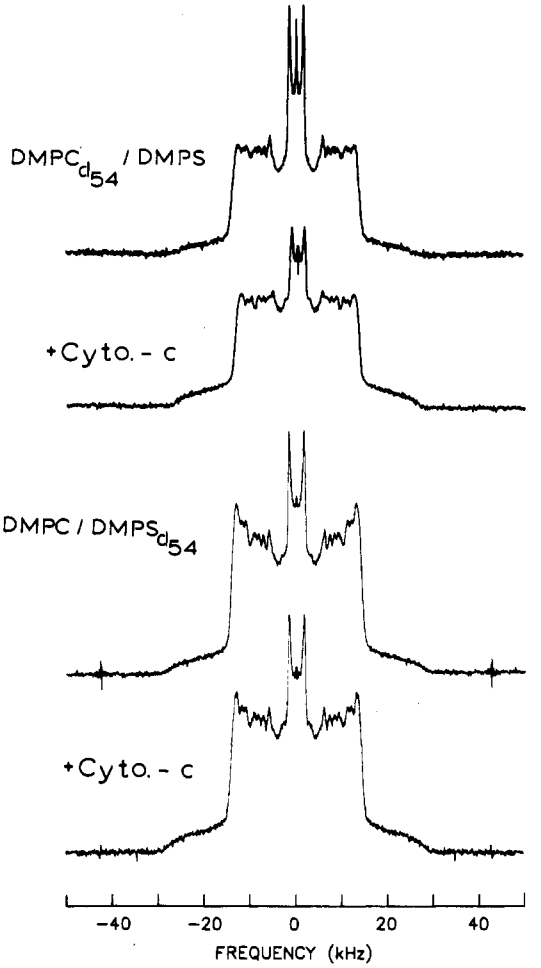

FIGURE 3: Deuterium NMR spectra of membranes composed of a 1:1 mole ratio of DMPC–DMPS in the absence and presence of cytochrome *c*. Each sample contains approximately 50 mg of each lipid and 40 mg of bound protein. A total of 36 000 scans were collected at 310 K by using a pulse spacing of 40 μs, a recycle time of 400 ms, and a dwell time of 5 μs.

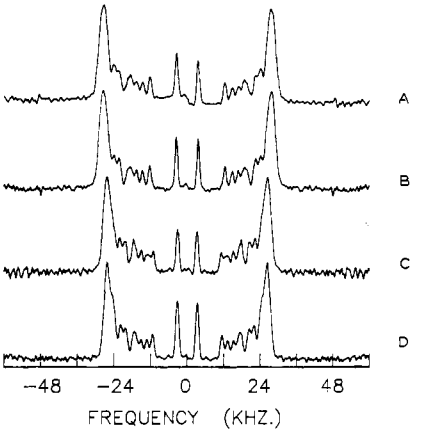

FIGURE 4: De-Paking of the spectra presented in Figure 3 provides the oriented spectra (0°). Note that the splittings are twice those obtained conventionally from the 90° edges of the powder pattern. 1:1 DMPC-d_{54}–DMPS (A) without protein and (B) with protein; 1:1 DMPC–DMPS-d_{54} (C) without protein and (D) with protein.

(Bloom et al., 1981; Sternin et al., 1983). For both lipids, the de-Paked spectra with and without protein are superimposable, indicating that the orientational averaging of the quadrupole interaction for each segment of the chain is not affected by the presence of the protein. Spectra were also obtained for all samples below the phase transition, and Figure 5 from DMPC–DMPS-d_{54} shows that protein does not influence the

Table I: Summary of Relaxation Times (T_{2e} and T_1) for Acyl Chain Perdeuterated Lipids in a 1:1 Mole Ratio of DMPC–DMPS Mixed Liposomes[a]

	T = 292 K			T = 310 K		
		T_1 (ms)			T_1 (ms)	
sample	T_{2e} (μs)	average[b]	slow	T_{2e} (μs)	average[b]	slow
DMPC-d_{54}–DMPS	160	35	53	392	34	63
DMPC-d_{54}–DMPS–cytochrome c	190	30	57	310	30	45
DMPC–DMPS-d_{54}	132	34	53	460	34	53
DMPC–DMPS-d_{54}–cytochrome c	210	52	176	340	33	176

[a] Results from samples with or without bound cytochrome c are provided for comparison. [b] The average value of T_1 is defined as the slope at short relaxation delays, $\langle 1/T_1 \rangle^{-1}$.

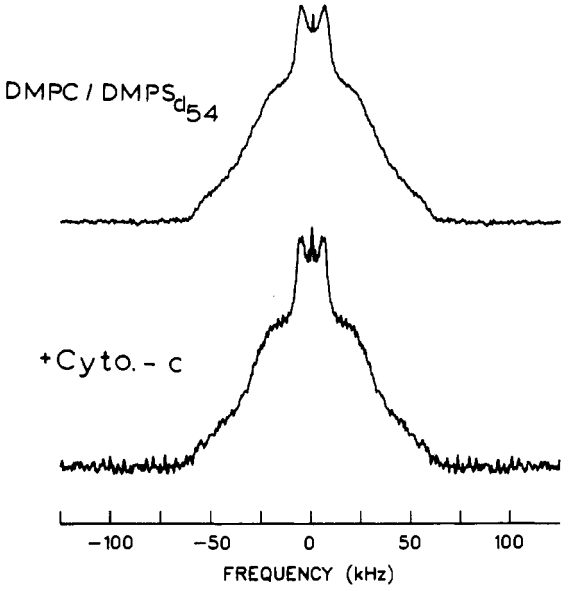

FIGURE 5: ^{2}H NMR spectra, at 292 K, of the gel phase of 1:1 DMPC–DMPS-d_{54} with and without bound cytochrome c.

^{2}H NMR line shape appreciably in the gel phase.

For the samples containing DMPS-d_{54} molecules, spectra were obtained in the temperature range 288–310 K in the presence and absence of cytochrome c. Spectra moments analysis was performed on all line shapes, yielding M_1, M_2, and Δ_2. The results show little change upon addition of protein as illustrated for the second moment, M_2, of the DMPC–DMPS-d_{54} samples with and without cytochrome c which are displayed as a function of temperature in Figure 6.

While the binding of cytochrome c had little or no observable effect on the quadrupole splittings of the perdeuterated acyl chains, it did have a selective effect on the relaxation rates. The values of both spin–lattice (T_1) and transverse (T_{2e}) relaxation rates are summarized in Table I. It was found that all samples, in both the liquid-crystalline and gel phases, exhibit a single-exponential decay of the quadrupolar echo amplitude with pulse separation. For both DMPC-d_{54} and DMPS-d_{54}, protein binding significantly decreased T_{2e} in the liquid-crystalline state and increased it in the gel phase.

Spin–lattice relaxation rates were measured by using an inversion–recovery sequence with a recycle delay of 400 ms. Although it was known that the very inefficiently relaxed sites, in particular the terminal methyls, had longer T_1 values, this choice of recycle delay enabled the measurement of spin–lattice relaxation rates of deuterons having T_1 less than 100 ms and saved a considerable amount of time.

Representative decay curves for DMPC-d_{54}–DMPS and DMPC–DMPS-d_{54} in the liquid-crystalline phase both with and without cytochrome c are displayed in Figure 7. For the 54 deuterons on the hydrocarbon chain, there is a wide distribution of relaxation times, simply due to the different

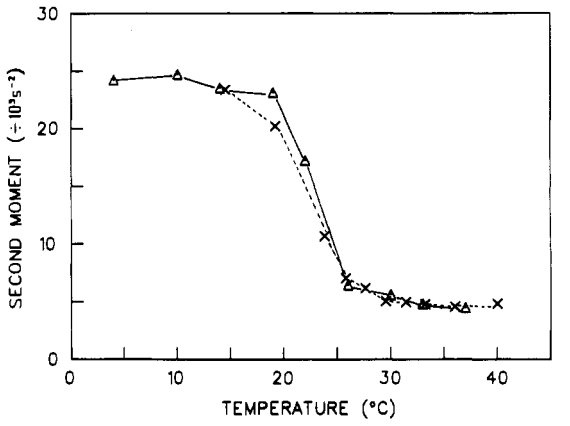

FIGURE 6: Second moment of the ^{2}H NMR line shape as a function of temperature for a 1:1 mole ratio of DMPC–DMPS-d_{54} (×) without and (△) with cytochrome c bound to the lipid membranes.

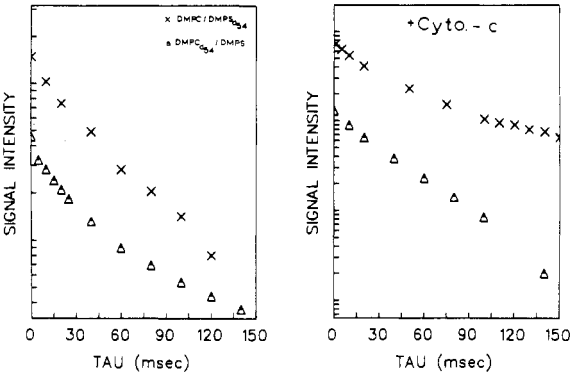

FIGURE 7: Inversion–recovery spin–lattice relaxation results for acyl chain perdeuterated lipids in 1:1 mixtures of DMPC–DMPS. Semilogarithmic plots of signal intensity vs. relaxation delay time are shown for (△) DMPC-d_{54} and (×) DMPS-d_{54} in the absence and presence of cytochrome c.

geometrical and motional properties of each chain segment and the absence of strong cross-relaxation between neighboring segments (Brown et al., 1979). It was therefore decided to characterize each decay curve by its slope at early times (1–20 ms), which represents $\langle 1/T_1 \rangle$ averaged for all deuterons, and the slope at longer times (60–140 ms), which represents an average over the more inefficiently relaxed deuterons. Note that while $\langle 1/T_1 \rangle$ is a well-defined property of the spin system, the slope at longer times will depend on the range of times considered.

On addition of cytochrome c to both the liquid-crystalline and gel phases of DMPC–DMPS-d_{54} bilayers, the initial slope of the relaxation curve was found to be essentially unaffected while the value of the slow component was increased considerably. DMPC-d_{54}–DMPS showed almost no change in relaxation rates on protein binding; if there is an effect, it is much smaller and in the opposite direction to that observed for DMPC–DMPS-d_{54}.

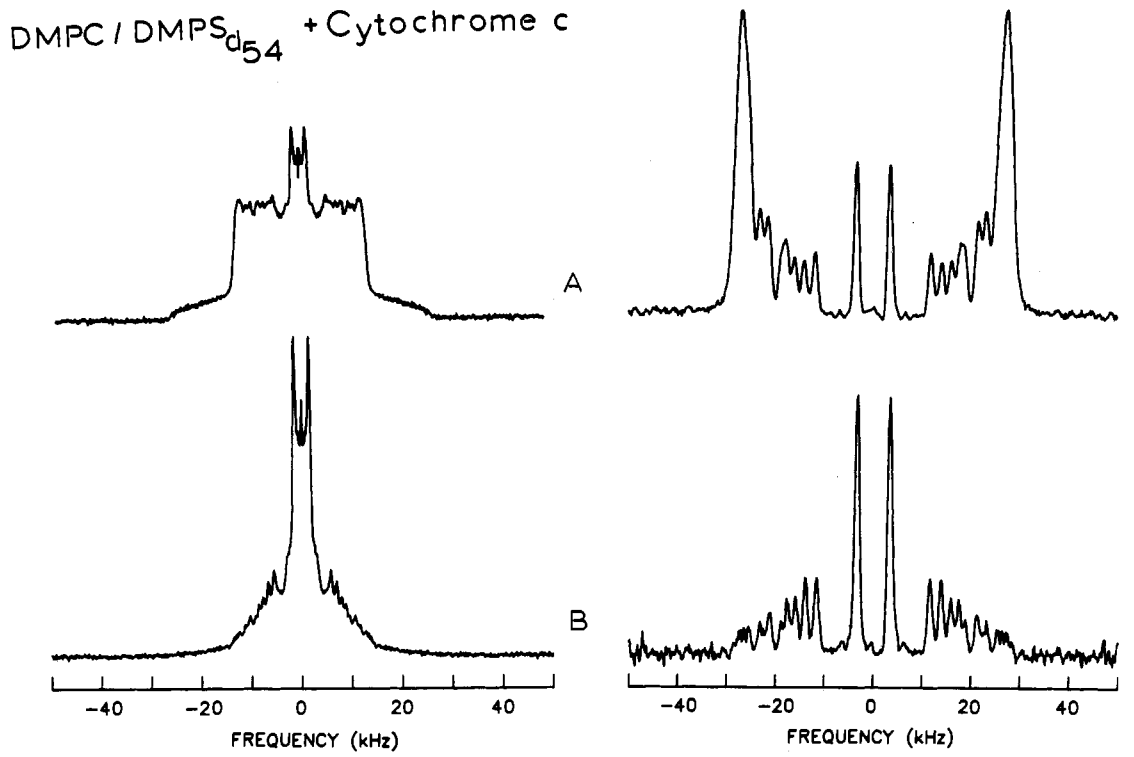

FIGURE 8: (A) Comparison of the normal and partially relaxed spectra for 1:1:1 DMPC–DMPS-d_{54}–cytochrome c at 312 K. (B) The (0°) oriented spectra obtained by de-Paking the line shapes. A total of 42 000 scans were collected with a repetition time of 400 ms, a pulse spacing time of 40 μs, and an inversion–recovery delay of 90 ms.

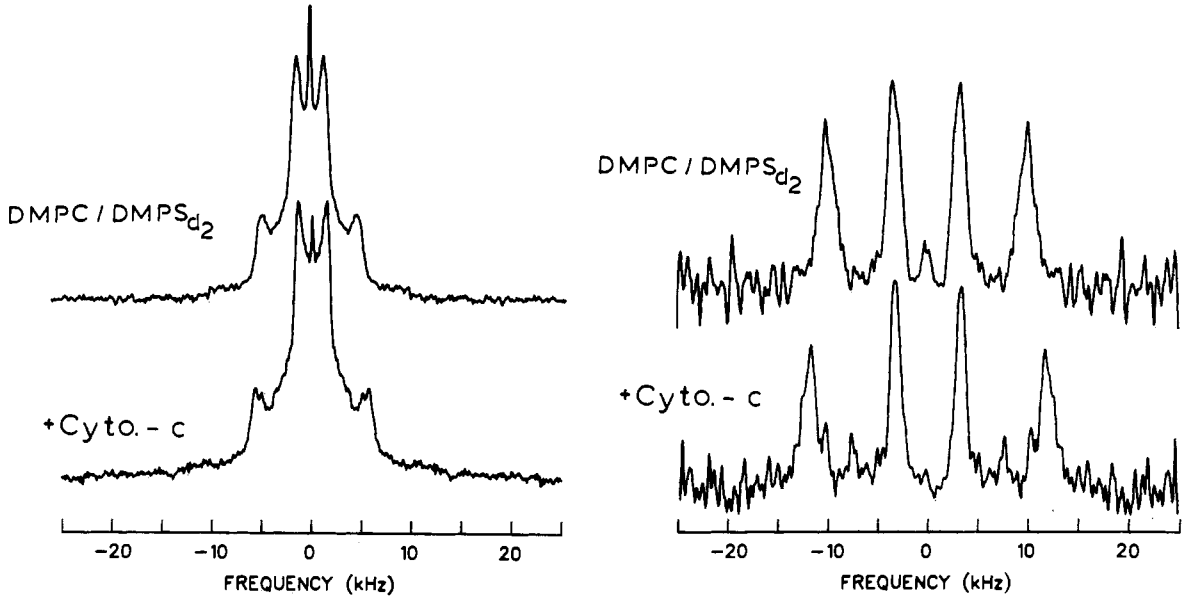

FIGURE 9: ^{2}H NMR spectra of the α-position head-group label in 1:1 DMPC–DMPS-d_2 with and without bound cytochrome c. Powder patterns and their corresponding de-Paked oriented spectra (0°) are presented. The samples contain approximately 30 mg of each lipid with 25 mg of bound protein. The repetition time on 100 000 scans was 150 ms with a pulse spacing of 40 μs.

Partially relaxed spectra permit the correlation of T_1 rates with the order parameter profile responsible for the line shape. The striking results given in Figure 8 show that the fast relaxers are predominantly in the plateau region which has the largest quadrupolar splittings and that the slow relaxers, which are the most affected by protein addition, are at the end of the hydrocarbon chains and have smaller order parameters.

^{2}H NMR of Head-Group-Labeled Lipids. The specifically labeled compound DMPS-d_2 contains deuterium at the α-position of the serine moiety. The L,L isomer contains inequivalent deuterons and thus gives rise to two quadrupolar splittings in the NMR spectrum (Browning & Seelig, 1980). Binding of the protein results in a 15% increase of the larger splittings but leaves the smaller one unchanged. This protein-induced modification is demonstrated in Figure 9 which shows both powder pattern and de-Paked spectra from the liquid-crystalline phase of the equimolar mixture of DMPC–DMPS-d_2 in the presence and absence of cytochrome c.

In cognizance of ESR and NMR experiments on intrinsic proteins, it seemed essential to establish conclusively whether binding of cytochrome c produced a strongly immobilized fraction of lipid on the NMR time scale (Bienvenue et al., 1982; Devaux, 1983). Using samples having 10:1 and 5:1 ratios of DMPC to DMPS, corresponding to a reduced number

Table II: Deuterium Quadrupolar Splittings (kHz) of α-Position Head-Group-Labeled DMPS-d_2 Obtained from de-Paking the Liquid-Crystalline Phase Spectra[a]

	DMPC:DMPS mole ratio		
	1:1 at 310 K	5:1 at 302 K	10:1 at 299 K
no protein	3.3	3.6	3.5
	10.0	9.4	9.7
protein	3.3	3.7	3.5
	12.0	10.9	10.0

[a] Splittings are tabulated as a function of the DMPC:DMPS-d_2 mole ratio.

of DMPS molecules interacting with cytochrome *c*, we have optimized the experimental conditions to detect a low intensity, slowly relaxing, and very broad NMR signal. None of the experiments gave any indication of the existence of such a protein-modified lipid component.

Relaxation measurements gave single-exponential decays for both T_1 and T_{2e}; T_{2e} was 420 ± 20 μs both with and without the protein cytochrome *c*, while T_1 changed from 6.5 ± 2.0 to 9.5 ± 2.0 ms. These results are different from those of the chain-perdeuterated lipid molecules where the protein was observed to modify the relaxation but not the quadrupolar splittings.

As indicated in the section on binding studies, experiments have been conducted on lipid bilayers with different ratios of DMPC to DMPS. One of the major motivations of the dilution investigations was to maximize the fraction of DMPS molecules which interact directly with the protein and hence modifies the strength of binding. Results on the influence of cytochrome *c* on the quadrupolar splittings of the head-group-labeled DMPS-d_2, as measured from the de-Paked spectra, are shown in Table II for DMPC:DMPS-d_2 mole ratios of 1:1, 5:1, and 10:1. All these samples gave rise to spectra in which one splitting increased upon protein binding, but the magnitude of this change in splitting decreased with increased dilution.

Discussion

Binding Studies. Many investigators have demonstrated that the water-soluble protein cytochrome *c* binds to negatively charged lipid interfaces (Nicholls, 1977). The high affinity of this protein to cardiolipin, phosphatidic acid, phosphatidylinositol, and phosphatidylserine has been studied by using both optical and magnetic resonance techniques [for a review, see Devaux & Seigneuret (1985)]. Similarly, it has been shown that cytochrome *c* does not bind to pure phosphatidylcholine or phosphatidylethanolamine vesicles. While all authors agree qualitatively on the requirement of a net negative charge, estimates of the number of phospholipid molecules bound to cytochrome *c* vary considerably from one study to another. Such a variation may originate from a range of possible causes: differences in ionic strength, physical nature of the interface, chain length, degree of unsaturation, head-group charge distribution, total number of charged lipids, and lipid to water ratios. Some of the observed discrepancies may well be associated with differences in the method and the time scale of the detection techniques employed.

In order to achieve consistency, independent determinations of the amount of cytochrome *c* binding to the lipid mixtures were performed. Because it was necessary to have large vesicles for the NMR experiments, all binding studies were carried out with liposomes. Since unsonicated vesicles form multilayers, it was not clear, a priori, that all lipids would be accessible to the soluble proteins. Figure 1A demonstrates that, provided freeze–thawing was used, it was possible to reach an apparent saturation in the binding of cytochrome *c* to the 1:1 mixture of DMPC–DMPS. From this plateau, it was estimated that 1 molecule of protein interacts with approximately 18 DMPS molecules implying a total of 36 phospholipids. Since the 3:1 water to lipid weight ratio used corresponded to a substantial excess of water, it is unlikely that a cytochrome *c* molecule interacted with more than one bilayer surface.

The three-dimensional solid-state structure of cytochrome *c* has been determined by X-ray crystallography (Dickerson et al., 1971). To a first approximation, the protein is an ellipsoid with dimensions 30 × 34 × 34 Å^3, corresponding to a cross section of approximately 1000 Å^2. Such an area corresponds to ≈20 phospholipids in the liquid-crystalline state and increases to ≈30 in the gel phase (Tardieu et al., 1973). For the high protein to lipid ratios used, it seems likely that the protein molecules cover a substantial fraction of the surface, thus suggesting that simple steric problems may be primarily responsible for the observed saturation levels.

The presence of a simple steric saturation mechanism was supported by results from the dilution of DMPS molecules with DMPC where it was possible to achieve higher ratios of protein to charged lipid while maintaining approximately constant protein concentration. Since the physical dimensions of the surface appeared to limit the protein binding, it seems unlikely that cytochrome *c* disrupted the bilayer structure. ^{31}P NMR spectroscopic results showed that protein binding caused no change in either the magnitude or the sign of the phosphate chemical shielding anisotropy and thus unequivocally demonstrated that the integrity of the bilayer is preserved. In summary, it is clear that for samples with high protein concentration all lipid molecules in the bilayer were in close contact with a cytochrome *c* molecule.

^{2}H NMR Studies. The binding studies do not distinguish between two limiting alternative descriptions of the nature of the binding: (i) specific binding, a strong attachment, for relatively long periods of time, of the individual negatively charged DMPS molecules to the positively charged lysine regions known to be distributed over the surface of the cytochrome *c* molecules (Dickerson et al., 1971); (ii) nonspecific binding, associated with the average attractive Coulomb force between the negatively charged liposome and the positively charged cytochrome *c* molecules.

The ^{2}H NMR spectroscopic studies presented under Results do have the potential to distinguish between these two limiting cases over the ^{2}H NMR time scale of 10^{-5} s. Indeed, the results strongly favor the limiting case (ii) described above. It is difficult to imagine how DMPS molecules could be strongly attached to individual proteins without producing some sort of measurable change in the ^{2}H NMR spectrum of the acyl chains of the DMPS molecules. As shown in Figures 3–5, the acyl chain spectra of DMPS-d_{54} in DMPC–DMPS mixtures with and without large concentrations of cytochrome *c* are indistinguishable both in the liquid-crystalline (310 K) and in the gel phases (292 K). This was consistent with the observation (Figure 6) that the moments of the ^{2}H NMR spectrum, which are sensitive to line shape and width, were found to be unchanged by the addition of protein.

There should not be any direct contact between lipid acyl chains and the extrinsic protein cytochrome *c*. However, any strong binding between the cytochrome *c* molecules and the head group of an individual DMPS molecule would, in general, be accompanied by a substantial torque on the rest of the molecule. If this torque persisted for a time long on the NMR time scale, its effect would be to produce a systematic mod-

ification of the acyl chain order parameters similar to that observed on soap molecules due to the local water structure at the soap–water interface (Abdolall et al., 1977).

Although corresponding studies of the influence of intrinsic proteins on acyl chain ^2H NMR spectra [see, e.g., Bienvenue et al. (1982)] gave little change in the average chain orientational order parameter, they did indicate a substantial spread of the order parameters due to the lipid–protein interaction, i.e., an increase in the inhomogeneous broadening. In addition, the intrinsic proteins gave a profound decrease in T_{2e} of the ^2H labels in the acyl chains, indicating a very large increase in the correlation time for orientational averaging of lipid chains in contact with the protein surface. While no measurable perturbations of the acyl chain ^2H NMR spectra were produced by the lipid–cytochrome *c* interactions, small changes in the spectra of the head groups were detected. These changes are too small to analyze unambiguously, as they could be produced by twists of the polar head group of only a few degrees. Since such small head-group twists would be expected to generate even smaller twists of the glycerol backbone, these results are quite compatible with limiting case ii discussed above.

The observed small changes in T_{2e} and T_1 of the acyl chain ^2H NMR signals could also be due to the same type of interaction. For example, the changes in T_{2e} require that the Coulomb interaction between the lipid head group and the protein slow down the overall lipid reorientation rate, τ_c^{-1}; i.e., the coupling increases the effective viscosity of the medium (membrane plus water) in which the lipid molecules move. Note that a slowing down of the lipid reorientation rate would explain both the decrease in T_{2e} in the liquid-crystalline phase and the increase in the gel phase since $T_{2e} \propto \tau_c^{-1}$ in the liquid-crystalline phase and $T_{2e} \propto \tau_c$ in the gel phase (Pauls et al., 1985). This explanation requires that τ_c be sufficiently long ($M_2\tau_c^2 >> 1$) in the gel phase as seems to be the case in many lipid systems (Wittebort et al., 1982). A calculation of the magnitude of the small but measurable influence of the lipid–protein interaction on quadrupolar interactions and the lipid reorientation rate requires quantitative treatment of the shielding effect of the aqueous buffer solution on the Coulomb attraction between the negatively charged lipid head groups and the positive charged on the protein surface and will not be attempted here.

It is noteworthy that the value of $\langle 1/T_1 \rangle \approx 34$ ms^{-1} obtained for dipalmitoylphosphatidylcholine-d_{62} at 45 °C [see Table II of Davis (1979)] is very close to our values (see Table I), indicating that the average relaxation rate of ^2H nuclei on acyl chains is not sensitive either to the type of head group or to the chain length. It is not possible to interpret the observed influence of cytochrome *c* on the more slowly relaxing ^2H nuclei (see Figure 8) in a definitive manner on the basis of the present understanding of T_1 relaxation measurements. The most serious attempt to characterize T_1 in membranes has been carried out by Brown (1979, 1984), who has classified the relaxation rate in terms of two contributions [see, e.g., eq 2 in Brown et al. (1983)]; one component ($T_{1f} = A\tau_f$) is associated with fast, single-molecule reorientations, and the second ($T_{1s}^{-1} = BS^2/\omega_o^{1/2}$) is due to collective slower motions having a wide spectrum of correlation times [see the discussion in section II.3C of Bloom & Smith (1985)]. The fast, single-molecule motions do not influence the average relaxation rate appreciably but do make a substantial contribution to relaxation rate of the more slowly relaxing ^2H nuclei at the ends of the acyl chains. In terms of Brown's classification of relaxation mechanism, we would be forced to conclude that the protein influences the "fast, local motions" but not the "slow, collective motions". A more definitive study would involve relaxation time measurements over a wide range of frequencies.

To this point, our discussion has assumed that the presence of the cytochrome *c* in the DMPC–DMPS mixtures does not modify the phase behavior of the lipid system. In fact, acyl chain ^2H NMR spectra are extremely sensitive indicator of phase separation for such systems when one of the separated phases is gellike and the other liquid-crystalline-like (Bienvenue et al., 1982; Huschilt et al., 1985), and the experimental spectra of Figures 3 and 5 demonstrate quite conclusively that no phase separation of this type is induced by the protein. Phase separation into two components, both of which are liquid-crystalline phase but having different ratios of DMPC to DMPS, cannot be ruled out in this manner since we have found that DMPC-d_{54} ^2H NMR spectra in the liquid-crystalline phases of pure DMPC and in mixtures of DMPC and DMPS are essentially indistinguishable. However, such a phase separation would presumably result in a change in the gel–liquid-crystalline phase transition, and the calorimetric data of Figure 2B,C provide no evidence for such an effect. More convincing is the close quantitative similarity of the temperature dependence of M_2 (Figure 6) for DMPC–DMPS-d_{54} mixtures with and without protein. A similar result was obtained with DMPC-d_{54}–DMPS mixtures but is not included here.

It would be of greater interest to carry out alternative, independent measurements which are sensitive to the time scale of specific lipid–protein interactions. One such experiment would be to compare the lateral diffusion constants of the extrinsic proteins and the charged lipids. These could be measured by using well-known NMR (Lindblom et al., 1981) and optical (Axelrod et al., 1976) methods. Strong binding would give rise to similar values of the diffusion constant for bound lipids and proteins even though one is in the membrane and the other in the aqueous medium. The conclusions drawn from our experiments imply that the diffusion constants of the two types of molecules should be different.

Conclusions

Studies of the interaction of cytochrome *c* with other lipid systems indicate that although a negative charge is a sufficient requirement to bind cytochrome *c*, the actual mechanism of interaction between the protein and lipid interface may depend critically on the exact nature of the lipids. For example, de Kruijff and Cullis (1980) have used ^{31}P NMR to show that cytochrome *c* is capable of inducing a nonbilayer structure in cardiolipid–phosphatidylethanolamine mixtures, and Brown and Wüthrich (1977) found, using ^1H and ^{13}C NMR, that cytochrome *c* induced phase separation in cardiolipin–lecithin bilayers. For cytochrome *c* with mixed DMPC–DMPS bilayers, there is no evidence for phase separation.

The main conclusions to be drawn from the experimental studies reported here are that although cytochrome *c* molecules are bound to membranes containing DMPS phospholipid molecules, whose head groups are negatively charged, a specific DMPS molecule is not bound to a particular cytochrome *c* molecule for a time long on the ^2H NMR time scale ($>10^{-5}$ s) and the orientational order of either of the phospholipids is not affected appreciably by the presence of the protein.

It is interesting that this conclusion differs from that drawn from the only other published study which deals explicitly with an extrinsic protein–phospholipid interaction using the ^2H NMR technique. Sixl et al. (1984) have performed ^2H and ^{31}P NMR investigations of the interaction of myelin basic protein with DMPG in DMPG–DMPC mixtures. They in-

terpret an observed decrease of the quadrupolar splittings for the DMPG head group and no variation of DMPC quadrupolar splittings with increasing protein concentration as an indication of a specific lipid–protein interaction.

It therefore appears that while the interaction of intrinsic proteins with lipid bilayers, as measured by NMR, is relatively uniform among different systems, the interaction of extrinsic proteins with lipids varies considerably from system to system and promises to be a very rich research area.

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