Reversible Unfolding of Cytochrome *c* upon Interaction with Cardiolipin Bilayers. 2. Evidence from Phosphorus-31 NMR Measurements†

Paul J. R. Spooner* and Anthony Watts

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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ABSTRACT: ^{31}P NMR measurements were conducted to determine the structural and chemical environment of beef heart cardiolipin when bound to cytochrome *c*. ^{31}P NMR line shapes infer that the majority of lipid remains in the bilayer state and that the average conformation of the lipid phosphate is not greatly affected by binding to the protein. An analysis of the spin–lattice (T_1) relaxation times of hydrated cardiolipin as a function of temperature describes a T_1 minimum at around 25 °C which leads to a correlation time for the phosphates in the lipid headgroup of 0.71 ns. The relaxation behavior of the protein–lipid complex was markedly different, showing a pronounced enhancement in the phosphorus spin–lattice relaxation rate. This effect of the protein increased progressively with increasing temperature, giving no indication of a minimum in T_1 up to 75 °C. The enhancement in lipid phosphorus T_1 relaxation was observed with protein in both oxidation states, being somewhat less marked for the reduced form. The characteristics of the T_1 effects and the influence of the protein on other relaxation processes determined for the lipid phosphorus (spin–spin relaxation and longitudinal relaxation in the rotating frame) point to a strong paramagnetic interaction from the protein. A comparison with the relaxation behavior of samples spinning at the "magic angle" was also consistent with this mechanism. The results suggest that cytochrome *c* reversibly denatures on complexation with cardiolipin bilayers, such that the electronic ground state prevailing in the native structure of both oxidized and reduced protein can convert to high-spin states with greater magnetic susceptibility.

The specialization found in natural membranes with respect to their lipid types and composition has led to numerous attempts to define a direct role for lipids in membrane function. These studies have been largely directed into the field of lipid–protein interactions and have provided quite precise details on the dynamic behavior of lipids at the interface with proteins, both in the hydrophobic core and at the membrane surface (Watts, 1987; Marsh & Watts, 1988). However, any general rules describing the specific involvement of lipids in membrane protein function have remained elusive. It may be that these effects are highly protein dependent and cannot be discussed in such general terms (Watts, 1989) or that lipids only promote functional activities in a few, rather unique cases (Devaux & Seigneuret, 1985).

Cardiolipin (disphosphatidylglycerol) is an attractive candidate for such a unique role in membrane function. As a phospholipid, cardiolipin has interesting chemical and structural characteristics (Ioannou & Golding, 1979), being highly acidic and having a headgroup (glycerol) which is esterified to two phosphodiglyceride backbone fragments rather than one. Cardiolipin also has a highly specialized physiological distribution being mostly localized in the inner membranes of mitochondria (Ioannou & Golding, 1979). A strong case may be made for some role for cardiolipin in the functioning of the mitochondrial proteins involved in physiological energy transduction. Cardiolipin is required for optimal cytochrome *c* oxidase activity (Robinson et al., 1980; Marsh & Powell, 1988), a dependence that may be related to the lipid being able to promote binding with the cytochrome *c* substrate (Vik et al., 1981). The suggestion that cardiolipin provides the inner membrane receptor for the mitochondrial creatine kinase enzyme (Müller et al., 1985) is evidence that this lipid may exhibit functional characteristics quite unique for a phospholipid species. The antigenic properties of cardiolipin, as utilized in the Wasserman test (Brady & Trams, 1964), further show that this lipid can interact in an apparently specific fashion with a variety of protein species.

In order to describe better these specific interactions of cardiolipin, we have analyzed the binding of this lipid to cytochrome *c* by ^{2}H NMR, by labeling with deuterons at various sites in the protein (Spooner and Watts, preceding paper in this issue). We now extend the use of solid-state NMR techniques to observations on lipid phosphorus for evaluating the structural and chemical environment of the lipid headgroups when complexed with the protein.

^{31}P NMR is a convenient and powerful approach to investigating the structural and dynamic characteristics of model and natural membranes (Smith & Ekiel, 1984). This application of ^{31}P NMR was largely developed by Cullis and de Kruijff (1979), who determined by this means that cytochrome *c* can induce nonbilayer states to form in hydrated cardiolipin dispersions (de Kruijff & Cullis, 1980). These were interpreted as inverted micellar states of lipid which show spectral characteristics resembling the hexagonal H_{II} phase. Waltham et al. (1986) also used ^{31}P NMR to characterize the effects of cytochrome *c* binding to hydrated bilayers of cardiolipin, as well as using a variety of other anionic phospholipids. These workers found no detectable change in the macroscopic structure of bilayer lipid but report that the protein induced a marked enhancement in the lipid phosphorus spin–lattice (T_1) relaxation rate. On the basis of this effect, it was concluded that cytochrome *c* restricted the motion of phosphates in the headgroup of cardiolipin.

The purpose of the current study was first to determine the nature of any macroscopic changes in lipid organization upon

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* To whom correspondence should be addressed.

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complexation with cytochrome *c*, under conditions used for the preceding ^2H NMR study. More importantly, the work seeks to investigate more closely any effects on nuclear magnetic relaxation of the lipid that can describe the motional state and nature of the phospholipid environment within the protein–lipid complex. This has been accomplished by studying the thermal dependence of T_1, measuring a variety of other magnetic relaxation processes for lipid phosphorus, and comparing the magnetic relaxation properties of samples when static and during MASS[1] experiments. Rather than describing an effect of cytochrome *c* on the motional behavior of cardiolipin, results reveal an effect on the protein conformation which allows efficient paramagnetic interaction with the lipid headgroup.

Materials and Methods

The cytochrome *c* and cardiolipin materials and their pretreatment were as described previously (Spooner & Watts, preceding paper). The protein and lipid were combined after hydration with 20 mM cacodylate buffer solution at pH 6.0, containing 0.1 M NaCl and 0.5 mM EDTA, normally in the proportions used for the preceding study. The complex was preequilibrated and fractionated from unbound protein by ultracentrifugation, as described (Spooner & Watts, preceding paper). Precautions were again taken to avoid contact with atmospheric oxygen during sample preparation and analysis.

Ferrocytochrome *c* was prepared by treating the purified protein with an excess of the reductant, sodium dithionite. Reduced protein was used either in the presence of excess reductant (10 mM) or after its removal by dialysis into solutions saturated with nitrogen to prevent reoxidation of the protein. For complexes prepared with excess reductant (10 mM sodium dithionite in buffer solution), the lipid component was also rinsed with buffer containing sodium dithionite in order to eliminate any contaminating oxidizing species.

Ferricytochrome *c* was combined at a molar ratio of around 1:15 with cardiolipin, as in the preceding study, while the stoichiometry of ferrocytochrome *c* binding was varied as described under Results and Discussion.

Samples were sealed under an atmosphere of nitrogen in glass tubes or MASS sample rotors and analyzed according to the NMR procedures described below which employed variable temperature and total measuring times of up to 5 h. After these measurements, the protein and lipid components were dissociated by washing the complex with 2 M KCl, and these showed no evidence of deterioration as determined by spectrophotometric analysis of fractionated cytochrome *c* and TLC of fractionated cardiolipin.

^{31}P NMR Measurements. All ^{31}P NMR measurements were made at 161.98 MHz on a Bruker MSL 400 spectrometer. MASS was performed by using Bruker double-bearing probe heads for 7 and 4 mm sample rotors. Usually, no special procedures were necessary to enable MASS on the hydrated lipid or protein–lipid complex, other than to seal the samples under an atmosphere of nitrogen with solid (nonvented) rotor caps.

Routine powder and MASS spectra were recorded by using a 5-μs pulse width for phosphorus and applying a 10-G proton-decoupling field during aquisition. Spin–lattice (T_1) relaxation times were measured by using the inversion–recovery pulse sequence with a recycle time of at least $5T_1$. Spin–spin (T_2) relaxation was recorded from the Hahn spin echo generated from a CPMG sequence of pulses which were used to minimize pulse-width errors and effects from molecular diffusion in the samples. During MASS operation, the CPMG pulses were synchronized with the rotation period set for the sample rotor. Longitudinal relaxation in the rotating frame ($T_{1\rho}$) was measured in the conventional manner by using a sustained B_1 field to spin-lock phosphorus magnetization in the rotating frame. All ^{31}P chemical shifts were referenced to 85% H_3PO_4.

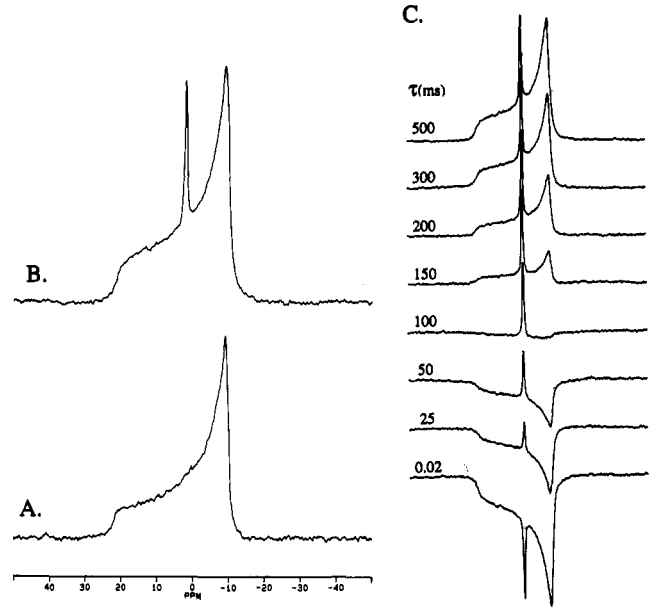

FIGURE 1: Proton-decoupled ^{31}P NMR powder patterns of hydrated cardiolipin bilayers alone (A) or complexed with ferricytochrome *c* (B) at a mole ratio of 15:1 (lipid:protein). Data were averaged from 64 transients recorded over a spectral width of 50 kHz, using a 1-s recycle time, and were processed with 30 Hz of exponential filtering. (C) Spectra obtained from the inversion–recovery experiment on the protein–lipid complex showing the various delay times, τ, used between inversion and observe pulses.

Results and Discussion

Measurements on Static Samples. Powder pattern ^{31}P spectra recorded from samples of hydrated cardiolipin and cardiolipin complexed with cytochrome *c* are shown in Figure 1 panels A and B, respectively. The intense high-field edge (at $\sigma_\perp$) and shallow low-field shoulder (to $\sigma_\parallel$) in both powder patterns are characteristic of an axially symmetric shielding tensor and typical of line shapes recorded from bilayers of hydrated phospholipid, for which the phosphorus shielding tensor is axially averaged by whole molecule rotation around the bilayer normal (Smith & Ekiel, 1984).

The only major difference between the spectra shown in panels A and B of Figure 1 is the appearance of narrow component at around +2 ppm upon complexing the lipid bilayers with cytochrome *c*, which is indicative of a small population of lipid in a more isotropic lipid environment. Similar narrow components have been observed in phosphorus spectra from bilayers of pure phospholipid, particularly the anionic lipids, and have been attributed to vesicular or inverted micellar structures which are intermediate between the lamella and hexagonal H_{II} phase states (Farren & Cullis, 1980). The nonbilayer component from the protein–lipid complex occasionally appeared broader and more pronounced than shown in Figure 1B (see Figure 7 in the preceding article) but produced no regular line shape indicative of a well-defined H_{II} phase, as reported previously for a cardiolipin complex with cytochrome *c* (de Kruijff & Cullis, 1980; Rietveld et al., 1983).

[1] Abbreviations: MASS, magic-angle sample spinning; DOPC, dioleoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine.

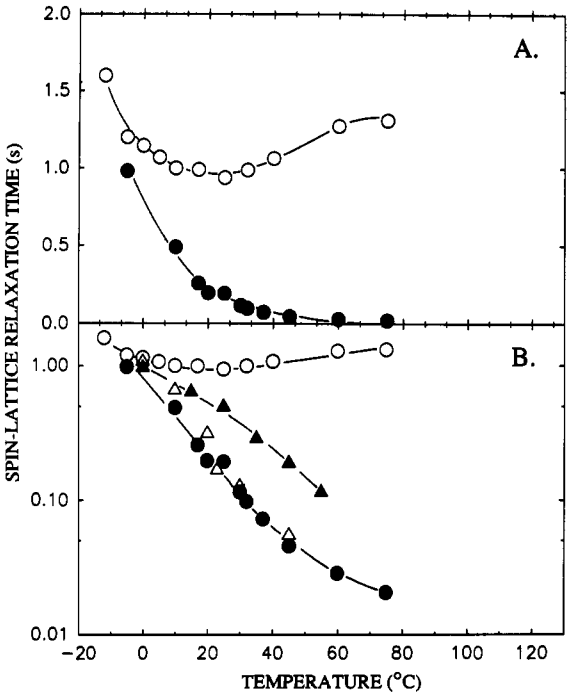

FIGURE 2: Variation in spin–lattice relaxation time with temperature for bilayers of cardiolipin alone (open circles) and when complexed with ferricytochrome *c* (closed circles) or ferrocytochrome *c* at a lipid to protein mole ratio of 8:1 (open triangles) or 26:1 (closed triangles). Relaxation data displayed on a linear scale in (A) and a logarithmic scale in (B).

The residual chemical shift anisotropy of lipid phosphorus was estimated from the breadth of the powder pattern ($\sigma_{\parallel} - \sigma_{\perp}$) to be −43 and −48 ± 3 ppm for cardiolipin and the protein–lipid complex, respectively, at 25 °C. The "bilayer" spectral component for the protein-containing complex shows a somewhat greater intensity over the low-field shoulder (Figure 1B). However, these protein effects on the phosphorus spectra are far too subtle to infer any distinct structural or motional change for the lipid phosphate and may well be ascribed to the relaxation phenomena discussed below. These ^{31}P NMR spectra therefore provide no indication that the average orientation of phosphate in the lipid headgroup is greatly affected by binding with the protein, in agreement with previous ^{31}P NMR analyses (Waltham et al., 1986).

Presented in Figure 2 are phosphorus spin–lattice (T_1) relaxation times for hydrated cardiolipin and the protein–lipid complex as a function of temperature. For bilayers of hydrated cardiolipin alone, a shallow but nonetheless well-defined T_1 minimum is observed at around 25 °C. T_1 minima, although quite rare in biological systems, have been recorded previously from ^{31}P NMR measurements on lipid bilayers (Seelig et al., 1981; Tamm & Seelig, 1983). From basic NMR relaxation theory, a T_1 minimum occurs when $\tau_c = 0.7/\omega_o$, where τ_c is the correlation time for the fast motions responsible for spin interaction with the lattice and ω_o is the Larmor frequency of the nucleus. For the cardiolipin phosphorus in these experiments, where $\omega_o = 1.02 \times 10^9$ rad·s^{-1}, we obtain $\tau_c = 0.71$ ns at the T_1 minimum. This is close to the value of 0.93 ns for phosphorus correlation times obtained from T_1 minima of DOPC at around 0 °C (Seelig et al., 1981) and POPC at around 15 °C (Tamm & Seelig, 1984). The minimum T_1 values are also similar at around 1 s for all these phospholipids. The shorter correlation time for phosphates of cardiolipin, compared with phosphatidylcholines at similar temperatures, is rather surprising in view of the particular geometry of this lipid in which the glycerol headgroup is anchored at both ends by phosphoglyceride backbone segments. Deuterium relaxation measurements have indicated that the glycerol headgroup portion of cardiolipin is indeed more rigid than the monoesterified headgroup of phosphatidylglycerol (Allegrini et al., 1984). The motional behavior of lipid phosphate groups, however, may occur rather independently of motions in the rest of the headgroup region, at least in regard to the high-frequency motions which affect T_1.

The T_1 minimum is also of value in providing an unambiguous definition of the motional regime for lipid phosphorus with respect to the NMR observation frequency and is exploited in this way to interpret the following effects of cytochrome *c* on phosphorus relaxation. As shown in Figure 2A, the protein markedly enhances spin–lattice relaxation of the phosphorus in bilayer lipid and the time constant for this process decreases precipitously with increasing temperature. The semilogarithmic plot of these data in Figure 2B shows that the T_1 decreases almost exponentially with increasing temperature, providing no suggestion of a minimum up to 75 °C. After data for this temperature were recorded, the T_1 at 25 °C could be reproduced to within 10%, showing the effect to be reversible and not significantly influenced by thermal decomposition in the sample. The "bilayer spectra" from both complex and hydrated lipid relax uniformly, revealing no anisotropic effects in T_1 for this component as illustrated in Figure 1C. The exchange between free and protein-bound lipid in the bilayers is therefore rapid on that time scale for averaging of the chemical shift anisotropy (10^{-4}–10^{-3} s), such that the technique measures the average relaxation rate for these two states. The small narrow component, however, relaxes more rapidly than the powder spectrum, suggesting that this represents lipid in a more mobile state.

Data in Figure 2B show that reduced cytochrome *c* can induce an equivalent enhancement in lipid phosphorus T_1 relaxation when bound in higher amounts compared with the oxidized form. A smaller reduction in T_1, obtained at quite low mole ratios of bound ferrocytochrome *c* (1:28 protein:lipid), is also shown in Figure 2B. This sample also contained a large excess of reductant (see Materials and Methods) to ensure that ferrocytochrome *c* had not reverted to the oxidized protein during sample preparation or recording of the relaxation data.

Waltham et al. (1986) have also observed a pronounced reduction in cardiolipin phosphorus T_1 values when bound to either oxidized or reduced cytochrome *c*. They conclude from this that the effects do not originate from a paramagnetic influence of protein on phosphorus relaxation but that the reduction in T_1 was due to a restriction of lipid headgroup motion by the protein. This was based on the assumption that the heme group in ferrocytochrome *c* remains in a d^6 low-spin electronic configuration and is therefore diamagnetic. The data presented here define well the dynamic state of the lipid phosphorus with regard to fast motions and are therefore well disposed to evaluate the mechanism of these relaxation phenomena, as given in the following discussion.

A reduction in the spectral density of fast motions for lipid phosphorus would cause a characteristic displacement in the T_1 minimum to higher temperatures and would also be expected to provide higher values of T_1 in the region of the minimum due to less efficient spin interaction with the lattice. Both these effects were observed from proteins of the sarcoplasmic reticulum on ^{31}P spin–lattice relaxation in DOPC (Seelig et al., 1981). The effects of cytochrome *c* reported here deviate strongly from this behavior. Simply, the much faster relaxation observed over the entire temperature range cannot be rationalized in terms of the motional model described

Table I: Relaxation Data for Hydrated Cardiolipin and the Protein–Lipid Complex at 25 °C[a]

	T_1 (s)	T_{2s} (ms)	$T_{1\rho}$ (ms)	T_{2r} (ms)	$\Delta\omega_r$ (Hz)
cardiolipin	0.94 ± 0.01	5.9 ± 0.4	71.9 ± 4.0	17.8 ± 0.5	14
cardiolipin–cytochrome *c* (15:1 mole ratio)	0.128 ± 0.003	3.9 ± 0.2	15.8 ± 0.5	4.9 ± 0.2	73

[a] Time constants for spin–lattice (T_1), spin–spin (T_2), and spin–lattice relaxation in the rotating frame ($T_{1\rho}$) with subscript "s" for static samples and subscript "r" for samples rotating at the "magic angle" (3.0 kHz) which gave line widths $\Delta\omega_r$. Error limits based on the variance from a nonlinear fit to 8 data points in each case.

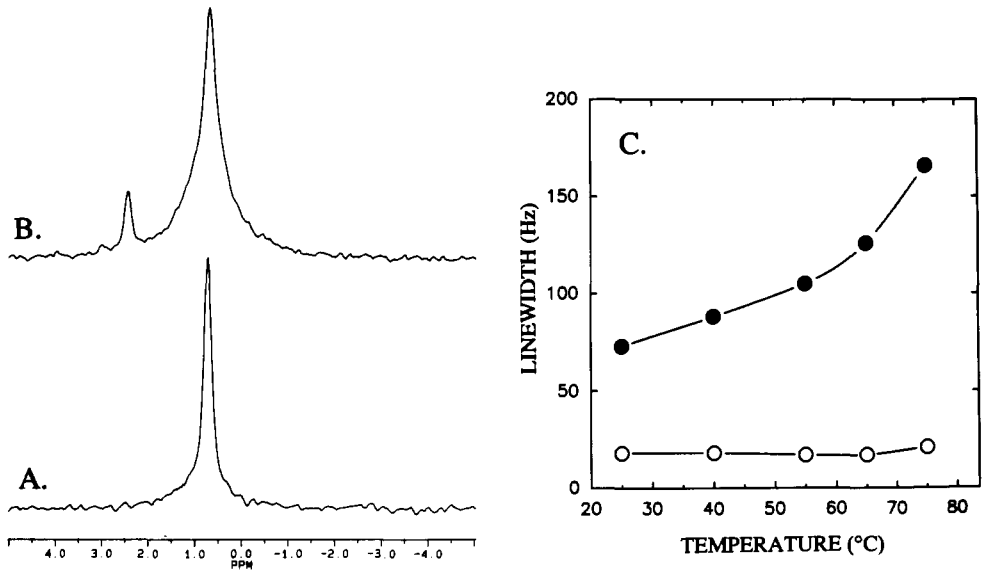

FIGURE 3: Proton-decoupled ^{31}P MASS NMR spectra from bilayers of cardiolipin (A) and the protein–lipid complex (B), obtained from 128 acquisitions recorded with a recycle time of 1 s and using a sample rotation speed of 3.0 kHz. Transients were apodized with a trapezoidal function prior to Fourier transformation. (C) Variation in line width of the MASS NMR spectra from cardiolipin (open circles) and the protein–lipid complex (closed circles) as a function of temperature.

above. Instead, it appears that the T_1 minimum for the fast lipid reorientation is eclipsed by a much more efficient relaxation mechanism which is associated with a very short correlation time. The only process which can satisfy these criteria, and is plausible for the cardiolipin–cytochrome *c* system, involves strong dipolar coupling with the unpaired spin of electrons in the protein heme. A similar effect has been described for cytochrome *c* oxidase on the phosphorus T_1 relaxation of POPC in bilayers (Tamm & Seelig, 1983), although in this instance the paramagnetic effect was only observed after the onset of T_1 minimum had occurred with increasing temperature—where the enzyme presumably became denatured.

Although spin–lattice relaxation is the single most useful nuclear magnetic property for quantifying fast molecular motions and electronic effects in NMR, it is instructive, and particularly important for establishing mechanisms of magnetic interaction, to combine information from a variety of relaxation processes and other NMR techniques. In Table I, the rates of T_1 relaxation are compared with other relaxation processes for lipid phosphorus, with and without cytochrome *c*. These data show that the protein also enhances spin–spin relaxation (T_{2s}) and longitudinal relaxation in the rotating frame ($T_{1\rho}$). This consistency in protein effects across the spectrum of motional frequencies of the nucleus is also characteristic of high-frequency electron interaction which provide an essentially flat spectral density profile over this range of correlation times down to 10^{-10} s (Sullivan & Maciel, 1982). The protein effects are greatest on the slower relaxation processes (and least for the short T_{2s}), which suggests that the electron–nuclear interaction is to some extent regulated by finite exchange processes involving molecular or spin diffusion. Further evidence in support of the above mechanism is obtained from ^{31}P MASS NMR measurements as described below.

^{31}P MASS NMR Experiments. MASS of hydrated cardiolipin bilayers and the protein–lipid complex at moderate rotation speeds (3.0 kHz) provides the high-resolution ^{31}P NMR spectra shown in Figure 3A,B. Spin–spin relaxation rates times measured under these conditions of sample rotation (T_{2r}) are entered in Table I with spectral linewidths corrected for static field inhomogeneity (~15 Hz). The T_{2r} for cardiolipin bilayers is equivalent to a spectral line width of 9 Hz—close to the measured value of 14 Hz. The T_{2r} for the protein–lipid complex is smaller than for the lipid alone, but only accounts for about half the observed line width of 73 Hz. The rotationally narrowed spectrum is therefore inhomogeneously broadened, presumably from magnetically distinct environments of the lipid phosphorus which are incompletely averaged by molecular exchange processes. This inhomogeneity imposes a broadening of only ~0.2 ppm on the ^{31}P NMR line widths and therefore was not detectable in the powder patterns which extend over a spectral width of around 50 ppm. The isotropic chemical shift measured at the peak of the resonance line was +0.72 and +0.67 ppm for the pure lipid and protein–lipid complex, respectively, and therefore was not significantly altered by interaction with the protein. The spectrum in Figure 3B also shows a small component at +2.5 ppm which does not appear in rotational sidebands (outside displayed frequency range) and therefore only appears to have isotropic shift component. This evidently corresponds to the narrow component observed around this chemical shift in the powder spectra from static samples (Figure 1B).

The data show that MASS efficiently averages the ^{31}P chemical shift anisotropy and dipolar interaction that contribute to the T_2 relaxation for the lipid bilayer system alone. MASS, however, fails to average effectively the interactions responsible for T_2 relaxation in the protein-containing complex, which is almost as rapid as that observed in the static sample

(Table I). This relaxation process cannot therefore be determined to any great extent by motional fluctuation in the shift or dipolar interactions experienced by the phosphorus spin, as in the case for the uncomplexed lipid. Instead, the predominant mechanism occurs on a time scale outside the effective range of the MASS technique ($>10^4$ Hz), a criteron which is again fulfilled by high-frequency electron–nuclear couplings.

The spectral line width for the complex also increases strikingly with increasing temperature as shown in Figure 3C, while the spectral line shape for cardiolipin alone is not significantly altered over this temperature range. This T_2 effect also distinguishes the predominant relaxation mechanism from one determined by conventional motional averaging and, furthermore, is consistent with the thermal dependence observed in T_1.

The results have some interesting implications concerning the spin state of the protein. For a dominant paramagnetic contribution to relaxation, the nucleus must be in spin contact with electrons which, from available thermal energy, can fluctuate rapidly between low-lying excited (high-spin) states and the ground (low-spin) state in the protein. These requirements are not met by the heme in the "native" structure of ferricytochrome *c*, which, through strong bonding with backbone residues, is held largely in the low-spin state (Smith & Williams, 1970). Furthermore, the electronic excited states of ferrocytochrome *c* are not of sufficiently low energy so as to be populated to any detectable levels under normal conditions (Smith & Williams, 1970). The effects observed here therefore indicate that complexation with cardiolipin induces quite profound changes in the configuration of the heme in cytochrome *c*, resulting in appreciable levels of low-lying electronic excited states in both the oxidized and reduced protein. Some direct evidence of this has been obtained by ESR (Vincent et al., 1987) which detected a small proportion (~5%) of high-spin species in a ferricytochrome *c*–cardiolipin complex at temperatures (7 K) where the protein should appear to exist solely in the ground state. Electronic relaxation in the protein heme is too rapid to allow any similar measurements at temperatures in any way approaching those used in the studies here. However, it is not unreasonable to expect that, under these conditions, the excited states will be populated to a much greater extent than observed at 7 K by ESR. Information on the electronic configuration of heme proteins at typical experimental temperatures can be obtained from their electronic spectra recorded in the visible and near-IR regions (Smith & Williams, 1970). Unfortunately, the cytochrome *c*–cardiolipin complex could not be sufficiently well dispersed in an aqueous system to enable any reliable determination of spin state by this means. Recently, however, this technique has been effectively employed to show that the binding of SDS to cytochrome *c* can convert both oxidized and reduced protein to a high-spin state (Yoshimura, 1988). These effects are indicative of changes in heme coordination with the polypeptide chain and can be expected from a variety of denaturants which have been shown to induce a large increase in the magnetic susceptibility of cytochrome *c* (Margoliash & Schejter, 1966). Yoshimura (1988) proposed a mechanism whereby, following electrostatic interaction at the protein surface, SDS can penetrate and interact hydrophobically in the core of the protein to displace heme ligands with the polypeptide chain. A denaturant effect is also plausible for cardiolipin—this being another amphipathic anion which interacts strongly with the protein, although a mechanistic interpretation of such an effect is not available from the results presented here. However, the relaxation data indicate that the polar headgroup region of cardiolipin must be able to come into close contact with the heme group in cytochrome *c* and do not preclude a direct interaction for lipid phosphate at this site.

Other studies have indicated that cardiolipin can change the conformation in and around the heme group in cytochrome *c*. Apart from detecting high-spin protein in the complex at 7 K by ESR, Vincent et al. (1987) also report that the low-spin spectra show a distortion in the heme structure. From fluorescence measurements, Jori et al. (1974) suggest that the aromatic residues within the heme environment of ferrocytochrome *c* realign when the protein is complexed with cardiolipin. These workers also provide circular dichroic evidence to support this contention and show that the effect can be induced with just equimolar amounts of bound cardiolipin.

The relaxation studies and their interpretation given here are in general agreement with previous evidence that cardiolipin can induce profound structural changes in and around the heme in cytochrome *c*. The current results specifically implicate electronic changes which relate directly to observed alterations in the redox potential of cytochrome *c* on binding with cardiolipin (Kimbelberg & Lee, 1970) and should therefore affect reactivity of the protein. Taken with observations from the preceding ^2H NMR study, solid-state NMR has shown that cardiolipin can induce extensive structural perturbations throughout the protein. It would be useful from a mechanistic point of view to be able to specify to what extent the effects on backbone and heme structure, observed separately, are interdependent in the action of cardiolipin on the protein.

The relaxation effects observed here were found to be highly temperature sensitive, suggesting a progressive exposure of the heme group at increased temperature, and also appeared quite reversible in these thermal effects. The reduced influence of ferrocytochrome *c* is probably related to the more compact structure of the protein in this oxidation state (Dickerson & Timkovich, 1975) which would be less readily perturbed by binding with cardiolipin. The primary effect of cardiolipin may involve a destabilization of the backbone structures in the protein such that the heme environment becomes exposed via opening of the heme cleft, although a subsequent interaction with the heme could presumably have a cooperative influence on these structural changes.

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Registry No. Cytochrome *c*, 9007-43-6; heme, 14875-96-8.

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High-Resolution ^{13}C NMR Study of the Topography and Dynamics of Methionine Residues in Detergent-Solubilized Bacteriorhodopsin†

Michel Seigneuret,* Jean-Michel Neumann, Daniel Levy, and Jean-Louis Rigaud
URA-CNRS 1290 and Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette, Cedex, France
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ABSTRACT: The proton transport membrane protein bacteriorhodopsin has been biosynthetically labeled with [*methyl*-^{13}C]methionine and studied by high-resolution ^{13}C NMR after solubilization in the detergent Triton X-100. The nine methionine residues of bacteriorhodopsin give rise to four well-resolved ^{13}C resonances, two of which are shifted upfield or downfield due to nearby aromatic residues. Methionine residues located on the hydrophilic surfaces, on the hydrophobic surface, and in the interior of the protein could be discriminated by studying the effects of papain proteolysis, glycerol-induced viscosity increase, and paramagnetic broadening by spin-labels on NMR spectra. Such data were used to evaluate current models of the bacteriorhodopsin transmembrane folding and tertiary structure. T_2 and NOE measurements were performed to study the local dynamics of methionine residues in bacteriorhodopsin. For the detergent-solubilized protein, hydrophilic and hydrophobic external residues undergo a relatively large extent of side chain wobbling motion while most internal residues are less mobile. In the native purple membrane and in reconstituted bacteriorhodopsin liposomes, almost all methionine residues have their wobbling motion severely restricted, indicating a large effect of the membrane environment on the protein internal dynamics.

Bacteriorhodopsin (BR),[1] the light-driven proton pump from *Halobacterium halobium*, appears as the best-characterized membrane transport protein to date [for a review, see Dencher (1983)]. However, many aspects of its structure are still a matter of debate, including the polypeptide chain transmembrane folding (Huang et al., 1982; Fimmel et al., 1989), the secondary structure (Jap et al., 1983; Nabedrik et al., 1985), the tertiary structure (Agard & Strout, 1982; Trewhella et al., 1986), and the protein internal dynamics (Herzfeld et al., 1987; Bowers & Oldfield, 1988). New data on BR tridimensional structure have recently been obtained from mutagenesis (Mogi et al., 1989; Soppa et al., 1989) and neutron (Popot et al., 1989) or electron (Henderson et al., 1990) diffraction experiments and need to be confirmed and extended by other approaches. Nuclear magnetic resonance (NMR) is a promising technique for investigation of the structure and dynamics of BR due to the large possibilities of stable isotope labeling of the protein. Presently, most NMR studies have used solid-state techniques to investigate the protein in the

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* To whom correspondence should be addressed.

[1] Abbreviations: BR, bacteriorhodopsin; [Met, ^{13}C]-BR, [*methyl*-^{13}C]methionine-labeled BR; ^{13}C-^{1}H COSY, ^{13}C-^{1}H heteronuclear chemical shift correlation spectroscopy; DEPC, dielaidoylphosphatidylcholine; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

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