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Reversible Unfolding of Cytochrome *c* upon Interaction with Cardiolipin Bilayers. 1. Evidence from Deuterium NMR Measurements†

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ABSTRACT: Deuterium NMR has been used to investigate the structure and dynamic state of cytochrome *c* complexed with bilayers of cardiolipin. Reductive methylation was employed to prepare [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c*, and deuterium exchange provided labeling of backbone sites to give [*amide*-^{2}H]cytochrome *c* or more selective labeling of just histidine residues in [ϵ-^{2}H]histidine cytochrome *c*. Deuterium NMR measurements on [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c* in the solid state showed restricted motions, fairly typical of the behavior of aliphatic side-chain sites in proteins. The [*amide*-^{2}H]cytochrome *c* provided "immobile" amide spectra showing that only the most stable backbone sites remained labeled in this derivative. Relaxation measurements on the aqueous solution of [*amide*-^{2}H]cytochrome *c* yielded a rotational correlation time of 7.9 ns for the protein, equivalent to a hydrodynamic diameter of 4.0 nm, just 0.6 nm greater than its largest crystallographic dimension. Similar measurements on [ϵ-^{2}H]histidine cytochrome *c* in solution showed that all labeled histidine residues were also "immobile" compared with the overall reorientational motion of the protein. The interaction with cardiolipin bilayers appeared to create a high degree of mobility for the side-chain sites of [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c* and perturbed backbone structure to instantaneously release all deuterons in [*amide*-^{2}H]cytochrome *c*. The [ϵ-^{2}H]histidine cytochrome *c* derivative, when complexed with cardiolipin, failed to produce any detectable wide-line ^{2}H NMR spectrum, demonstrating that the overall reorientational motion of bound protein was not isotropic on the NMR time scale, i.e., $\tau_c > 10^{-7}$ s. Additional measurements on the deuterium-exchanged protein–lipid complex, prepared in ^2H_2O, did not reveal any stable amide sites in the protein backbone, providing the lipid remained in its normal liquid-crystalline state. However, stable backbone sites were detected at reduced temperatures where, according to ^{31}P NMR observations, the lipid component was becoming immobilized in the complex. A strong binding of protein with liquid-crystalline bilayers of cardiolipin disorders the lysine sites of interaction on the surface of the protein and appears to cause an extensive derangment of secondary structure, such that no stable α-helices can exist in the protein backbone with a lifetime longer than around 10^{-6} s.

The organization of electron-transfer systems within mitochondrial membranes is known to play an important role in the compartmentalization of these processes and in exerting directional control over electron transfer. Apart from this "physical" influence, other components of mitochondrial membranes may become directly involved in the mechanisms of electron transfer between cytochrome proteins and complexes. For instance, cardiolipin, an anionic diphosphoglyceride localized in the inner mitochondrial membrane, is known to be required for optimal activity of the cytochrome *c* oxidase enzyme (Robinson et al., 1980; Marsh & Powell, 1988). The

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origin of this dependence is unclear, but there is evidence that cardiolipin facilitates binding of the cytochrome *c* substrate molecule to the enzyme (Vik et al., 1981). Cytochrome *c* has been described as an *extrinsic* membrane protein (Brown & Wüthrich, 1977; Devaux et al., 1986) although it can diffuse freely in the mitochondrial intermembrane space and under physiological conditions may not be directly associated with the inner membrane (Gupte & Hackenbrock, 1988). Opportunities for membrane interactions of cytochrome *c* may therefore be confined to rather specific types of association with cardiolipin segregated at the boundary of cytochrome complexes within the inner membrane.

Evidence of specific types of lipid interactions of cytochrome *c* has been sought from its binding with model membrane systems. The protein interacts strongly with anionic lipids (Reitveld et al., 1983; Devaux et al., 1986; Waltham et al., 1986) and particularly cardiolipin (Kimbelberg & Lee, 1969; De Kruijff & Cullis, 1980; Demel et al., 1989) in lipid bilayers. When bound to cardiolipin, the protein also exhibits some physical characteristics of the mitochondrial protein (Kimbelberg & Lee, 1969) and has a reduced redox potential (Huang & Kimura, 1984). There is some evidence (Brown & Wüthrich, 1977; Szerbeni & Tollin, 1988) that not only does this binding involve electrostatic interactions with surface lysines in the protein but also cytochrome *c* can penetrate, at least partially, into bilayers containing cardiolipin to interact hydrophobically with the membrane interior. This behavior conflicts with the conventional view of how *extrinsic* membrane proteins bind at membrane surfaces but supports the contention that cytochrome *c* may have some quite specific membrane interactions involving cardiolipin.

In most studies, measurements have been made to determine the influence of cytochrome *c* on the membrane lipid, and these data are used to provide information on the characteristics of the binding. While focusing in this way on the lipid component, it has generally been assumed that the protein retains its well-described water-soluble structure. In only a few cases (Jori et al., 1974; Vincent & Levin, 1986; Vincent et al., 1987) have attempts been made to detect structural changes in the protein upon binding with cardiolipin. The work reported here explores the utility of ^2H NMR for describing the influence of binding with cardiolipin bilayers on the structure and motional behavior of cytochrome *c*.

Solid-state NMR methods have proved a particularly useful source of structural and dynamic information on complex proteins (Torchia, 1984; Opella, 1986) as well as for the lipid components of membranes (Seelig & Seelig, 1978; Griffin, 1981; Watts, 1987). The deuterium nucleus is especially attractive for such studies since the magnitude of its quadrupole spectral splitting has a direct relationship with bond orientation and motion. Also, the nuclear magnetic relaxation of deuterons occurs by a well-defined mechanism and can be interpreted in terms of motional rates in a fairly straightforward manner. For this study, ^2H nuclei are introduced into amino acid side chain and backbone sites in cytochrome *c*, in order to observe the protein sites of interaction with cardiolipin bilayers and attempt to monitor the overall reorientational motion of cytochrome *c* when combined with these bilayers. The behavior of protein side-chain sites of interaction with the lipid and of exchangeable backbone sites in the protein shows that the binding induces an extensive disordering of protein structure, indicating that the close-packed configuration of cytochrome *c* is not fully retained on complexation with bilayers of cardiolipin.

Materials and Methods

Cytochrome *c* from horse heart (Grade V1, Sigma Chemical Co., St Louis), with or without chemical modification, was purified by ion-exchange chromatography on Whatman CM-32, eluted with 65 mM phosphate buffer at pH 7.0 (Brautigan et al., 1978) or at pH 6.3 with 100 mM phosphate buffer. Eluent containing the purified protein was concentrated by ultrafiltration using Amicon YM-5 ultrafiltration membranes and then dialyzed extensively to remove phosphate. Protein concentrations were measured spectrophotometrically by using a molar absorptivity of 2.95×10^4 at 550 nm and pH 7.0 for the protein reduced with sodium dithionite (Margoliash & Walasek, 1967). Cardiolipin 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 form within 2 weeks of receipt.

[N^{ϵ},N^{ϵ}-C^2H_3]Lysyl Cytochrome c. Deuteriomethyl substitution at amino groups of lysines and at the N-terminus in cytochrome *c* was achieved by reductive methylation based on the procedure of Jentoft and Dearborn (1980). Paraformaldehyde-$^2H_2$ (Merk Sharp & Dohme) was hydrolyzed to form a 2 wt % aqueous solution of deuterioformaldehyde by heating in a sealed ampule overnight at 110 °C. A solution of protein (0.42 mM) at 4 °C in 20 mM sodium cacodylate (pH 7.0) was combined with sodium cyanoborodeuteride (32 mM) and nickel chloride (10 mM) before incorporating the deuterioformaldehyde (32 mM). The reaction mixture was kept at 4 °C for 24 h and then dialyzed against double-distilled water for a further 24 h before purification by ion-exchange chromatography at pH 7.0. Purified protein was concentrated before dialysis and lyophilization. Simultaneous incorporation of ^{14}C-labeled formaldehyde showed that all 19 lysines and the terminal amino of cytochrome *c* were completely dimethylated under these conditions. Despite this extensive derivatization, the protein showed an identical UV/visible spectrum to the purified native form (Margoliash & Walasek, 1967).

[amide-^2H]Cytochrome c. A 1–2 mM solution of protein in ^2H_2O was flushed with oxygen to convert residual ferrocytochrome *c* to the oxidized protein and then adjusted to a p^{2}H 10.5 with NaO^{2}H to catalyze exchange of amide protons within the protein. After incubating for 18 h at 37 °C, the solution of deuterium-exchanged protein was adjusted to a p^{2}H of 6.3 with ^{2}HCl and purified at the same value of pH by ion-exchange chromatography at 4 °C. The purified fraction was concentrated, dialyzed at 4 °C against 20 mM phosphate buffer at pH 6.3, and then concentrated for sample preparation or crystallized as described below. Simultaneous incorporation of ^3H_2O showed that when freshly prepared, the deuterium-exchanged and purified product retained 40–50 deuterons per protein molecule, corresponding to 20–25% of total amide sites in cytochrome *c*.

[ϵ-^2H]Histidine Cytochrome c. A solution of the deuterium-exchanged protein in ^2H_2O was adjusted to a p^{2}H of 6.0 with ^{2}HCl and then lyophylized. The dried protein was back-exchanged first by dissolving in deuterium-depleted water followed by lyophilization and then by heating in deuterium-depleted water at 80–85 °C for 5 min. The protein was finally purified by ion-exchange chromatography at pH 6.3 and then dialyzed to remove phosphate. Around 2–4 deuterons per protein molecule were retained following this procedure as indicated by ^3H_2O incorporation.

Sample Preparation. Samples of derivatized protein alone were studied by ^2H NMR in their lyophilized, crystalline, or solution state. Crystallization was conducted directly after

purification, from a 2 wt % solution of protein at pH 6.3, containing 20 mM phosphate and 1.5 M NaCl and at 90–95% saturation with respect to ammonium sulfate. Solutions of purified protein for direct measurement or for complexing with cardiolipin were successively concentrated to a small volume (~2–4 mL containing 200–500 mg of protein) followed by dilution with 20 mM cacodylate buffer at pH 6.0 or 6.3, prepared in deuterium-depleted water with 0.1 M NaCl and 0.5 mM EDTA.

Protein–lipid complexes were prepared and maintained in a nitrogen atmosphere to prevent the protein catalyzing oxidation of lipid fatty acyl chains. Usually, 200 mg of cardiolipin was dried down by evaporation under reduced pressure from its stock solution in ethanol and then treated under high vacuum for 5 h. The dry lipid residue was then hydrated with 0.3 mL of the buffer and then mixed vigorously with protein solution (1 mL at 10 mM) in buffer. The suspension was allowed to equilibrate (2 h at 25 °C) and then centrifuged (2 × 10^5 g for 5 h). The clear supernatant was decanted for spectrophotometric analysis of free protein, and the dense pellet of protein–lipid complex was removed for analysis. Typically, over 90% of the added protein was bound to the lipid under these conditions. It was not necessary to rinse the protein–lipid complex with fresh buffer solution since, from the low concentration of protein remaining in the aqueous phase and the low amounts of this phase retained in the pelleted complex, we estimate that less than 2% of protein in the complex was in the unbound state. Although [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c* showed a reduced affinity for ion-exchange resin, the binding of this analogue to cardiolipin was quantitatively indistinguishable from that of native cytochrome *c* as well as the other protein derivatives used. For some studies, the protein–lipid complex was prepared in a ^2H_2O buffer solution by using ^{2}H-exchanged materials. Cardiolipin residues were ^{2}H-exchanged by dissolving in CH_3O^2H, evaporated to dryness, and then were treated under high vacuum. The lipid was then hydrated with ^2H_2O buffer solution, combined with ^{2}H-exchanged protein in the same buffer, and pelleted according to the procedure described above for other samples. Samples of protein–lipid complex were sealed in tubes under a nitrogen atmosphere and examined immediately by the NMR procedures outlined below. Following this analysis, the components of the complexes were dissociated by rinsing with 2 M KCl and showed no evidence of decomposition of lipid as determined by TLC and no indication from spectrophotometric analysis of any irreversible denaturation of the cytochrome *c*.

Nuclear Magnetic Resonance. Deuterium NMR measurements were carried out at 55.3 MHz on a home-built spectrometer based on Nicolet hardware or at 60.1 MHz on a Bruker MSL-400 spectrometer. Pulse widths were 3–4.5 μs for solid-state measurements and 6–8 μs for measurements on solutions. Transients were aquired from the solid protein or protein–lipid complex by using the quadrupole echo (Davis et al., 1976) with appropriate phase cycling and were processed by using 50–500 Hz of exponential filtering. Spectra showed only small imperfections in symmetry about the central Larmor frequency and were normally aliased to improve signal to noise in the spectra. Spin–lattice relaxation times on solutions were measured by using the π–τ–$\pi/2$–*aqu* inversion–recovery pulse sequence with a recycle time of at least $5T_1$. This technique was also used to record spectra for line width measurement, by selecting τ to suppress the signal from residual ^{2}HHO in the solution samples. Line width values were then obtained from fitting Lorentzian line shapes to experimental spectra.

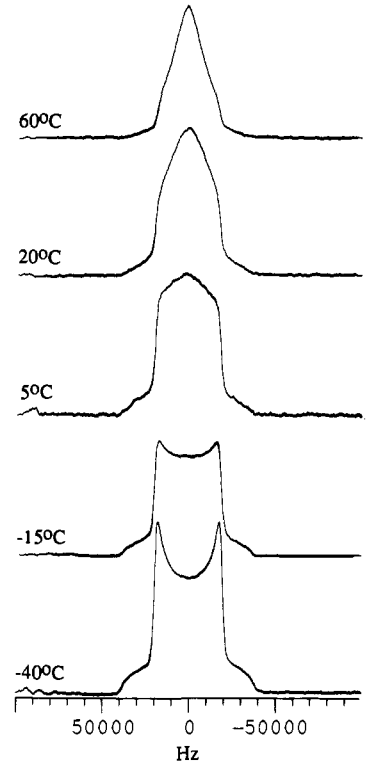

FIGURE 1: ^{2}H NMR spectra from [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c* in the lyophilized solid state as a function of temperature as indicated. Spectra recorded over 500-kHz spectral width from 960 acquisitions, a pulse width of 4 μs, and a recycle time of 0.5 s.

Conditions used for ^{31}P NMR measurements are given in the appropriate figure legend.

Results and Discussion

Solid-State NMR of Deuterated Cytochrome c Analogues. The majority of information on deuterated proteins has been obtained from C^2H sites located in amino acid side chains. The behavior of these aliphatic deuterons has been well described in terms of motional models (Torchia, 1984; Opella, 1986; Leo et al., 1987; Colnago et al., 1987). These models should be applicable to the methylated lysine derivative of cytochrome *c*, since the bond coordination at this amino cation site will exhibit the same tetrahedral symmetry as the aliphatic side chain. Spectra obtained from a lyophilized sample of [N^{ϵ},N^{ϵ}-C^2H_3]lysyl cytochrome *c* as a function of temperature are shown in Figure 1. At low temperatures (−40 °C) motion is essentially confined to fast rotation around the 3-fold symmetry axis, providing a well-defined quadrupolar splitting ($\Delta\nu_q$) close to the theoretical value of 40 kHz. A similar splitting was also observed at low temperatures from C^2H_3 substituted into the ε-position in both methionine residues of cytochrome *c* (data not shown). Spectra at higher temperatures show the onset of motions around the εN–δC bond in the lysine side chain. Comparison with the theoretical line shapes for motions of aliphatic deuterons (Griffin, 1981; Torchia, 1984) indicates that these rotations occur via continuous diffusion rather than in jump motions or hopping between discrete sites around this N–C bond. The line shape narrows at the highest temperature at which spectra were recorded, indicating that further motional averaging is occurring around additional side-chain bonds closer to the backbone in the protein. Despite the large number of sites labeled in this material there is no indication of heterogeneity in the line shapes. Although the measurements on this amino acid chain in cytochrome *c* are confined to protein in the amorphous solid state, the motions described

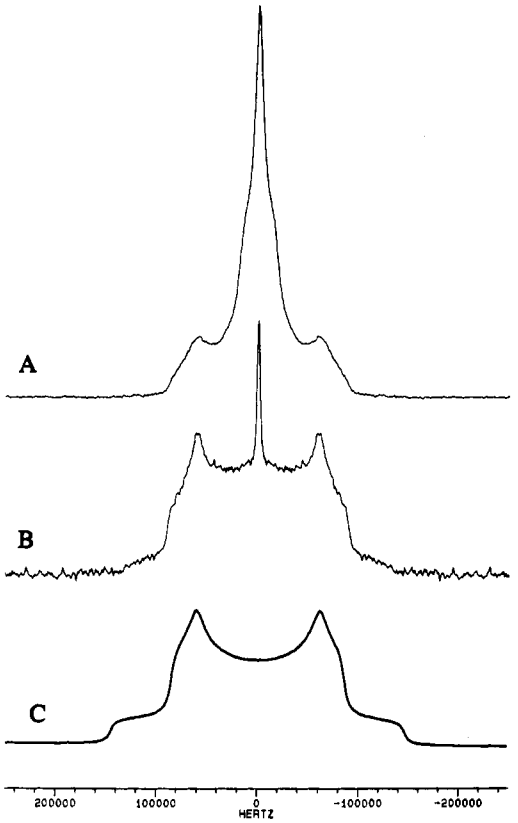

FIGURE 2: ^2H NMR spectra of deuterium-exchanged sites in cytochrome *c* showing the following: (A) all exchangeable sites in the protein, from 5000 acquisitions over a 1-MHz spectral width, a pulse width of 4 μs, and a recycle time of 1 s; (B) microcrystalline sample of [*amide*-^2H]cytochrome *c* recorded as for (A) but from 25 000 acquisitions and a pulse width of 4.5 μs; and (C) spectral simulation using a quadrupole splitting of 148 kHz and $\eta = 0.18$.

here are generally similar in degree to those observed from aliphatic side chains in proteins constrained within a membrane environment (Leo et al., 1987; Colnago et al., 1987).

Spectra obtained from deuterium-exchanged protein are shown in Figure 2. Following p^2H-catalyzed exchange (Figure 2A), the NMR spectrum shows some narrow components superimposed on a broad signal about 150 kHz wide which is characteristic of deuterons hydrogen bonded within stable amides within the proteins (Pauls et al., 1985; Colnago et al., 1987). The narrower components are from more mobile amides, ^2H-exchanged amino groups of lysine residues which form shoulders in the spectrum around 40 kHz wide and from the most mobile exchangeable sites on the protein which provide the narrow central spike in the spectrum. Spectra from crystalline [*amide*-^2H]cytochrome *c* in Figure 2B show retention of only stable amide sites in the protein with an isotropic signal from ^2H_2O in water of crystallization. In Figure 2C the broad signal is closely simulated by using values of the quadrupole parameters typical of amide deuterons hydrogen bonded to oxygen atoms in solids (Hunt & Mackay, 1976) and very similar to values measured for amide deuterons hydrogen bonded within the linear α-helix of a model peptide (Pauls et al., 1985). Lyophilized [*amide*-^2H]cytochrome *c* showed the reappearance of less stable sites in the protein, and therefore this derivative was always used fresh from column purification to avoid ^2H exchange within the protein. In the freshly purified state, deuterons only reside in stable backbone sites and therefore can be used to monitor overall molecular reorientation of the protein, as described below. No attempts were made to obtain solid-state spectra of the weakly labeled [ε-^2H]histidine cytochrome *c*. However, confirmation of labeling at these sites was obtained from proton NMR by using reported assignments (Wand et al., 1989; Feng et al., 1989).

Table I: Values of Spin–Lattice Relaxation Time (T_1) and Spectral Line Widths ($\Delta\omega$) Measured from Aqueous Solutions of Deuterated Cytochrome *c* Analogues at 15 °C with Values of Rotational Correlation Time (τ_r) and Quadrupole Coupling Constant (e^2qQ/h) Calculated from the Data According to Equations 3 and 4

	T_1 (ms)	$\Delta\omega$ (Hz)	τ_r (ns)	e^2qQ/h (Hz)
[*amide*-^2H]cytochrome *c*				
6 mM; pH 6.3	3.3	537	7.8	221
3 mM; pH 6.3	3.6	517	8.0	213
1 mM; pH 6.3	3.3	507	7.7	216
solid state[a]				222[a]
[ε-^2H]histidine cytochrome *c*				
10 mM; pH 6.0	6.2	369	8.9	171
[ε-^2H]histidine hydrochloride[b]				167[b]

[a] From solid-state measurements (Figure 2). [b] Measured by Scramm and Oldfield (1983).

Deuterium NMR of Cytochrome c in Solution. The rotational correlation times for cytochrome *c* in aqueous solution are obtained from an analysis of the relaxation for motionally restricted sites in the protein, according to the following treatment. For isotropic reorientation of a rigid lattice of spin = 1 nuclei, we can write (Abragham, 1962)

$$\frac{1}{T_1} = \frac{3\pi^2}{10}\left(\frac{1+\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)^2\left(\frac{\tau_c}{1+\omega_o^2\tau_c^2} + \frac{4\tau_c}{1+4\omega_o^2\tau_c^2}\right) \quad (1)$$

$$\frac{1}{T_2} = \frac{3\pi^2}{20}\left(\frac{1+\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)^2 \times \left(3\tau_c + \frac{5\tau_c}{1+\omega_o^2\tau_c^2} + \frac{2\tau_c}{1+4\omega_o^2\tau_c^2}\right) \quad (2)$$

where e^2qQ/h is the quadrupole coupling constant for the nuclear species, η is the asymmetry parameter, and τ_c is the rotational correlation time describing overall molecular reorientation. Equations 1 and 2 are quite generally applicable to relaxation processes without regard to the particular motional rate or observational frequency (ω_o). However, outside the *region of extreme narrowing* and in the limit where $\omega_o^2\tau_c^2 \gg 1$, eqs 1 and 2 simplify to

$$\frac{1}{T_1} = \frac{3}{5}\left(\frac{e^2qQ\pi}{h\omega_o^2}\right)^2\frac{1}{\tau_c} \quad (3)$$

and

$$\frac{1}{T_2} = \frac{9\pi^2}{20}\left(\frac{e^2qQ}{h}\right)^2\tau_c \quad (4)$$

These expressions may be solved simply with measured values of T_1 and spectral line width ($\Delta\omega = 1/2\pi T_2$) to provide values for the coupling constant (e^2qQ/h) and τ_c without relying on any other experimental information. This approach has been shown to be reliable for measurements on aqueous solutions of lysozyme also labeled at stable amide and histidine sites in the protein (Schramm & Oldfield, 1983).

Representative spectra obtained for the relaxation measurements on [*amide*-^2H]cytochrome *c* and [ε-^2H]histidine cytochrome *c* are shown in Figure 3, with a spectral simulation for line width determination. Spin–lattice relaxation times (T_1) are given in Table I with line widths corrected for static magnetic field inhomogeneity (~20 Hz). Values of e^2qQ/h and τ_c in Table I were calculated from the experimental data

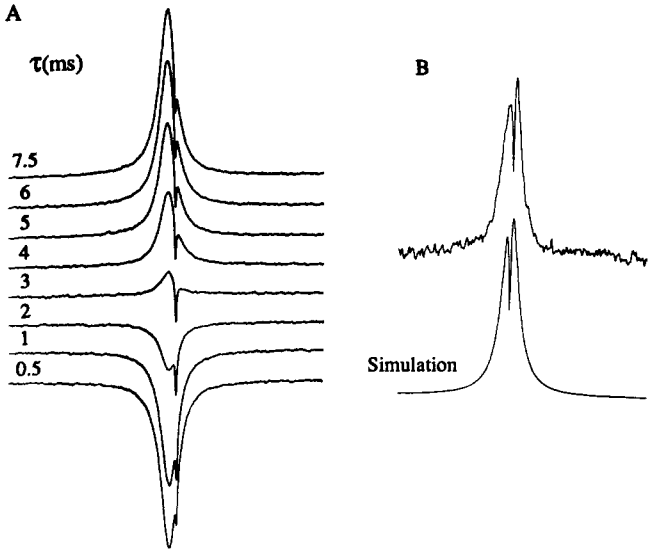

FIGURE 3: Spectra used in the relaxation analyses on aqueous solutions of deuterated cytochrome *c* analogues at 15 °C. (A) Inversion–recovery measurements for spin–lattice relaxation time of 6 mM [*amide*-^{2}H]cytochrome *c*; (B) spectrum of 10 mM [ϵ-^{2}H]histidine cytochrome *c*, also from the inversion–recovery pulse sequence (τ = 100 ms) with a spectral simulation used to determine line width.

by using eqs 3 and 4. Results for [*amide*-^{2}H]cytochrome *c* showed good reproducibility with no systematic variation between 1 and 6 mM protein concentration. The average value for the coupling constant of 217 kHz is in close agreement with the solid-state measurements (e^2qQ/h = 222 kHz) on this derivative and similar to the coupling constant of 210 kHz measured for lysozyme labeled in stable amide sites (Schramm & Oldfield, 1983). The mean value for τ_c of 7.9 ns from this data set compares with a value of 13 ns measured for [*amide*-^{2}H]lysozyme by using these methods (Schramm & Oldfield, 1983), consistent with the lower molecular mass of cytochrome *c* and, perhaps more importantly, the highly efficient packing of residues around the heme center in this protein. This packing also results in a near-spherical structure with a semiaxis ratio of just 1:1.13 (Dickerson et al., 1971) compared with 1:1.5 for lysozyme (Blake et al., 1965), making these measurements of rotational correlation time more appropriate for the near-spherical cytochrome *c*.

A quantitative relation between motion and molecular size was attempted by applying the simple Debye relation: $\tau_c = 4\pi v r^3/3kT$, where for v we use the bulk aqueous viscosity and r is the effective spherical radius. According to this simple model, a τ_c of 8 ns is equivalent to a hydrodynamic diameter of just 4.0 nm—close to the value of 3.4 nm for the larger subaxis dimension determined for horse heart cytochrome *c* by X-ray crystallography (Dickerson et al., 1971). This is indeed a surprisingly small effective size in view of the extent to which the influence of protein surface interactions is normally considered to extend into the surrounding water (Karpus et al., 1983). These effects generally result in values of τ_c much greater than predicted by the Debye model (Yudin et al., 1987).

The slightly larger value of τ_c measured for [ϵ-^{2}H]histidine cytochrome *c* (an increase of 12.5%) shows that the location of a histidine (residue 18) at the fifth coordination site of the heme iron can only have a nominal influence on the net relaxation observed, and this difference probably does not exceed the combined experimental error in the relaxation measurements. The coupling constant of 171 kHz determined for these sites agrees very well with the value of 167 ± 3 kHz reported for [ϵ-^{2}H]histidine hydrochloride (Schramm & Oldfield, 1983)

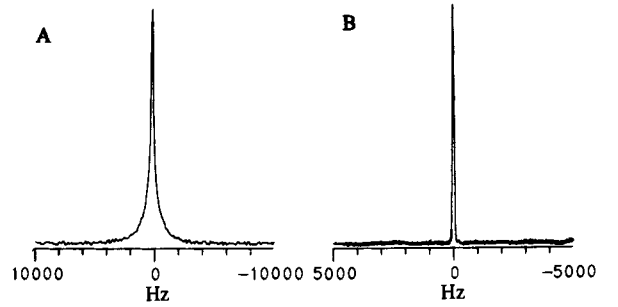

FIGURE 4: ^{2}H NMR spectra from (A) [N^ϵ,N^ϵ-C^2H_3]lysyl cytochrome *c* complexed with cardiolipin bilayers at 20 °C from 160 aquisitions using a 0.5-s recycle time and (B) [*amide*-^{2}H]cytochrome *c* complexed with cardiolipin showing a narrow signal from back-exchanged ^{2}H, recorded with 32 acquisitions and a 1-s recycle time.

and 168 kHz reported for the imidazolium ion (Wooton & Cohen, 1979). All histidine residues of cytochrome *c* are presumably restrained via stable hydrogen bonds to other sites in the protein. Therefore, the labeled histidines and the amides labeled in the backbone of the protein both satisfy the "rigid rotor" condition for monitoring the overall reorientation of protein in these studies.

Protein–Lipid Complexes. The conditions described under Materials and Methods for combining cytochrome *c* derivatives with cardiolipin yielded lipid–protein molar ratios of between 15:1 and 20:1. This binding is greater than the apparent saturation value of 43:1 observed under identical conditions of ionic strength and pH (de Kruijff & Cullis, 1980). The binding observed here would appear to be more consistent with reports that cytochrome *c* can complex with cardiolipin up to a stoichiometry of 4 lipid molecules per protein (Azzi et al., 1969; Waltham et al., 1986). Lipid stoichiometries selected for the present study were appropriate for ensuring that most of the lipid remained in a bilayer state on binding with the protein. These stoichiometries are very similar to those employed in a ^{31}P NMR study with bilayers of cardiolipin and phosphatidylserine (Waltham et al., 1986) in which it was demonstrated that the complexed lipid retained a bilayer configuration, as also shown here (see later). Similar stoichiometries were also employed in a ^{2}H NMR study of the cytochrome *c* binding with phosphatidylserine mixed 1:1 (by weight) with phosphatidylcholine, using deuterated lipid analogues (Devaux et al., 1986). A pH of 6.0 or 6.3 was used in the current study to avoid perturbing deuterium-exchanged sites within the protein and corresponds to the range of pH measured in unbuffered protein–lipid complex (Kimbelberg & Lee, 1969).

As shown in Figure 4A, [N^ϵ,N^ϵ-C^2H_3]lysyl cytochrome *c* produced a narrow ^{2}H NMR signal when combined with cardiolipin. The line width of around 100 Hz indicates that the labeled side chains are highly mobile in the complex. There is some suggestion however, from broadness in the base of the signal, of a population of somewhat less mobile sites. The surface lysine groups on the protein can be presumed to play a dominant role in initiating and stabilizing complexation with the anionic lipid. However, rather than resulting in a motional restriction of these side-chain sites involved in the binding, the strong interaction would appear to create a highly mobile state, at least in these regions in the protein. The information available from such extensive local motional averaging is obviously rather limited. This observation for the side-chain sites needs to be contrasted with the behavior of backbone sites in the protein which can describe a more general influence of the lipid binding on the overall protein dynamics and structure. The ^{2}H NMR spectrum obtained from combining [*amide*-

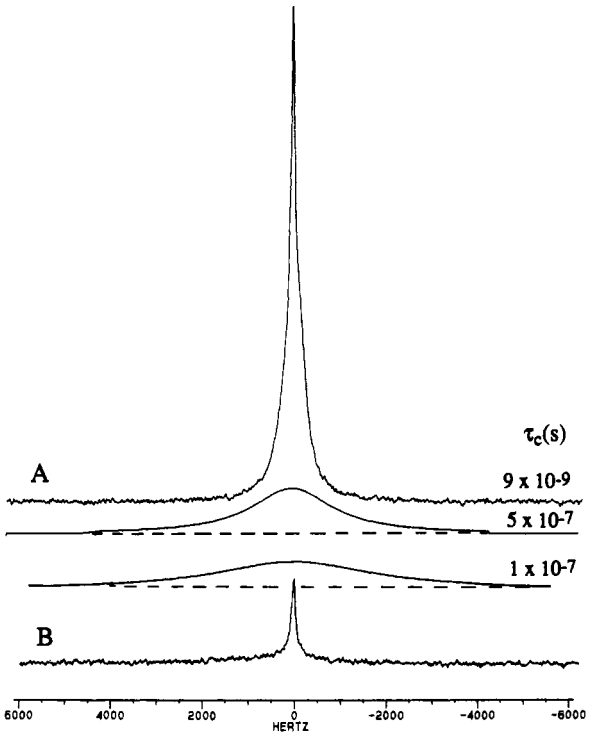

FIGURE 5: ^{2}H NMR spectra from 7.2 μmol (89 mg) of [ε-^{2}H]histidine cytochrome *c* (A) in aqueous buffer solution or (B) complexed with cardiolipin bilayers, both recorded from 10000 acquisitions under identical conditions (4-μs pulse width, 1-s recycle time) at 15 °C. Simulations were made by using eq 2 given in the text and assuming Lorentzian line shapes for indicated values of rotational correlation time (τ_c).

^{2}H]cytochrome *c* with cardiolipin was however fully isotropic with a line width of a few tens of hertz as shown in Figure 4B. Extended NMR measurements on the pelleted complex failed to show any broad signal around the isotropic component, indicating that no ^{2}H remained in stable backbone sites after combination with the lipid. The isotropic signal, when observed from the dilute protein–lipid suspension rather than the pelleted complex, had a spin–lattice relaxation time of 0.44 s, the same as for ^2H_2O. This signal therefore arises from deuterium released from the stable amide sites in the protein. Furthermore, this complete back-exchange of deuterons was observed within minutes of sample preparation and therefore appeared to be instantaneous with binding.

To demonstrate that protein could not be reorienting on the time scale of the isotropic signal ($\tau_c \sim$ nanosecond), the complex was prepared by using [ε-^{2}H]histidine cytochrome *c* which was also shown to have motionally restricted sites of deuteration from solution measurements (see above). Figure 5A shows the broad-line spectrum for this protein in solution which corresponded to a τ_c for reorientation of 8.9 ns. The spectrum in Figure 5B was recorded under identical conditions and from an equal mass of this protein bound to cardiolipin. This shows no detectable ^{2}H signal other than a small exchange-broadened isotropic component from residual ^{2}HHO in the sample. The line shape simulations shown in Figure 5 as a function of τ_c were generated by assuming that these approximate to a Lorentzian form and then applying the relation to line width provided by eq 2, using the coupling constant of 171 kHz from solution measurements and $\eta = 0$ for these sites (Schramm & Oldfield, 1983). Simulated spectra show that there is sufficient signal intensity to detect correlation times up to at least 10^{-7} s. According to the simple Debye model, this correlation time for a 4.0-nm diameter spherical protein corresponds to an effective viscosity for the environment of the protein of just 0.12 P. Much higher microviscosities of around 1–10 P are typically measured in membranes from translational and reorientational correlation times for lipids and proteins (Edidin, 1974; Peters & Cherry, 1982). If effective over the whole protein surface, these values would limit reorientational correlation times to the 10^{-6}–10^{-5}-s range. It would appear though that cytochrome *c* can be only partially embedded in lipid bilayers, with perhaps much of its surface remaining exposed to the aqueous environment at the bilayer surface (Brown & Wuthrich, 1977). However, on the basis of the above results, it appears that the histidine residues remain immobile on the $\tau_c = 10^{-7}$ s time scale and that the weak signal from these residues was not observed due to a restricted overall reorientation of the protein which may be 1 or 2 orders of magnitude slower. These slower reorientational motions should be best observed from averaging of the large static quadrupolar interaction from backbone sites in the protein, as was intended from using [*amide*-^{2}H]cytochrome *c*. The loss of deuterons from these sites implies that the lipid strongly perturbs the secondary structure in the protein. The label in this material is largely confined to the more stable sites in the prominent secondary structural features in the protein, i.e., the α-helical regions. The α-helices are estimated to involve 35% of amides in cytochrome *c* (Takano & Dickerson, 1981) whereas up to 25% of amides remained labeled in [*amide*-^{2}H]cytochrome *c*. The extensive and rapid release of this label from the protein indicates that lipid binding perturbs even the most stable α-helical sites in the protein. Since the loss of ^{2}H from [*amide*-^{2}H]cytochrome *c* was irreversible under the conditions used in the binding experiments with this derivative, it is not possible to conclude how this observation reflects the state of the protein at equilibrium binding with the bilayers. It is plausible that a distortion of the macromolecule occurs upon the initial interaction of the curved protein surface with a planar bilayer surface and that this is relieved by bilayer components reorganizing to accommodate the protein within the bilayer. Certainly, the rather unique geometric characteristics of the diphosphoglyceride, cardiolipin, with its relatively small headgroup and highly mobile polyunsaturated chains would appear to be favorable for accommodating at least a portion of the globular protein surface within the bilayer. De Kruijff and Cullis (1980) considered that the whole protein surface may even be completely enveloped within an inverted micelle of cardiolipin molecules, providing a means by which this hydrophilic protein can translocate the bilayer. Clearly, it is necessary to employ more stable backbone sites in order to illuminate these issues or, alternatively, to enable these sites to be labeled in situ as achieved in the following experiments conducted in ^2H_2O.

Experiments in ^2H_2O and ^{31}P NMR. Protein–lipid complexes were prepared as before except that protein and lipid were deuterium exchanged before combining in ^2H_2O buffer, as described under Materials and Methods. Time domain data were collected by using the echo-pulse sequence and processed according to the procedure of Callaghan et al. (1984) for extracting weak, broad deuterium signals in the presence of a large solvent peak. The basis of signal separation by this technique lies in the fact that, a short while after commencement of aquisition, the solid echo will have decayed leaving just the slowly relaxing solvent signal. A low-order polynomial function is fitted to this solvent signal, extrapolated back to before the echo, and then subtracted from the total FID to leave just the solid component of interest. This method was tested for the current experiments on a sample of the pentadecapeptide, gramicidin D, incorporated into di-

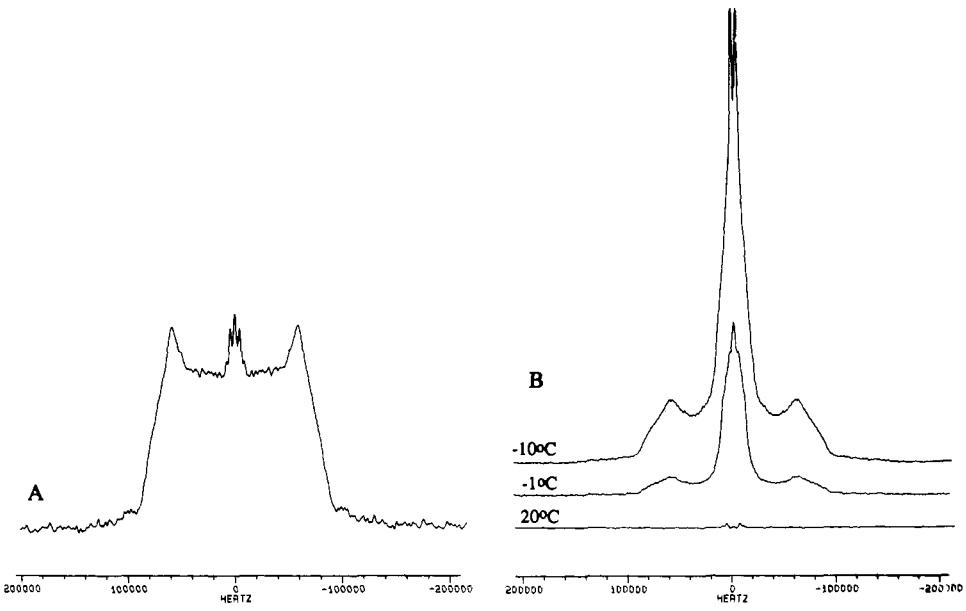

FIGURE 6: Solvent-subtracted ^2H NMR spectra of protein–lipid complexes prepared in ^2H_2O. (A) Spectrum of exchangeable sites in gramicidin D at 13 wt % in dipalmitoylphosphatidylcholine bilayers and measured at 40 °C from 80000 acquistions with a 4-μs pulse width and 1-s recycle time. (B) Spectra recorded from the cytochrome *c*–cardiolipin complex with 70000 acquisitions at 20 °C and 5000 acquisitions at −1 and −10 °C and using a 4-μs pulse width and 1-s recycle time.

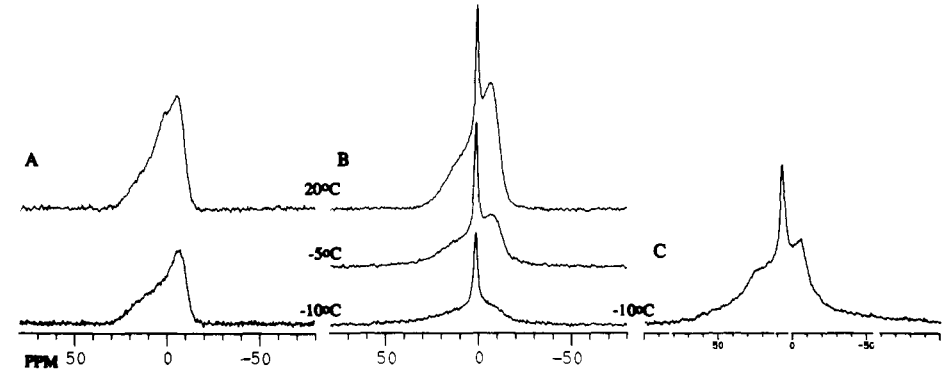

FIGURE 7: ^{31}P NMR spectra from (A) cardiolipin hydrated with aqueous buffer and (B) cardiolipin complexed with cytochrome *c*. Measurements at temperatures indicated from 320 acquisitions recorded over a 50-kHz spectral width with a 15-μs pulse width and a 1-s recycle time. (C) Low-temperature spectra from the protein–lipid complex recorded from 800 acquisitions over a 100-kHz sweep width using a Hahn echo with a 5-μs pulse width and a 15-s recycle time.

palmitoylphosphatidylcholine bilayers hydrated with ^2H_2O as described by Datema et al. (1986). The spectrum obtained after subtraction of the solvent signal is shown in Figure 6A and is typical of deuterons at stable amide sites in proteins, as described earlier. The spectrum also compares well with that shown by Datema et al. (1986) for this system in which the peptide is presumed to span the bilayer as a dimer of two linear helices. Spectral distortions arising from errors in the solvent subtraction appear to be confined to about ±5 kHz around the central Larmor frequency, which agrees well with the reliability specified by Callaghan et al. (1984) for this technique. This method therefore does not impose any serious limitations for observing the normal range of deuterium frequencies in solid or anisotropic systems. More importantly for this work, this small error means that the method should be effective for recording ^2H signals corresponding to all possible correlation times above the lower limit of 10^{-7} s set from observations on [ε-^2H]histidine cytochrome *c* in the complex and thus will be sensitive to any stable amide sites in the complexed protein.

Spectra obtained from the cytochrome *c*–cardiolipin complex in ^2H_2O buffer are shown in Figure 6B. At close to ambient temperatures, no broad component indicative of motionally restricted sites was detected in the samples. Upon decreasing sample temperature to just below 0 °C, the broad amide spectrum appears with good sensitivity and became more intense with further decreases in sample temperature. The full width of the "rigid" amide spectra appears as a sharp transition without any partial averaging of the deuterium signal. These observations were made above temperatures at which bulk ^2H_2O buffer freezes (<−10 °C) and far above the normal chain-melting temperature of the cardiolipin alone (<−30 °C). However, changes in the state of the complexed lipid are apparent from ^{31}P NMR spectra as shown in Figure 7B. . Using conditions normally adequate for measurements on lipids in the liquid-crystalline state, the signal intensity from mobile lipid is seen to fall dramatically around the temperature at which the amide signal was observed from the complex. In contrast, only a nominal decrease in signal intensity is observed over the same temperature range with bilayers of cardiolipin alone, as shown in Figure 7A. Employing conditions appropriate for detecting phosphorus in solids revealed the existence of an underlying broad spectral component at reduced temperatures which is indicative of phosphates that are immobile on the millisecond time scale of the NMR measurement, as shown in Figure 7C.

An immobilization of phosphate groups on the ^{31}P shift anisotropy time scale has been observed in dipalmitoylphosphatidylcholine bilayers, below their subtransition temperature (Füldner, 1981), and by the action of Ca^{2+} on hydrated bilayers of the acidic phospholipids, phosphatidylserine (Hope & Cullis, 1980) and phosphatidylglycerol (Farren & Cullis, 1980). It is possible that the surface of cytochrome *c* can behave similarly to multivalent cations in restricting mobility for some of the phosphates in the acidic headgroups of cardiolipin. However, it is not possible to entirely rule out any contribution from the aqueous region in this effect. A more detailed study of the protein–lipid complex by ^{31}P NMR is given in the following paper (Spooner & Watts, 1991).

The detection of stable amide spectra from the complexed protein at reduced temperatures appeared to be accompanied by an immobilization of the lipid component. This indicates that lipid mobility has a profound influence on the structure and dynamics of the protein. Strong interactions with the mobile lipid in the liquid-crystalline state resulted in a pronounced mobilization of surface lysine groups on the protein and appeared to disrupt backbone sites to the extent of destroying stable α-helical structure within the protein. Particular residues such as the histidines may however remain sufficiently well bonded within the protein so as to reflect more its overall reorientational motion. It should not be assumed that cytochrome *c* is particularly prone to these changes. This protein is generally viewed as having a relatively rigid conformation, stabilized through covalent linkages and strong interactions with the heme moiety (Mathews, 1985). However, it is reasonable to assume that some structural reorganization would be thermodynamically required if this hydrophilic globular protein is to become inserted into a lipid bilayer as described by others (Brown & Wüthrich, 1977; Szerbini & Tollin, 1988). The relationship between protein structure and membrane interactions has been considered an important factor in processing, targeting, and compartmentalization of the mitochondrial cytochrome proteins (Demel et al., 1989). For instance, the lack of well-defined structure in *apo*-cytochrome *c* would be favorable to its penetration into the outer mitochondrial membrane, and then, once inserted in the membrane, this protein may adopt a conformation that promotes its translocation to the inner surface of this membrane for processing (Reitveld et al., 1986). The results here suggest the opposing situation for the *holo*protein which must lose some of its well-ordered hydrated structure in order to penetrate into the lipid bilayer.

The effects described here contrast with the way membrane proteins are generally considered to interact with the membrane environment. It is customary to view the membrane proteins as quite rigid structures that impose some restraining influence on molecular motions in the membrane. Consequently, studies have concentrated on describing lipid dynamics in these systems according to a variety of physical techniques (Watts & Pont, 1985, 1986). This approach is also fairly straightforward compared with monitoring changes in the physical state of the protein—the methodology required to achieve this is in comparison poorly developed. The solid-state NMR techniques described here can make useful contributions to this challenging task and could be made amenable to a variety of membrane assemblies.

There is little additional evidence from the literature that cardiolipin can induce extensive changes in the secondary structure of cytochrome *c*. Jori et al. (1974) showed changes in the circular dichroic properties of cytochrome *c*, suggesting that stoichiometic levels of bound cardiolipin (4:1) influence the backbone structure in the protein. This study and recent work (Vincent & Levin, 1986; Vincent et al., 1987) provide more convincing evidence of structural changes occurring around or within the heme moiety. It would be interesting to establish if these changes at the reaction center in cytochrome *c* are a consequence of some specific perturbation at this site or are related to the more general effects on backbone structure implied in the work reported here. The current results disagree with a very recent report (Soussi et al., 1990) which concludes from an observed increase in the p*K* of the alkaline isomerization of cytochrome *c* upon binding with bilayer vesicles containing cardiolipin that the lipid stabilizes the native structure of the protein. Since it is expected that the membrane-bound protein will exist in a different dielectric environment than in aqueous solution, it is unlikely that changes in p*K* between these two states will be a reliable indication of structural changes in the protein.

The functional relevance of these structural changes in cytochrome *c* is dependent upon the extent to which the protein can be considered to interact with components of the inner mitochondrial membrane other than its redox partners. Results from fluorescence photobleaching experiments at physiological ionic strength (Vanderkooi et al., 1985) showed that iron-free porphorin cytochrome *c* exists in two motional states at the inner membrane: one with a quite restricted lateral diffusion ($D = \sim 10^{-10}$ cm^2 s^{-1}) and the other comprising an "immobile" pool at this site. On the other hand, Gupte and Hackenbrock (1988) report that, at physiological ionic strengths, cytochrome *c* is not immobilized to any significant extent at the inner membrane, and therefore, they assume a diffusion coefficient for the free protein in aqueous solution ($\sim 10^{-6}$ cm^2 s^{-1}). Although cytochrome *c* may not be associated with bulk components of the inner mitochondrial membrane, it could interact in a more specific way with some components, particularly cardiolipin, which become segregated from the membrane bulk. This type of selective interaction is implicit in the proposal of Vick et al. (1981) that cardiolipin assists in the binding of cytochrome *c* to its site of action on the oxidase enzyme. It is also worth noting here that this role for cardiolipin would not be dissimilar to its receptor function for the mitochondrial creatine kinase enzyme as proposed by Müller et al. (1985), since this also implies a specific interaction with lipid sites that are segregated from inner membrane bulk components.

Registry No. Cytochrome *c*, 9007-43-6.

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