Cytochrome *c* Interactions with Cardiolipin in Bilayers: A Multinuclear Magic-Angle Spinning NMR Study†

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ABSTRACT: The influence of cytochrome *c* binding to cardiolipin bilayers on the motional characteristics of each component has been analyzed by magic-angle spinning (MAS) NMR. Observations were made by NMR of natural abundance ^{31}P, ^{13}C, and ^{1}H nuclei in the lipid as well as sites enriched with ^{13}C in the protein. Analysis of methyl carbons enriched in ([ϵ-$^{13}CH_3$]*methionine*)cytochrome *c* at residues 65 and 80 reveal quite different behavior for these sites when the protein was bound at a 1:15 molar ratio with hydrated cardiolipin. Cross-polarization (CP) shows a single broad resonance downfield in the methyl region which corresponds to the spectral characteristics of methionine 65 in the solution protein when subjected to moderate thermal perturbations. These observations suggest that although methionine 65 remains motionally restricted when the protein binds to the lipid bilayers, this residue becomes less shielded and exposed to more chemically distinct environments than in the native state of the protein. In contrast to its behavior in native oxidized protein, the methionine 80 methyl could be detected following direct $\pi/2$ pulse excitation, and this residue is assumed to be released from the axial ligand site on the heme iron to become more exposed and highly mobile in the protein–lipid complex. An analysis of the CP response for natural abundance ^{13}C nuclei in the lipid reveals a general increase in motions with slower rates (tens of kilohertz) on binding with cytochrome *c*, except for sites within the region of fatty acyl chain unsaturation which appear to be selectively mobilized in the complex with protein. It is concluded that, aside from effects on the unsaturated segments, the bound protein induces new modes of slow motions in the lipid assemblies rather than restricting the overall reorientation freedom of the lipid. The strong paramagnetic effects observed previously on the relaxation of phosphorus in protein–bound lipid [Spooner, P. J. R., & Watts, A. (1991) *Biochemistry 30*, 3880–3885] were not extended to any carbon and proton sites observeable by MAS NMR in the lipid, and this infers a specific interaction of lipid phosphate groups with the heme. However, when protein was bound to cardiolipin mixed at a 1:4 mole ratio with dioleoylphosphatidylcholine in bilayers, no direct interaction with the heme was apparent from the phosphorus NMR relaxation behavior in this component, resolved by MAS. Instead, the spectral anisotropy of cardiolipin phosphorus was determined to be reduced, indicating that, on binding with cytochrome *c*, the headgroup organization was perturbed in this component. Although these organizational effects appeared confined to cardiolipin in the mixed system, this component evidently could not have phase separated sufficiently well on binding with cytochrome *c* to reproduce the more profound effects on the protein observed from undiluted cardiolipin.

One well-documented example of a specific phospholipid type required for functioning of membrane proteins is in the tetraacyl phospholipid CL[1] (diphosphatidyl glycerol) which in mammals is exclusively localized in the membranes of mitochondria. This highly specialized distribution alone implies some functional involvement for CL at this site, and there is good evidence to support this view. For instance, CL is known to be essential for optimal activity of the cytochrome *c* oxidase (Robinson et al., 1980, 1990; Marsh & Powell, 1988). An explanation given for this dependence is that CL promotes binding of the substrate cytochrome *c* at a site of action on this enzyme (Vik et al., 1981) and thereby facilitates the processes which complete the mitochondrial respiratory chain.

Studies using lipid model membranes have shown that CL interacts strongly with cytochrome *c* (Kimbelberg & Lee, 1969; de Kruijff & Cullis, 1980; Demel et al., 1989). Magnetic resonance studies on these systems [reviewed in Watts (1987)] have indicated that cytochrome *c* binding can perturb the lipid so as to induce the formation of nonbilayer states (de Kruijff & Cullis, 1980; Reitveld et al., 1983) or cause CL to phase separate within the bilayer when mixed with a neutral, inert phospholipid (Birrell & Griffith, 1976; Brown & Wüthrich, 1977). Overall, these physical studies with the model membrane systems are suggestive of a rather specific type of interaction between cytochrome *c* and CL, as emphasized recently in a comparison with the binding behavior of the heme-free *apo*cytochrome *c* (Demel et al., 1989). Such specificity is rather difficult to justify from considering just peripheral electrostatic interactions between these species and without the involvement of other modes of interactions. However, Brown and Wüthrich (1977) and more recently Szerbini and Tollin (1988) have argued that cytochrome *c* can penetrate, at least partly, into bilayers containing CL to interact hydrophobically with the membrane interior. Since the native structure of cytochrome *c* is folded to avoid surface exposure of hydrophobic protein segments, these phenomena could be expected to be linked with structural transformations occurring within the protein itself.

In general, it has been tacitly assumed that cytochrome *c* retains its overall native structure on interaction with com-

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[1] Abbreviations: CL, cardiolipin; MAS, magic-angle spinning; DOPC, dioleoylphosphatidylcholine; CP, cross-polarization; T_{1z}, laboratory frame spin–lattice relaxation time; $T_{1\rho}$, rotating frame spin–lattice relaxation time.

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ponents of the inner membrane. In only a few cases have attempts been made to detect any structural changes in the protein which might accompany binding with CL (Jori et al., 1974; Vincent & Levin, 1986, Vincent et al., 1987). Recently, we have used solid-state NMR techniques to address this issue and provide evidence that CL perturbs the backbone structure of cytochrome *c* (Spooner & Watts, 1991a) and also changes the heme configuration to promote conversion to high-spin states for both oxidized and reduced protein (Spooner & Watts, 1991b). These findings strongly suggest that cytochrome *c* reversibly unfolds on binding with CL, causing a loss in the coordination responsible for electronic stabilization of the heme center in the protein. Although these general conclusions were well supported by the work, the solid-state NMR techniques used were not adapted for obtaining specific details on the mechanism underlying these changes in protein structure. The purpose of the current study was to enable observation of particular sites in cytochrome *c* that might report in a more specific way on the nature of these structural perturbations. Of particular interest is the methionine 80 residue which occupies the sixth coordination site on the heme group. This bond between the methionine sulfur and the heme iron is highly strained in the oxidized protein and is particularly sensitive to configurational changes and interactions occurring in the vicinity of the active site (Moore et al., 1982). Detection of this site in the protein is approached here by enrichment of the methionine methyls with ^{13}C and performing MAS[1] to generate spectral resolution for the solid protein or protein–lipid complex in the NMR experiment.

The effectiveness of MAS NMR, combined with isotopic enrichment, for selective observation of sites in membrane proteins is well illustrated by extensive studies recently completed by Griffin and co-workers (de Groot et al., 1989; Smith et al., 1989) on the conformation of the retinal side chain of bacteriorhodopsin in purple membrane preparations. MAS has also been reported to have advantages for obtaining high-resolution natural abundance NMR spectra from multibilayers of membrane lipids (Oldfield et al., 1987; Forbes et al., 1988). The use of MAS NMR was consequently extended in the current study to include an extensive analysis of the behavior of various sites in CL on binding with cytochrome *c*.

Techniques used in the previous studies (Spooner & Watts, 1991a,b) were also not readily amenable to the investigation of cytochrome *c* binding with CL in mixed lipid systems, due to a limited sensitivity or resolution. The current introduction of MAS in this study should enable these problems to be at least partly alleviated, and the technique is applied here in ^{31}P NMR studies on CL mixed in bilayers at a 1:4 mole ratio with DOPC.[1] Results show that MAS NMR can achieve successful observation of specific sites enriched in cytochrome *c* when bound to CL bilayers and provides opportunities for examining a range of lipid sites from natural-abundance nuclei, in the hydrated pure and mixed lipid systems as well as in the protein–lipid complex.

MATERIALS AND METHODS

Cytochrome *c* from horse heart (Grade VI, Sigma Chemical Co.) was purified by ion-exchange chromatography on CM-52 cellulose eluted with 65 mM phosphate buffer at pH 7.0 (Brautigan et al., 1978). The main purified fraction, comprising of nondeamidated monomeric protein, was concentrated by ultrafiltration (Amicon YM-5 membranes) and then extensively dialyzed against distilled water before use. CL from beef heart (Sigma Chemical Co.) was found to contain less than 1 wt % impurity by TLC and was used as supplied in its sodium salt form within 2 weeks of receipt. DOPC (99%; Sigma Chemical Co.) was also used as supplied.

Cytochrome *c* was methylated to form ([ϵ-$^{13}CH_3$]-*methionine*)cytochrome *c* by a procedure similar to that used by Jones et al. (1976) for derivatizing myoglobin. A solution of the protein (0.5 mM) was adjusted to pH 3.0 with HCl and stirred in the dark with a 100-fold molar excess of $^{13}CH_3I$ (99 atom % ^{13}C; Aldrich). After 24 h, a further 100-fold excess of $^{13}CH_3I$ was added and stirred for an additional 24 h, after which the solution of alkylated protein was then dialyzed against distilled water for 24 h. The protein was then demethylated at elevated pH in the presence of dithioerythritol (0.5 M) according to Jones et al. (1976) and then dialyzed extensively to remove the thiol reagent. The protein was finally purified by ion-exchange chromatography and dialyzed to remove phosphate. An aqueous solution of the purified protein showed an identical UV/visible spectra to the native protein (Margolish & Walasek, 1967), and high-resolution NMR on the reduced protein confirmed that ^{13}C enrichment was confined to the methyls of both methionine residues, by comparison with previous assignments (Schejter et al., 1978). Simultaneous incorporation of ^{14}C from radiolabeled iodomethane showed that exchange of the ϵ-methyl in the methionines of cytochrome *c* was within 5% of the 50% theoretically possible by this technique.

Stock solutions of lipids in organic solvent were evaporated under reduced pressure, and the resulting lipid residues were treated under high vacuum for at least 5 h. Aqueous solutions of purified protein were prepared in cacodylate buffer (10 mM) at pH 6.0, containing 0.1 M NaCl and 5 mM EDTA. All solution were saturated with nitrogen prior to combining the protein and lipid, and all sample manipulations were conducted under an atmosphere of nitrogen in order to avoid any oxidation of the lipid species. Lipid residues (typically 150–200 mg) were hydrated with buffer solution (0.2–0.3 mL) and then mixed vigorously with protein solution (1 mL at $\sim$10 mM). The binding with pure CL bilayers was allowed to proceed for 2 h with intermittent mixing, whereas suspensions of cytochrome *c* with mixed lipid bilayers (DOPC/CL) required freeze–thawing three times to ensure a homogeneous mixing of protein and lipid. The protein–lipid complex was pelleted by ultracentrifugation (2×10^5 *g*; 2–5 h), and the clear supernatant was removed for spectrophotometric analysis of free protein. Typically, less than 10% of the protein remained unbound, and at these low aqueous concentrations we estimated that less than 2% of the protein in the pelleted complex was not bound directly to the lipid. Cytochrome *c* was bound at a mole ratio of 1:15 with CL in the pure or mixed lipid bilayers, as used for the binding stoichiometry in previous experiments (Spooner & Watts, 1991a,b). Samples of the protein–lipid complex were sealed under an atmosphere of nitrogen in MAS sample rotors for analyses by the NMR procedures described below.

Nuclear Magnetic Resonance. MAS NMR measurements were carried out using a Bruker MSL spectrometer operating at 400 MHz for protons, 161.98 MHz for phosphorus, and 100.63 MHz for the carbon-13 nuclei. Sample spinning was accomplished in Bruker double-bearing MAS probeheads for 4- or 7-mm sample rotors with the bearing air supply being controlled at 25 °C unless otherwise specified. Kel-F® inserts were sometimes used to facilitate uniform packing of samples in the larger 7-mm rotors, and all rotors were sealed with nonvented caps to prevent access to air. CP MAS experiments for ^{13}C detection were conducted using the conventional

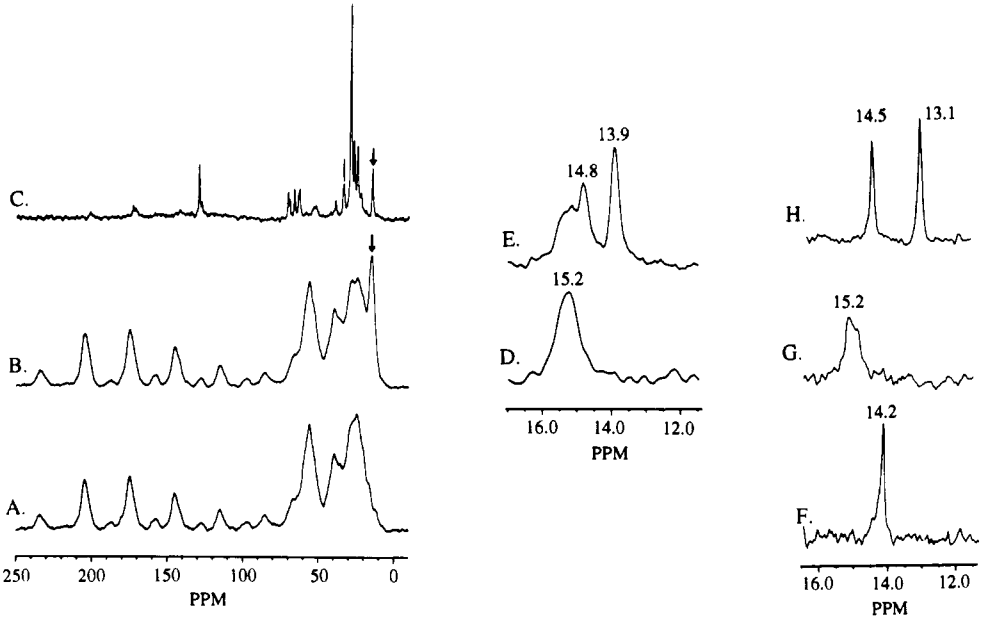

FIGURE 1: Proton-decoupled ^{13}C MAS spectra of native cytochrome *c* in the lyophilized state (A) and ([ϵ-$^{13}CH_3$]*methionine*)cytochrome *c* either lyophilized (B) or combined at a 1:15 mole ratio with CL (C) and showing resonances from ^{13}C-enriched sites (arrowed), all having been recorded from CP with a 2-ms contact time and a 3-s recycle time. Methyl regions from NMR spectra show the resonance detected by CP (D) and the net methyl spectral intensity observed from simple $\pi/2$ pulse excitation (E) with a recycle time of 8 s and from 2500 aquisitions for both spectra. High-resolution proton-decoupled ^{13}C NMR spectra from ([ϵ-$^{13}CH_3$]*methionine*)cytochrome *c* in solution show the methyl regions recorded in buffer at pH 6.0 and 25 (F) or 55 °C (G) and at 25 °C from the reduced protein (H). Spectra from solutions shown here were recorded from around 3 mM concentrations of protein and with 1600–4000 aquisitions using a 1-s recycle time. Chemical shifts are referenced to TMS using the lipid chain methyl as an internal reference for the MAS spectra (13.9 ppm in panel E).

pulse sequence in which a field strength of 30 kHz was applied to protons in the sample for the initial $\pi/2$ pulse as well as for the subsequent spin-locking and decoupling periods. The field applied to the carbon spins to establish contact with spin-locked protons was adjusted until the optimal Hartmann–Hahn match was observable under these conditions for the sample of interest. The duration of this contact period was varied in order to monitor CP dynamics in particular samples. These power levels typically resulted in $\pi/2$ pulse widths of 8–9 μs for both ^1H and ^{13}C, and the CP conditions were established on the individual samples themselves, since there are no standards appropriate for these "lossy" samples. This task is feasible with the sensitivities afforded at high-field and by the CP probeheads currently available. The relatively mild conditions used to establish CP avoided excessive sample heating (Spooner et al., 1990) and are not expected to significantly affect the behavior or stability of samples during the measurements. The same power levels were also used to conduct measurements on ^1H and ^{13}C from single-pulse excitation. ^{31}P MAS spectra were recorded following a 5-μs $\pi/2$ pulse and using a ~25 kHz proton decoupling field. ^{31}P measurements on static samples were made using the same pulse width and proton decoupling in the Hahn echo pulse sequence ($\pi/2$–τ–π–τ–aquire). Spin–lattice relaxation times (T_1) were measured from the π–τ–$\pi/2$–aquire inversion–recovery pulse sequence, using a recycle time of at least 5 times T_1. NMR transients were co-added within a sequence of phase cycling appropriate for mimimizing artifacts in detection and were processed with 5–50 Hz of exponential filtering prior to Fourier transformation. Carbon-13 NMR measurements on protein in solution were conducted at 90.57 MHz (360 MHz for protons) using a home-built spectrometer with conventional high-resolution probeheads.

Carbon-13 chemical shifts from MAS NMR were routinely calibrated according to an internal reference of 13.9 ppm (versus TMS) for the chain methyl resonance recorded from the lipid in the bilayers or protein–lipid complexes. The use of an internal reference was preferable for the MAS NMR experiments which were conducted without any provision for locking the field frequency. High-resolution NMR chemical shifts from solution samples were directly referenced to external TMS, and all phosphorus chemical shifts are reported with respect to 85% H_3PO_4.

RESULTS

^{13}C-Enriched Cytochrome c. The proton-decoupled ^{13}C CP MAS spectrum of unmodified cytochrome *c* in the lyophilized solid state is shown in Figure 1A. This merely displays broad and featureless resonances with associated rotational side bands that extend over a wide frequency range. The spectrum from lyophilized ([ϵ-$^{13}CH_3$]*methionine*)cytochrome *c* in Figure 1B is similar to the spectrum in Figure 1A but shows pronounced intensity in the upfield region (arrowed) from enrichment of methyl sites in methionine residues. When the labeled protein was complexed with hydrated CL bilayers, this high-field intensity could be well resolved from natural abundance ^{13}C resonances which arise mainly from the lipid component, as shown in Figure 1C. This CP, using moderately long contact times (2 ms), was confined to a single methyl resonance as shown in the expanded region of the spectrum in Figure 1D. This resonance is, however, quite broad, having a line width of around 80 Hz. The labeled protein in aqueous solution also produced a single intense methyl resonance from conventional ^{13}C high-resolution NMR analysis although, under the same conditions of temperature and aqueous pH, this resonance is much narrower and appears farther upfield than that from the complex, as shown in Figure 1F. As previously reported by Schejter et al. (1978), this resonance arises from the methyl of methionine 65 alone, while the NMR signal from enrichment in methionine 80 is assumed to be shifted and broadened beyond detection due to a *contact* effect from the heme to which methionine 80 is coordinated. Both sites of enrichment are, however, observeable from the reduced protein in solution, as shown in Figure 1H, in which

the methionine 80 methyl remains well shielded and appears upfield in the spectrum at 13.1 ppm, as also reported previously (Schejter et al., 1978).

On heating the oxidized protein in solution, the methionine 65 resonance shifts downfield and broadens until at 55 °C it resembles closely the resonance detected from the lipid-complexed protein by CP, as shown in Figure 1G. At intermediate temperatures, the broadened methyl intensity often appeared heterogeneous, comprising of a number of overlapped resonances, although no significant changes in spectral intensity within the methyl region (within ±10%) were noted under conditions expected to provide essentially equilibrium magnetization (recycle time = 8 s). Therefore, these changes in the spectrum from the solution protein still do not appear to involve the participation of methionine 80 methyl which can be assumed to remain obscured by ligation to the heme. At temperatures in excess of 60 °C where dissociation of this ligand becomes significant (Angström et al., 1982), the protein did not remain sufficiently stable for the analysis by solution state NMR. On the basis of the above observations, it is concluded that the methyl resonance detected from the complexed protein by CP (Figure 1D) comprised of intensity from the methionine 65 methyl and that this residue becomes exposed to less shielded and more heterogeneous environments than in the native state of the protein. However, the CP data were not amenable to rigorous quantitative interpretation of spectral intensities and so could not be confirmed in this way to represent a measurement of methionine 65 alone. Evidence for this and a more accurate reflection of the coordination state of the heme is gained from a direct irradiation of the carbons by nonselective $\pi/2$ pulses, as used for the conventional solution-state NMR measurements. This provides a more complex methyl spectrum as shown in Figure 1E and consists of a downfield composite component and a narrow component resolved upfield which is readily assignable to methyls from the lipid chains (13.9 ppm). The remaining overlapped intensity from the protein methyls not only shows a broad component corresponding to that detected by CP but now also comprises of a narrow resonance located on the high-field edge (14.8 ppm) of this envelope. The ratio of total protein to lipid methyl spectral intensity was 1.7 ± 0.1, measured from three samples with recycle delays allowing essentially equilibrium magnetization (8 s). This is somewhat greater than the intensity ratio of 1.3:1 expected from enrichment of methionines in the protein complexed at this stoichiometry, indicating that both methionines are detected together with some nominal intensity from natural abundance sites in the protein. The recovery of the full intensity for methyl enrichment from the protein along with the discrimination of the methionine 65 methyl intensity, ostensibly provided by the CP spectrum, allows the additional narrow protein resonance to be attributed to methionine 80 becoming observable under these conditions of direct carbon irradiation. It can further be concluded that methionine 80 was not detected in the CP analysis. Successful CP is favored for the more rigid sites, and therefore methione 80 in the complexed protein, along with the lipid methyls, appeared too mobile for detection under these conditions.

The chemical shift of the narrow methionine 80 resonance varied between 14.7 and 15.0 ppm from the samples used for the above quantitation of intensities. Given the magnitude of the shift from the resonance position in reduced protein (13.1 ppm) and the sensitivity of the methionine 65 methyl shift to quite moderate thermal perturbations, this variation is considered to represent only small changes in the state of the bound protein. From one complex, this resonance appeared at 14.1 ppm, but this outlying sample did not allow any separate quantitation of the broad component, assigned to methionine 65, since this intensity alone was not well resolved by the direct irradiation method. Intensity in this region is greatly enhanced upon heating the complex to 55 °C, but this was estimated to be some 3-fold greater than expected from methionine 65 alone. Therefore, moderate heating introduced large natural abundance contributions from the protein, due presumeably to strong perturbations in the protein structure at this temperature. Nevertheless, the quantitation already specified for the composite peaks provides adequate justification for the assignments made for both methionine sites in the complexed protein.

As with the methionine 65 methyl, the methionine 80 methyl becomes substantially deshielded on binding the protein with the CL bilayers. This residue thus was removed from the strong shielding afforded by the protein heme, which was responsible for the highfield location of this methyl resonance in the reduced protein (13.1 ppm; Figure 1H). No spectral changes could be observed on heating the labeled ferrocytochrome *c* in solution, prior to loss of sample integrity upon reaching temperatures around 80 °C. However, this protein has been found to exhibit similar spectral characteristics as described for the oxidized state when bound to CL bilayers and examined by the MAS NMR techniques.

The combined use of CP and direct irradiation of carbon nuclei thus provided a strategy for observation and reasoned assignment of both methionine sites enriched in the protein and illustrates their diverse motional behavior in the protein–lipid complex.

Natural Abundance ^{13}C NMR of CL. The sensitivity of the MAS technique allows well-resolved natural abundance ^{13}C spectra to be obtained with relative ease from the lipid in pure hydrated bilayers or when combined with the protein. Complexation with cytochrome *c* has little effect on the lipid spectra recorded from a simple $\pi/2$ pulse, causing some line broadening, particularly for glycerol resonances in the region of 60–75 ppm. There is consequently no evidence from ^{13}C NMR analysis that the lipid has been chemically degraded on complexation with cytochrome *c*, in agreement with other analyses (see Materials and Methods). However, measurements made from a CP experiment, as used for the spectrum shown in Figure 1C, indicate that the protein influences rates of polarization transfer in the lipid. Some of these effects on CP to ^{13}C sites are shown by the spectra in Figure 2, recorded from using a 10-ms contact time for CP. These lengthy contact times improve the rather inefficient polarization transfer for lipid sites and suppress spectral contributions from the protein which decay more rapidly compared with those from the lipid. Consequently, the spectrum in Figure 2B from the protein–lipid complex shows very little resolvable protein contributions under these conditions. However, different rates of polarization transfer compared with the bilayers of pure lipid (Figure 2A) are evident, particularly for certain olefinic sites in the vicinity of 130 ppm. The natural CL from mammalian tissue used here is almost exclusively esterified with linoleoyl (*cis*-18:2-$\Delta^{9,12}$) fatty acyl chains (Smaal et al., 1985). The upfield olefinic component, which is strongly suppressed in the spectrum from the complex (Figure 2B), can be assigned to the pair of olefinic carbons located centrally within the linoleoyl unsaturated segment and designated $\Delta^{10,12}$, while the downfield component is assigned to the olefinic carbons at both ends of the unsaturated region, $\Delta^{9,13}$. Binding with cytochrome *c* also induces a different NMR spectral response

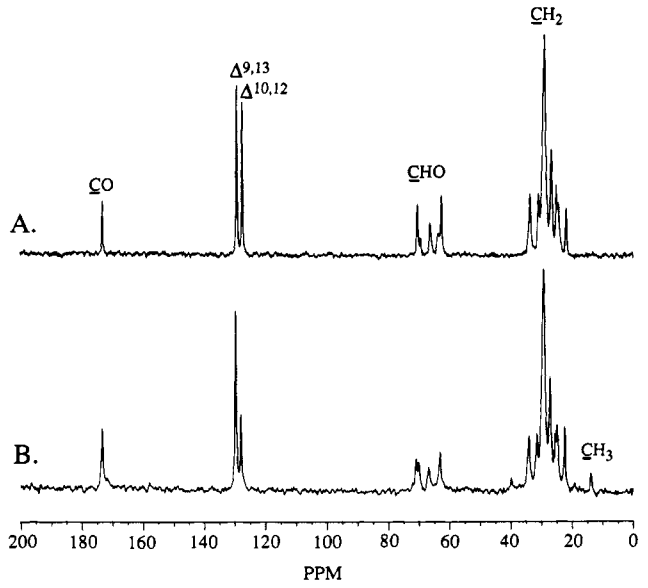

FIGURE 2: CP MAS spectra from natural abundance ^{13}C in hydrated CL alone (A) or bound to cytochrome *c* (B), recorded from 256 aquisitions at a 3.0-kHz rotation speed, using a 10-ms contact time and a 3-s recycle time. Assignments are for the fatty acyl chain methyl ($\underline{C}H_3$) and methylenes ($\underline{C}H_2$) and glyceryl ($\underline{C}HO$) and olefinic carbons at their respective chain positions ($\Delta^{10,12}$, $\Delta^{9,13}$) and the carbonyls ($\underline{C}O$).

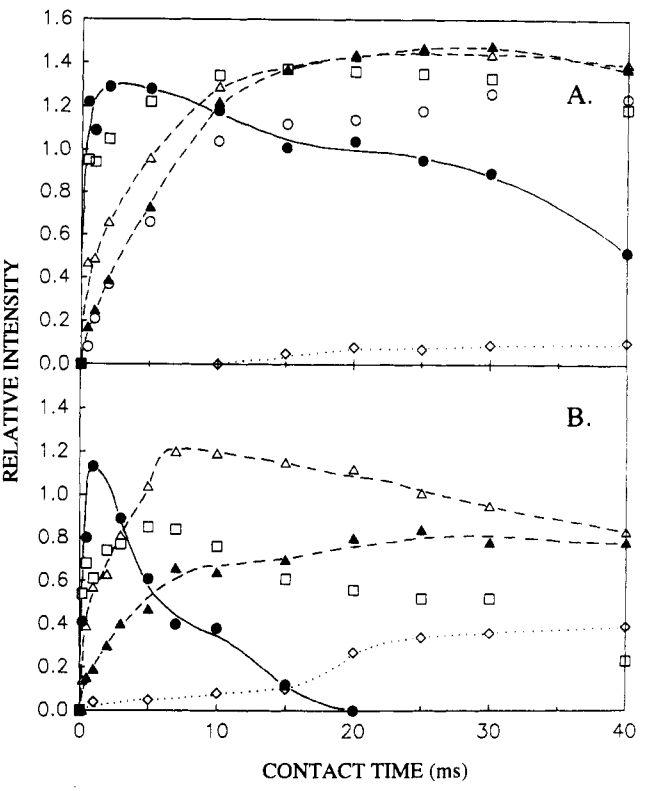

FIGURE 3: CP response as a function of contact time of the natural abundance ^{13}C nuclei in hydrated CL alone (A) and bound to cytochrome *c* (B). Intensities are expressed relative to those observed from using a simple $\pi/2$ pulse excitation to acquire the same number of transients (256). Data are for glyceryl carbons (filled circle, solid line), olefinic carbons $\Delta^{10,12}$ (filled triangle, broken line) and $\Delta^{9,13}$ (open triangle, broken line), methyl (diamond, dotted line), methylene (open square), and carbonyl carbons (open circle). The carbonyl data not included in panel B showed a similar initial rate of intensity increase as in panel A but decrease to coincide with the data for the methylenes in B.

from the glyceryl sites of CL and enhances detection of fatty acyl methyl resonances, which do not appear in the spectrum of pure CL bilayers (Figure 2A). To characterize more fully these changes, the CP dynamics were monitored over a wide range of contact times and are quantified in terms of spectral intensities in Figure 3 for CL bilayers alone (A) and complexed with cytochrome *c* (B).

The initial buildup in ^{13}C NMR spectral intensities reflect the CP rates for the respective sites. These rates are a function of the strength of dipolar interactions, which are in turn determined by the abundance of protons and degree of motional averaging at these sites. CP rates are slow for these mobile liquid-crystalline systems, as are the rates of polarization decay which are observable at long contact times where loss of magnetization to the lattice begins to dominate. The intensity maxima are consequently attained only at long contact times (20–30 ms for most sites) and barely exceed the sensitivity afforded by conventional single-pulse observation. This CP behavior can be assumed to be determined by the motional behavior of the lipid rather than by proximity of protons, which is usually the case for CP in less-mobile, solid-like materials. The contrasting CP behavior shown in Figure 3A for the glyceryl and terminal methyl segments of CL thus identifies these as being the least and most mobile sites, respectively, in the lipid molecule. Apart from these extreme cases, most other lipid sites show quite similar spectral intensity profiles in Figure 3A. Some irregular fluctuations observed in the CP profiles are probably indicative of difficulties in maintaining optimal spin temperatures for polarization transfer between proton and carbon populations (Schaefer et al., 1984), but these effects do not interfere greatly with comparisons made here of relative CP and relaxation rates.

Binding with cytochrome *c* has a profound effect on the CP behavior of CL as shown in Figure 3B. Most of the lipid sites detected in the protein–lipid complex show an earlier and more rapid loss of intensity due to enhanced relaxation during CP, compared with the pure hydrated lipid. Most CP rates, however, are not noticeably altered, and so intensity maxima are shifted to shorter contact times and provide a lower detection sensitivity in the presence of protein. These protein effects are particularly pronounced for the glyceryl regions where the optimal contact time is reduced to around 1 ms. Optimal contact times of this order are typical for the crystalline solids, although the sensitivity observed here is far less than achieved by CP in rigid materials. Apart from the terminal methyl groups which show an somewhat improved CP efficiency, there is no evidence that binding with protein enhances dipolar interaction by restricting reorientational motion in the lipid.

The response of the upfield olefinic resonance ($\Delta^{10,12}$) is quite distinct from the general effects of cytochrome *c* described above. Here, the CP rate is reduced while the relaxation rates are not noticeably altered, compared with the behavior of these sites in unbound CL. This results in a gradual buildup in spectral intensity over the whole range of contact times used, and these sites now show a pronounced lag in CP response compared with the other olefinic carbons ($\Delta^{9,13}$).

The data reported here were recorded under identical spectrometer conditions for generating CP within the samples. Separate experiments on duplicate samples show some quantitative differences in CP rate and sensitivity due to small variations in setting up the CP condition. However these repeat experiments always reproduce well the trends reported above for differences between the CP behavior at individual sites in the lipid and on binding with cytochrome *c*. A clearer interpretation of the mechanism responsible for these observations is possible with reference to the spin–lattice relaxation data presented below.

Table I: Laboratory Frame Spin–Lattice Relaxation Times (T_{1z}) for Natural Abundance ^{13}C, ^{1}H, and ^{31}P in the Hydrated Lipid Bilayers Alone and When Complexed with Cytochrome *c* at a Mole Ratio of 1:15 with CL[a]

	T_{1z} for hydrated pure lipid (s)		T_{1z} for lipid–protein complex (s)	
	25 °C	50 °C	25 °C	50 °C
^{13}C CL				
$\underline{C}H_3$	2.89		3.96	
$\underline{C}H_2$	0.72		0.59	
$\underline{C}HO$	0.22		0.20	
$\underline{\Delta}^{10,12}$	1.08		0.98	
$\Delta^{9,13}$	1.09		1.02	
$\underline{C}O$	1.67		1.64	
^{1}H CL				
$C\underline{H}_3$	1.22	1.39	0.43	0.63
$C\underline{H}_2$	0.68	1.04	0.35	0.50
$C^{8,14}\underline{H}_2$	0.63	0.86	0.37	0.48
$C^{11}\underline{H}_2$	0.59	0.83	0.34	0.52
$C\underline{H}O$	0.58	0.53	0.29	0.27
$\underline{\Delta}$	0.94	1.06	0.26	0.46
^{31}P CL[b]	0.94		0.13	
CL[c]	0.93		0.94	
DOPC[c]	1.02		1.03	

[a] All measurements were made using MAS NMR at the temperatures indicated. The various ^{13}C and ^{1}H lipid sites correspond to those identified in Figures 2 and 5. [b] From Spooner and Watts (1991b) on bilayers of pure CL. [c] In mixed bilayers of CL and DOPC (1:4 mole ratio) from the current study.

Spin–Lattice Relaxation. Laboratory frame spin–lattice relaxation times (T_{1z}) for nuclei in hydrated CL alone or bound with cytochrome *c* are shown in Table I. These measurements were made by combining MAS methods with a direct irradiation of spins from a conventional pulse sequence (see Materials and Methods). The protein induces only a small increase in relaxation rates for all carbon sites except for the terminal methyl group, which showed a decrease in relaxation rate. This loss of carbon magnetization to the lattice therefore remains slow and can be assumed to have no effect on the CP behavior described above.

MAS also provides fairly good spectral resolution for protons in hydrated CL as seen in Figure 4A. Laboratory frame spin–lattice relaxation times of these sites are included in Table I from measurements at 25 and 50 °C. Increasing temperature caused a reduction in relaxation rate for all except the glycerol segment, where a small decrease in T_{1z} is observed. This defines the correlation times (τ_c) of high-frequency motions responsible for T_{1z} relaxation as being fast with respect to ω_o, the proton Lamor frequency (i.e., $\tau_c < 1/\omega_o$), with the exception of motions in the glycerol segments. The ^{1}H MAS spectrum of the protein–lipid complex is not so well resolved as for the lipid alone, as shown in Figure 4B. The spectrum contains broad components from the protein showing that MAS at typical rotation speeds (<5 kHz) is much less effective for averaging the strong dipolar interactions at proton sites in the bound protein. This spectrum also contains a more pronounced water resonance which orignates from protons released from the protein, and this is exchange-broadened to overlap to some extent the glycerol and olefinic resonances from the lipid. Broad spectral contributions were subtracted where possible to assist in the deconvolution of the lipid intensities for integration. Although the relaxation data arrived at in this manner cannot be expected to be as reliable as from the better resolved spectra from pure lipid, each set could be fitted well to a single time constant, and the values of T_{1z} so obtained are shown in Table I to be all somewhat less than those for pure CL at the same temperature. The

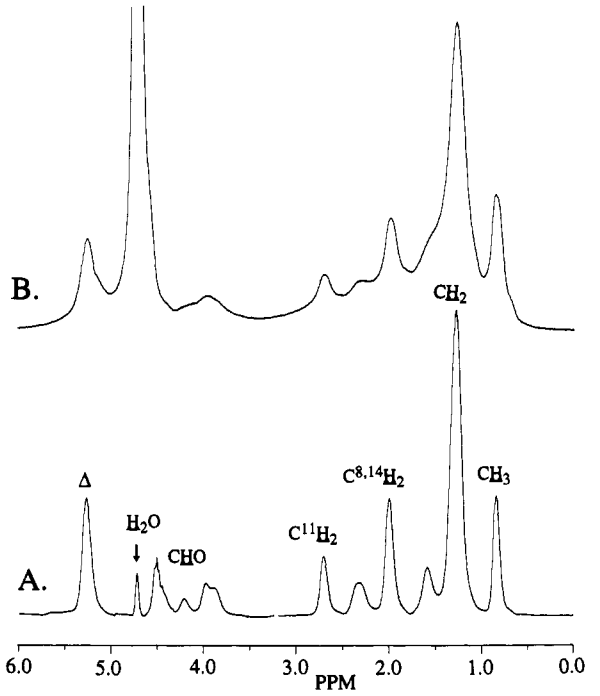

FIGURE 4: ^{1}H MAS NMR spectra from hydrated CL alone (A) and bound with cytochrome *c* (B) at a 15:1 mole ratio. Measurements were made at 4-kHz rotation speed from 16 aquisitions and using a 1-s recycle time. Assignments have identical nomenclature to those in Figure 2. The peak Δ is comprised of around 10% intensity from a nonresolved glyceryl proton resonance (from the *sn*-1 position on the side-chain segments), but this did not appear to significantly affect the analysis of T_1 data for this site.

increased temperature also yielded a similar increase in the lipid proton T_{1z} values from the complex, as observed for pure CL bilayers. Therefore, although the lipid motions which determine laboratory frame spin–lattice relaxation in the complex appear to remain in the fast regime, the contribution of these motions to spectral densities are somewhat decreased upon binding to the protein.

The moderate effects of protein binding on laboratory frame spin–lattice relaxation (T_{1z}) in the lipid again suggest no strong motional restriction of the lipid component but do point to an increase in the spectral densities of slower motions that enhance spin–lattice relaxation in the rotating frame ($T_{1\rho}$). It is this relaxation process that acts on the spin-locked protons in the contact period to deplete ultimately the available polarization for transfer to carbon spins. The slower motions affecting $T_{1\rho}$, which have frequencies on the order of the proton spin-locking frequency (tens of kilohertz), are thus enhanced on binding with the protein for all lipid sites detected, apart from the olefinic carbons, $\Delta^{10,12}$.

Lipid Mixtures: ^{31}P NMR Measurements. The ^{31}P NMR "powder-type" spectra shown in Figure 5 were recorded from static samples of hydrated mixtures of CL and DOPC (1:4 mole ratio) with (B) and without (A) cytochrome *c* bound at the 1:15 mole ratio with respect to the CL component. The broad distribution of intensity in both spectra is composed of an intense high-field peak and shallow low-field shoulder, which is typical of line shapes recorded from phospholipids in the bilayer configuration. The spectra from the two lipid components are shifted slightly with respect to one another to create some distortions in the overall line shape. However, this displacement is quite small compared with the overall chemical shift dispersion, such that the breadth of the spectrum in Figure 5A from the lipid mixture (–55 ppm), which provides a measure of the residual ^{31}P shielding ani-

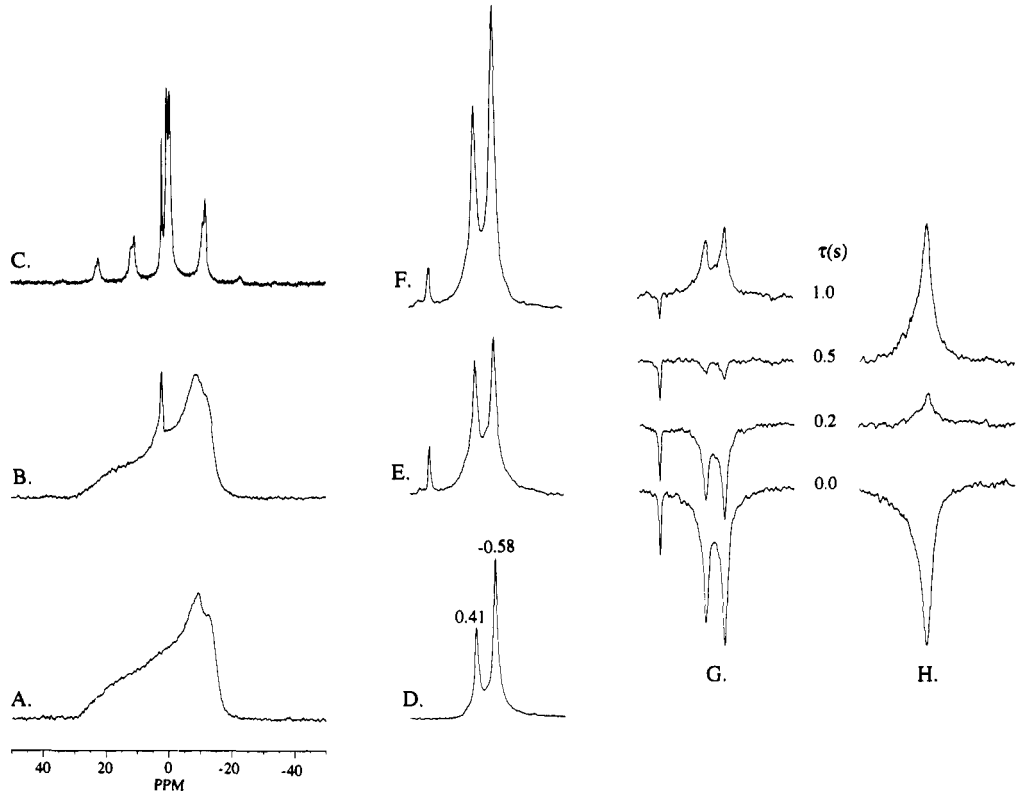

FIGURE 5: ^{31}P NMR spectra from static samples of a hydrated mixture of CL and DOPC (1:4) alone (A) and bound with cytochrome *c* at a 15:1 mole ratio (B). The change in the spectrum from conducting MAS on the protein–lipid complex at a slow rotation speed (1.8 kHz) is shown in panel C. Isotropic regions of the spectra from the lipid mixture alone (D) and complexed with cytochrome *c* (E), both at 4-kHz rotation speed and from the complex at 8.0-kHz rotation speed (F). The downfield component at 0.41 ppm is from CL, and all chemical shifts are referenced to 85% H_3PO_4. Examples are given of spectra from the inversion–recovery experiment on the bilayers of CL (H) or CL mixed with DOPC (G), both bound with an equivalent amount of cytochrome *c* with respect to the CL component (1:15 mole ratio) and with delay times (τ) used between the inversion and observe pulse, as indicated. The T_{1z} for CL phosphorus in the complex used for spectrum H was 0.23 s. All spectra were recorded at 25 °C from the following number of aquisitions: 128 (A), 256 (B, C, G. H), and 512 (D–F). Recycle times were 1 s, except for inversion–recovery spectra.

sotropy, is no greater than that measured for the lipid components separately in their nonmixed bilayers. Indeed, most phospholipid mixtures in bilayers reveal an overall chemical shift dispersion close to this value, including the complex mixtures of lipids present in natural membranes (Cullis & de Kruijff, 1979).

The breadth of the ^{31}P "powder" spectrum of the phospholipid mixture in fact shows no significant change (<5 ppm) on binding with cytochrome *c*. As shown in Figure 5B, the "powder" spectrum of the lipid with bound cytochrome *c* has somewhat less intensity in the low-field region and an isotropic peak, probably representing a small population of phospholipid executing less restricted motion and possibly existing within smaller structures.

MAS causes the "powder" spectra to collapse into a set of central isotropic components and an array of rotational side bands, located at multiples of the rotational frequency from the isotropic shifts. These components are well illustrated in the spectrum recorded at relatively low rotation speeds (1.8 kHz) and shown in Figure 5C. MAS at higher rotation speeds (~4 kHz) affords good resolution between the isotropic resonances from CL and DOPC in the pure lipid mixture as indicated in the expanded isotropic spectral region in Figure 5D. The intensities of these components also closely reflect their respective chemical proportions of 2:1 in favor of the DOPC phosphorus in the lipid mixture. As expected, there is therefore insufficient difference in the residual shielding anisotropy between the two phospholipids to cause a significant differential loss in intensity to rotational side bands. However, this close stoichiometric ratio of isotropic intensities is lost on binding with cytochrome *c*, as shown in the spectrum in Figure 5E. Here, the relative isotropic spectral intensity from the CL phosphorus is increased, indicating a change in the chemical shielding anistropy in the mixture. Since the breadth of the "powder" spectrum and thus the maximum residual anisotropy was not greatly effected upon binding with cytochrome *c*, the change must result from a decrease in anisotropy of the CL phosphorus, which will concentrate more intensity in the isotropic resonance of this component. It would obviously be preferable to confirm this interpretation by conducting a detailed analysis on the rotational side bands, whose intensities contain explicit information on the residual shielding anisotropies in the mixture. Unfortunately, the slow rotation speeds required for reliable determination of intensities over a number of orders of these side bands did not provide sufficient resolution between the two lipid resonances (see Figure 5C). However, the conclusion that cytochrome *c* binding causes a reduction in the residual shielding anisotropy of the CL phosphorus in the mixed bilayers receives additional support from observations at even greater rotation speeds (8 kHz). This further diminishes the influence of these anisotropic effects on the spectrum so that, as shown in Figure 5F, the isotropic intensities from the complex again approach the stoichiometric relationship of the two lipid phosphates.

Rather surprisingly, the small isotropic component superimposed on the "powder" spectrum from the protein-complexed lipid did not correspond to either isotropic shift but appeared as a minor resonance resolved at +2 ppm in the MAS spectrum (Figure 5E, F). It appears, therefore, that a small proportion of protein-bound lipid is isolated in an

environment which prevents molecular exchange on this time scale (10^{-2}–10^{-3} s) and where the phosphates are deshielded compared with the normal bilayer state.

The changes observed in the ^{31}P MAS NMR spectra were not influenced by phosphorus spin–lattice relaxation, which is unchanged in both components on binding with cytochrome *c*, as shown by the data included in Table I. This contrasts with previous observations on binding with bilayers of CL alone (data also in Table I), in which cytochrome *c* was shown to induce a marked enhancement in the phosphorus spin–lattice relaxation (Spooner & Watts, 1991b). These differences are illustrated by the spectra in Figure 5 from the inversion–recovery experiments on the complex prepared with the mixed lipid bilayers (G) or CL alone (H). While the relaxation behavior of CL phosphorus in the lipid mixture is indistinguishable from that observed without protein, the lipid phosphorus in the protein complex with CL alone clearly relaxes at a much faster rate and displays a much broader line shape.

DISCUSSION

Cytochrome c Dynamics. Previous results (Spooner & Watts, 1991b) suggest that binding with CL can convert cytochrome *c* to a high-spin state and that the lipid phosphorus interacts directly with the protein heme in this altered configuration. High-spin ferricytochrome *c* has been proposed as a labile intermediate formed during heme complexation with small anionic ligands (Sutin & Jandell, 1972) and arises from displacement of the labile axial ligand (methionine 80) to the heme iron. The analyses reported here on ([ϵ-$^{13}CH_3$]-*methionine*)cytochrome *c* provide strong evidence that the methionine 80 ligand is indeed diplaced from the heme on binding to CL. This site could be detected at high-resolution in the NMR measurements, demonstrating that the *contact* effect of the heme group is absent in the complexed state. Also, in common with the lipid methyls, the methionine 80 methyl did not cross-polarize detectably when employing moderate contact times for magnetization transfer. This site therefore experiences only weak dipolar interactions from associated protons as a result of its high mobility in the lipid-complexed protein.

Detachment of methionine 80 from its axial coordination site should allow its associated backbone domain to move away from the heme group to convert the protein to a *groove-open* state (Williams, 1989). The observation of freely mobile methionine 80 reported here is therefore fully consistent with the structural relaxation required to permit direct interaction of an exposed heme with the anionic headgroups of CL, as proposed in previous studies (Spooner & Watts, 1991b).

In contrast to the behavior of methionine 80, the methionine 65 methyl in the complexed protein appeared to be cross-polarized efficiently, and its large spectral line width rendered this site poorly resolved in single-pulse experiments at ambient temperatures. This broadening suggests that methionine 65, located within a short α-helical segment that abuts one face of the heme in the protein (Gao et al., 1989), is also perturbed by binding with CL so that this residue experiences chemically different environments in the complex. Evidently, methionine 65 in the complexed protein does not possess sufficient mobility to average these inhomogeneous interactions. The backbone structure is, however, clearly destabilized in the complexed state since appreciable motion was indicated at temperatures (55 °C) which do not strongly perturb free protein in solution (Moore & Williams, 1982). This conclusion is in agreement with recent evidence from FT-IR spectroscopy and calorimetry that the thermal stability of cytochrome *c* is substantially reduced on binding with bilayers of anionic lipid (Muga et al., 1991).

The finding that conformational perturbations from binding with CL extend to backbone regions of cytochrome *c* involved in the native secondary structure was to be expected from previous studies showing that no amide sites within the native helical segments of protein were immobile on a short time scale (10^{-6} s) following binding with CL bilayers (Spooner & Watts, 1991a). The methionine 65 motions, however, appeared to remain quite restricted, indicating that perturbations at least in this region of the backbone may be limited to short-range fluctuations rather than any extensive conversion to a random-coil configuration. The evidence given here for rather restricted perturbations in the protein backbone raises the possibility that some native structure is still retained in the complexed protein. Recently, Roder and co-workers (Jeng et al., 1990) determined that another perturbed state of cytochrome *c*, the acid-denatured or globular A-state, retains all the major native-like secondary structure while some elements of tertiary structure are lost within this compact configuration of the protein. This state also lacks the native methionine ligand with the heme (Goto et al., 1990). The majority of amide sites sites in the native α-helices of the protein were found to be still afforded protection by inter-residue hydrogen bonding (Jeng et al., 1990) although these protons generally exchange at a much faster rate than in the unperturbed protein. It is possible that our direct ^{2}H NMR observation on the amide sites in cytochrome *c* were much more sensitive to perturbations of the hydrogen-bonding network within helical segments of the protein than is net amide exchange with bulk solution deuterons as used to characterize the A-state of cytochrome *c* (Jeng et al., 1990). It is still feasible, therefore, that certain elements of native structure can exist in the lipid-perturbed cytochrome *c* although this is likely to be dynamically quite distinct from the fully folded configuration. Indeed, de Jongh et al. (1992) have recently proposed a highly dynamic folded state of cytochrome *c* associated with surfactant micelles, on the basis of the rapid rates of amide-exchange observed from the protein in this state.

In general, the results reported here are fully consistent with and reinforce previous findings that binding with CL induces extensive structural perturbations in cytochrome *c* and that the lipid headgroup can interact directly with the heme which has undergone conversion to a high-spin state. According to previous results (Spooner & Watts, 1991b), this interacting lipid is required to exchange on a time scale which is generally orders of magnitude faster than observed from binding of simple anionic species (Sutin & Jandell, 1972). It is reasonable to assume therefore that the kinetics of complexation with the heme is driven in this instance by motional processes that are unique to the protein complexed with the lipid in bilayers. Further details of these dynamic properties are evident from the examination of the lipid component in the complex conducted here and these results are discussed below.

CL Dynamics. The method employed here of monitoring the CP behavior in CL can detect modulations in the dipolar interactions at a range of sites in the lipid and was particularly sensitive to fluctuation on the slow time scale which affect the proton $T_{1\rho}$ relaxation rate. The CP profiles provide a particularly graphic illustration of how binding with cytochrome *c* specifically enhances these slower motions in the lipid. However, the CP rates show no enhancement in the

dipolar interactions on binding with cytochrome *c* to indicate that lipid became more immobile in the complex, and so the slow motions would appear to be introduced as an additional perturbation from the protein. Although slow in terms of reorientational motion within the bilayer lipid molecules, these motions would be sufficiently fast to reflect molecular exchange involving lipid complexed with the heme in cytochrome *c*, as predicted previously (Spooner & Watts, 1991b) and considered further in the preceding discussion.

Quite different effects are detected for the central olefinic sites, $\Delta^{10,12}$, which appear to experience a suppression of dipolar interactions in the protein–lipid complex. According to the CP profiles, dipolar interaction in this segment are even reduced compared with the other olefinic sites, $\Delta^{9,13}$ in the unbound lipid. Although small in the case of pure lipid, this effect is rather surprising since unsaturated regions of fatty acyl chains are generally viewed as rather rigid segments, such that segmental motions are expected to be quite reproducibly transmitted through these regions of the fatty acyl chain. Such restricted motions might also be expected to provide more rapid and efficient CP for olefinic segments than observed here. However, recent ^{2}H NMR measurements on a linoleoyl chain incorporated into a hydrated PC have revealed that the polyunsaturated segment undergoes quite unexpected motional behavior (Baenziger et al., 1988). The central methylene located within the polyunsaturated region, and labeled for this study, showed motional fluctuations of large amplitude and an average orientation that is essentially orthogonal to that expected for methylenes in the saturated region of the chain, suggesting that the polyunsaturated segment is exchanging rapidly between two conformers. Such behavior can be expected to attenuate dipolar interactions at the adjacent olefinic sites, $\Delta^{10,12}$, more effectively than at the more remote sites, $\Delta^{9,13}$, which are presumeably bonded to methylenes with more typical motional characteristics. Therefore, conformational fluctuations of this nature may be the reason why CP is generally poor for the olefinic segment and rather slower for olefinic carbons located centrally within the unsaturated segment ($\Delta^{10,12}$). The observation that binding with cytochrome *c* futher restricts the CP response at central olefinic sites implies that the protein specifically enhances these large amplitude motions that attenuate dipolar interactions within the polyunsaturated region.

The only other specific information reported on the effects of cytochrome *c* on the fatty acyl chain dynamics in CL is from resonance Raman spectroscopy (Vincent & Levin, 1986). From this study, the protein was adjudged to induce an overall increase in conformational disorder in the lipid chains and also appeared to have some selective influence on the olefinic chain segment. These conclusions do not conflict with the effects observed here but rather reflect the difficulty in interpreting an overall motional state from particular modes of molecular motion detected by any one technique. To assist in this process, it is desirable to employ techniques which are sensitive to a wide variety of motional modes and rates. We have attempted to achieve this here by a comparing rates of CP, laboratory frame relaxation, and rotating frame relaxation. The analysis indicates that an increase in motional disorder of the bound lipid does not necessarily imply a state in which overall motional rates are enhanced. Indeed, a disordering of the lipid by binding with cytochrome *c* may be more strongly associated with slower fluctuations of the type stressed here.

The MAS technique has enabled a more extensive analysis of the protein effects on laboratory frame spin–lattice relaxation in CL. The moderate changes observed in these relaxation rates show that the strong paramagnetic effect reported previously on the lipid phosphorus (Spooner & Watts, 1991b) is not extended to any other detectable site in the lipid molecule. There are therefore good grounds for believing that the CL phosphates can interact directly with the heme in the perturbed configuration of cytochrome *c*. However, this did not appear to apply to CL mixed with neutral lipid, as discussed below.

Lipid Mixtures. The ^{31}P MAS NMR measurements indicate that diluting CL in bilayers with neutral lipid impairs its ability to interact directly with the heme in cytochrome *c*. There was no direct evidence of this interaction when CL was diluted with neutral lipid to concentrations similar to those found in the inner mitochondrial membrane. Instead, the protein appears to perturb the organization of CL in this mixture, as deduced from a decreased shielding anisotropy of the lipid phosphorus. This does not simply imply that the CL headgroup is less ordered in the protein–lipid complex since it is not possible from the current results to exclude the possibility that ^{31}P NMR spectral changes result from a change in the average orientation of the CL phosphate in the bilayer surface on binding with the protein. Even so, the observations are rather the reverse of effects observed on binding with bilayers of pure CL, which was not considered to influence the organization of the lipid phosphorus but instead resulted in a strong perturbation of the protein structure (Spooner & Watts, 1991a,b).

The apparently selective influence of cytochrome *c* on the CL fraction in the mixture may provide some grounds for believing that the protein can induce a phase separation of CL as suggested by others (Birrell & Griffith, 1976; Brown & Wüthrich, 1977). It is clear, however, that this process cannot occur to the extent of allowing CL to exert its effects on cytochrome *c* as a pure lipid. DOPC may be viewed as influencing the cohesion between CL molecules in the heterogeneous bilayer, such that cytochrome *c* can more easily perturb the lipid than when bound with the pure CL bilayers, which themselves appear to exert the reverse effect on the protein structure. The apparent reduction in effects of CL on the protein when diluted with neutral lipid can be rationalized in terms of a diminished density of excess negative charge from the phosphates within the surface of the mixed bilayers with possibly some reduced opportunities for lipid chain interactions when mixed with the monounsaturated lipid. This acknowledges the likely role of both electrostatic and hydrophobic interactions in the effects on protein, although the individual importance of these factors in mixed bilayers cannot be established from the information currently available.

The above result suggests that bulk lipid in the mitochrondrial inner membrane would not perturb cytochrome *c* structure as strongly as did CL alone. However, the CL dispersed in this bulk compartment should not be of principal interest concerning any effects of this lipid on the functioning of mitochondrial proteins. In fact, there is evidence to indicate that cytochrome *c* is not bound to any significant degree to this bulk lipid fraction (Gupte & Hackenbrock, 1988). Instead, it is more important to consider the behavior of CL which may be segregated from the membrane bulk due to specific types of interaction with the other protein constituents. A selective segregation of CL in the inner membrane is implicit from its known functional association with cytochrome *c* oxidase (Robinson et al., 1980, 1990) and from evidence that CL can form a receptor surface in the inner membrane for the mitochondrial creatine kinase enzyme (Müller et al., 1981). Although rather small, this CL fraction will be localized at

functionally important sites and may be sufficiently ordered by these associations with the integral membrane proteins and complexes to perturb the structural and electronic configuration of cytochrome *c* and thereby modulate its physiological behavior. The most critical test of any influence of CL on the activity of cytochrome *c* in mitochondria would be to determine whether any of the effects discussed in this and previous work can be detected on the protein localized in the region of its site of action on the mitochondrial cytochrome complexes.

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Registry No. DOPC, 10015-85-7; cytochrome *c*, 9007-43-6.

CORRECTIONS

Solution Structure of Murine Epidermal Growth Factor Determined by NMR Spectroscopy and Refined by Energy Minimization with Restraints, by Gaetano T. Montelione, Kurt Wüthrich, Antony W. Burgess, Edward C. Nice, Gerhard Wagner, Kenneth D. Gibson, and Harold A. Scheraga*, Volume 31, Number 1, January 14, 1992, pages 236–249.

The previously published energy-refined structures of murine epidermal growth factor (mEGF) presented in the paper exhibit some peptide groups with significant deviations from planarity. These 16 mEGF structures were therefore refined again, starting from the coordinates calculated with the DISMAN program. The restrained energy minimization was repeated exactly as described in the paper, except that the peptide bond dihedral angles were also restrained using a penalty function weighted sufficiently to prevent deviations of greater than 10° from the planar trans conformation. Except for minor variations, these 16 energy-refined mEGF structures are largely identical to those reported previously in the paper. The atomic coordinates for this revised set of energy-refined structures replace the original set of energy-refined coordinates and have been deposited in the Brookhaven Protein Data Bank.