Dynamical and Temperature-Dependent Effects of Lipid–Protein Interactions. Application of Deuterium Nuclear Magnetic Resonance and Electron Paramagnetic Resonance Spectroscopy to the Same Reconstitutions of Cytochrome *c* Oxidase†

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ABSTRACT: ^{2}H NMR and EPR spectra have been obtained as a function of temperature and protein concentration from the same samples of beef heart mitochondrial cytochrome *c* oxidase reconstituted into 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine. At all temperatures, the EPR spectra show the characteristic "bound" and "free" components, while the ^{2}H NMR spectra show only a narrow distribution of orientational order parameters. At temperatures near the phase transition of the pure lipid, the dependence of the ^{2}H NMR average orientational order on protein concentration fits a two-state model in which the phospholipid molecules exchange rapidly between two states tentatively identified as sites either on or off the protein surface. From this model, the ^{2}H NMR spectra yield a value of 0.18 mg of phospholipid per mg of protein as necessary to cover the surface of cytochrome *c* oxidase, which is the same value as derived from the EPR spectra at −20 °C. Both the ^{2}H NMR and EPR spectra vary markedly with temperature. At temperatures well above the phase transition of the pure lipid, the average orientational parameters derived from the ^{2}H NMR spectra are independent of protein concentration and are the same as for the lipid alone. Qualitatively, the EPR spectra show large apparent decreases in the average orientational order with increasing temperature. Analysis of ^{2}H NMR relaxation rates indicates an additional motion in the presence of protein with a correlation time of 10^{-6}–10^{-7} s. If this new motion is associated with exchange between the two states, a minimum value of 10^6–10^7 s^{-1} for the exchange rate is obtained, assuming that the lipids on the protein surface are much more motionally restricted than the rest of the lipid. Such an exchange rate is compatible with the observed differences in the ^{2}H NMR and EPR spectra. These results are consistent with short-lived, energetically weak interactions between cytochrome *c* oxidase and the phospholipids used in this study.

The nature of the interaction between integral membrane proteins and their lipid environment has been the focus of considerable study in recent years. These interactions are undoubtedly important in determining the structural properties of the membrane. In addition, it is clear that the dynamical properties of the functional membrane are dependent on both the membrane lipids and the way in which those lipids interact with the proteins in the membrane. It has therefore become increasingly important to probe not only the specificity but also the range, strength, and dynamics of these interactions. The application of various spectroscopic techniques to these questions has begun to provide information about lipid–protein interactions.

In their original, pioneering work using electron paramagnetic resonance (EPR)[1] spin-label techniques, Jost et al. (1973a) clearly demonstrated the presence of a relatively motionally restricted component in the EPR spectra of lipid–protein particles which was not present in the spectra of pure lipid particles. This led to the proposal that membrane proteins restrict the motion of those lipid molecules which are in direct contact with the protein surface, and they proposed the term "boundary lipid" to describe these motionally restricted lipid molecules. This idea was supported by similar, subsequent EPR studies including other integral membrane proteins (Jost et al., 1973b, 1977; Warren et al., 1974, 1975; Hesketh et al., 1976; Boggs et al., 1976; Marsh et al., 1978; Knowles et al., 1979; Watts et al., 1979). Unfortunately, conventional EPR techniques are not sensitive to motions which occur at rates slower than about 10^8 s^{-1}.

Recently, new information about the molecular dynamics at the lipid–protein interface has emerged. Little or no evidence for two distinct motional environments is found in NMR studies, particularly those employing ^{2}H NMR, of intact membranes (Brown et al., 1977; Stockton et al., 1977; Smith et al., 1979a; Davis et al., 1979, 1980; Nichol et al., 1980; Kang et al., 1979b) or reconstituted systems (Seelig & Seelig, 1978; Oldfield et al., 1978; Kang et al., 1979a; Rice et al., 1979a,b). The conclusion of these studies is that it is not possible to identify two motional environments whose lifetime is longer than 10^{-4}–10^{-5} s. Further, recent EPR studies using spin-labeled fatty acids covalently attached to an integral membrane protein indicate that the degree to which the protein restricts the motion of the spin-label may be highly temperature dependent (Favre et al., 1979; Davoust et al., 1979, 1980). These NMR and EPR results raise important new questions about the dynamics and energetics of lipid–protein interactions, suggesting that lipid–protein interactions may, in general, be relatively weak and short-lived. They also question whether

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; ^{2}H NMR, deuterium nuclear magnetic resonance; $(CD_3$-P)(PO)PC, 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine; Tris, tris(hydroxymethyl)aminomethane.

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EPR and NMR reflect the same molecular phenomena at the lipid–protein interface.

In this communication, we describe a series of experiments in which both ^2H NMR and EPR techniques have been applied to the same samples of cytochrome *c* oxidase reconstituted with a single synthetic, deuterated phospholipid covering a wide range of lipid to protein ratio. All samples show two distinct components in their EPR spectra, while their ^2H NMR spectra have a narrow distribution of orientational order parameters. Both techniques show a strong temperature dependence, such that at high temperatures the perturbations introduced by a protein are substantially diminished. All of our results are both qualitatively and quantitatively consistent with rapid exchange of lipid between sites corresponding to free and protein-associated environments. An analysis of the deuterium relaxation behavior suggests an exchange lifetime of 10^{-6}–10^{-7} s or shorter.

Materials and Methods

Preparation of 1-(16,16,16-Trideuteriopalmitoyl)-2-palmitoleoyl-sn-glycero-3-phosphocholine. 1-(16,16,16-Trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine [$(CD_3$-P)(PO)PC] was prepared as described previously (Dahlquist et al., 1977).

Isolation of Cytochrome c Oxidase. Cytochrome *c* oxidase was isolated from beef heart mitochondria by using the method of Capaldi & Hayashi (1972). All isolations were checked for purity by using gel electrophoresis in 12.5% or 15% acrylamide, 0.10% sodium dodecyl sulfate, 5.3 M urea, 0.165 M Tris, and 0.68 M Na_2HPO_4, pH 6.8, gels and by spectrophotometrically measuring the heme aa_3 to protein ratio (Briggs & Capaldi, 1977; Lowry et al., 1951). Heme aa_3 to protein ratios were 8–9 μmol of heme per mg of protein. Enzymatic activity was measured with an oxygen electrode assay system in the presence of Tween 80 (Vik & Capaldi, 1977) and showed 100–140 mol of cytochrome *c* oxidized per mol of heme aa_3 per s at pH 7.4.

Partial Delipidation of Purified Cytochrome c Oxidase. To maximize the relative amount of deuterated to endogenous, undeuterated phospholipid in our reconstituted system—which is of great practical importance at low phospholipid to protein ratios—we removed much of the endogenous phospholipid coisolating with the purified cytochrome oxidase by using the general methods of Robinson & Capaldi (1977). Typically, to 2.5 mL of 40 mg/mL purified cytochrome *c* oxidase (100 mg of protein) in 250 mM sucrose and 100 mM K_2HPO_4, pH 7.4, was added 2.0 mL of 100 mg/mL sodium cholate (Aldrich and Sigma; recrystallized 3 times from 70% ethanol and neutralized with NaOH), 90 mM Tris-HCl, and 5% (w/w) sucrose, pH 7.4 (200 mg of sodium cholate, giving 2 mg of sodium cholate per mg of protein), to yield a solution approximately 44 mg/mL in sodium cholate and 22 mg/mL in protein. After mixing, this solution was allowed to incubate on ice for 1 h before being loaded onto a 0.9 × 42 cm Sepharose 4B-200 column (Sigma) equilibrated with 10 mg/mL sodium cholate, 90 mM NaCl, 20 mM Tris-HCl, and 5% (w/w) sucrose, pH 7.4. Elution of the visually detectable major peak containing cytochrome oxidase required 3–8 h, depending on reservoir height above the bed. All but the last three or four fractions of this main peak were pooled and assayed for protein (Lowry et al., 1951) and lipid phosphorus (Ames & Dubin, 1960). These assays typically showed 50 μg of lipid phosphorus per mg of protein, as compared to approximately 110 μg of lipid phosphorus per mg of protein before delipidation. This material is henceforth called partially delipidated cytochrome *c* oxidase.

Reconstitution of Partially Delipidated Cytochrome c Oxidase with Deuterated Phospholipid. Partially delipidated cytochrome *c* oxidase was reconstituted with deuterated phospholipid by using the procedure previously published (Dahlquist et al., 1977), with these exceptions: 25–40 mg of the deuterated phospholipid was used (less phospholipid for lower lipid to protein ratios) and was dissolved in a volume of the 20 mg/mL sodium cholate buffer to yield 1 mg of sodium cholate per mg of phospholipid. A volume of the partially delipidated cytochrome *c* oxidase in its 10 mg/mL sodium cholate buffer was added to give the desired lipid to protein ratio (meaning that from about 44 to 600 mg of protein was added). Dialysis was over 2–3 days, involving 8–11 changes of 1–2 L of dialysis buffer. Dialysates were spun at 10000–20000*g* for 10–20 min before being loaded onto sucrose gradients; 33-mL sucrose gradients were used for samples containing more than 200 mg of protein. All sucrose gradients were spun for 18 h. The tight bands of reconstituent from the sucrose gradients were diluted approximately 5-fold with 90 mM NaCl and 20 mM Tris-HCl, pH 7.4, layered onto 2–5 mL of 10% (w/w) sucrose, 90 mM NaCl, and 20 mM Tris-HCl, pH 7.4, in deuterium-depleted water (Aldrich), and spun at 20000*g* until pelleted (generally less than 30 min). The two samples with the lowest lipid to protein ratio (i.e., containing the most protein) were further reduced in volume by spinning at 234000*g* for 16 or 30 h. Aliquots from all samples were removed for protein, lipid, and enzymatic assays before transfer to 8- or 10-mm flat-bottomed NMR tubes. Lipid phosphorus (Ames & Dubin, 1960) and protein assays (Lowry et al., 1951) were run after sucrose had been removed by dialysis. The cytochrome *c* oxidase retained full or had slightly improved enzymatic activity after delipidation, reconstitution, and volume reduction; detergent-solubilized activities were in the range of 140–170 mol of cytochrome *c* per mol of heme aa_3 per s.

Preparation of Samples Containing Lipid Alone. Samples containing only the $(CD_3$-P)(PO)PC were prepared as dispersions in 10% sucrose, 90 mM NaCl, and 20 mM Tris-HCl, pH 7.4, in deuterium-depleted water (Aldrich).

Incorporation of Fatty Acid Spin-Label into ^2H NMR Samples. After all the ^2H NMR measurements were complete, the 18-carbon fatty acid spin-label 16-doxylstearic acid (Syva) was diffused into an aliquot of each sample as follows: 1 μL of an approximately 6 mg/mL solution of 16-doxylstearic acid in 95% ethanol (approximately 6 μg of 16-doxylstearic acid) was blown dry under a stream of dry nitrogen in a 1.5-mL snap-cap, conical, polypropylene centrifuge tube. To this tube was added a volume of the ^2H NMR sample containing approximately 4 mg of protein. Following centrifugation in an Eppendorf desk top centrifuge for 2–5 min, the water above the loosely packed pellet was removed, and the pellet was transferred to the capillary tubes used for taking the EPR spectra.

The 0.098 mg of phospholipid per mg of protein sample gave EPR spectra containing intensity arising from copper ions in a different chemical state than is normally found in the isolated enzyme ("bad copper"; P. C. Jost, private communication). Though this sample had enzymatic activity comparable to that of the other samples and the functional and structural consequences of these copper signals are unclear, we prepared another sample of 0.091 mg of phospholipid per mg of protein and obtained EPR spectra from it for use in the spectral subtractions. Use of either the 0.098 mg/mg or the 0.091 mg/mg sample in the spectral subtractions gave essentially the same results. However, to be strictly consistent and correct, the spectra from the 0.098 mg/mg sample are shown in Fig-

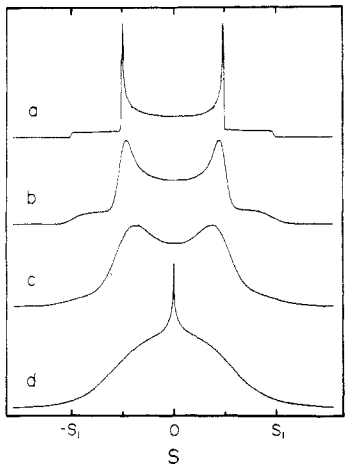

FIGURE 1: Effect of the width of a Gaussian distribution of order parameters on the peak to peak splitting of theoretical ^2H NMR spectra with the same average orientational order parameter S_1: (a) $(\Delta_2)^{1/2} = 0.01$, $\Delta\nu_{max} = (\omega_Q/2\pi)S_1$; (b) $(\Delta_2)^{1/2} = 0.10$, $\Delta\nu_{max} = 0.92(\omega_Q/2\pi)S_1$; (c) $(\Delta_2)^{1/2} = 0.25$, $\Delta\nu_{max} = 0.76\ (\omega_Q/2\pi)S_1$; (d) $(\Delta_2)^{1/2} = 0.50$, $\Delta\nu_{max} = 0$.

ures 8 and 9 with the other spectra from the ^2H NMR samples.

Nuclear Magnetic Resonance Methods. The NMR methods and instrumentation used here are similar to those described previously (Davis et al., 1976; Davis, 1979), with all ^2H NMR data being taken at 34.4 MHz except for the spectra of Figures 2 and 3 and the relaxation data of Figure 10. These were obtained at 23.3 MHz on a Nicolet NT-150 spectrometer with an Oxford Instruments superconducting solenoid. All spectra were acquired by using the Fourier-transform quadrupolar echo technique (Davis et al., 1976). As has been demonstrated elsewhere (Davis, 1979; Valic et al., 1979), this method is capable of giving nearly distortion-free spectra even for the broadest (120 kHz) ^2H NMR lines arising from deuterons on hydrocarbon chains. The ability to make precise statements about the wings of the ^2H NMR spectrum is of importance in this study since one of the possible effects of lipid–protein interactions might be to partially immobilize the hydrocarbon chains resulting in broad ^2H NMR lines.

The ^2H NMR "powder" spectra (Seelig, 1977) associated with molecules containing a single deuterium nucleus whose C–D bond is characterized by a unique orientational order parameter S have two peaks whose separation yields the magnitude of S directly. Systems having inequivalent phospholipid molecules [e.g., mixtures of gel and liquid-crystalline regions (Davis, 1979) or hypothetical "free" and "boundary" lipids suggested by EPR spin-label experiments (Jost et al., 1973a)] are characterized in general by a distribution $p(S)$ of orientational order parameters. The existence of a distribution can result in a broadening and shift in the intensity maxima of the ^2H NMR powder spectra. An example of this is given in Figure 1, which shows powder patterns for a system having various Gaussian distributions of order parameters but with the same mean orientational order parameter. That is, $p(S)$ has the form

$$p(S) = [1/(2\pi\Delta_2 S_1^2)]^{1/2} \exp[-(S - S_1)^2/(2\Delta_2 S_1^2)] \quad (1)$$

and Δ_2 is varied while S_1 remains constant. As may be seen from this figure, in spectra with the same average order parameter S_1, the peak splitting decreases as the width $(\Delta_2 S_1^2)^{1/2}$ of the distribution is increased.

One way to avoid the systematic errors arising from measurement of peak splittings in the presence of a broad distribution of order parameters would be to simulate the ^2H NMR spectra for some assumed form of $p(S)$ and with some assumed line broadening to account for relaxation effects, as has been done by Kang et al. (1979a). We have chosen a different, more direct method of obtaining information on the average value of the order parameter and, more generally, on the distribution of order parameters: the method of moments. For quadrupolar interactions in which the ^2H NMR absorption line shape is symmetric about the Larmor frequency, it is convenient to use the moments M_n of the half-spectrum (Davis, 1979; Davis et al., 1979):

$$M_n = \int_{\omega_0}^{\infty} (\omega - \omega_0)^n f(\omega)\, d\omega \Big/ \int_{\omega_0}^{\infty} f(\omega)\, d\omega \quad (2)$$

The M_n are simply related to the moments S_n of $p(S)$, defined by

$$S_n = \int S^n p(S)\, dS \quad (3)$$

For a system in which the molecular motions associated with the orientational averaging have axial symmetry, we have

$$M_n = A_n(\tfrac{3}{4}e^2qQ/\hbar)^n S_n \quad (4)$$

where $e^2qQ/\hbar = 2\pi(1.67 \times 10^5$ Hz) is the quadrupolar coupling constant in hydrocarbon chains (Burnett & Muller, 1971) and the coefficients $A_1 = 2/[3(3^{1/2})]$, $A_2 = 1/5$, etc. are easily calculated from the "Pake doublet" powder line shape (Seelig, 1977). The first two moments of the half-spectrum, M_1 and M_2, determine the mean orientational order parameter, S_1, and its mean squared value, S_2, and thereby provide a measure of the fractional mean squared width of the distribution of order parameters:

$$\Delta_2 \equiv (S_2 - S_1^2)/S_1^2 = [M_2/(1.35M_1^2)] - 1 \quad (5)$$

Hence, the method of moments allows a direct determination of S_1 and, in principle, $p(S)$ without any assumptions about the number of individual order parameters and their distribution, as is necessary in spectral simulations.

In addition to the information on orientational order provided by the ^2H NMR spectrum, it is straightforward to measure the nuclear spin relaxation times due to fluctuations of the quadrupolar interactions about their average values. Two types of relaxation times will be reported in this paper. One of them, the time constant for decay of the quadrupolar echo as a function of twice the separation of the two pulses in a quadrupolar echo experiment, T_{2e}, is sensitive to slow motions, i.e., to the spectral density of the fluctuating quadrupolar interaction near zero frequency, as will be discussed in the next section. We also report measurements of the nuclear spin–lattice relaxation time, T_1, which is the time constant for the approach of the nuclear magnetization to thermodynamic equilibrium. This time constant is determined from the amplitude of the quadrupolar echo signal as a function of the time between the application of an inverting rf pulse and the two-pulse quadrupolar echo sequence, and is sensitive to fluctuations near the Larmor frequency.

Electron Paramagnetic Resonance Methods. Spectra were recorded on a Varian E-9 spectrometer operating at 9.5 GHz equipped with a Varian field/frequency lock and interfaced with a 32K Varian 620/L100 minicomputer. Spectral subtractions and integrations were performed on a Nicolet 1180 minicomputer using Nicolet NTCESR software. Data analysis generally followed the procedures of Jost & Griffith (1978).

Theory

As mentioned in the introduction, one of our original motivations for carrying out these ^2H NMR experiments was to

examine critically the hypothesis that the phospholipids occupy at least two distinctly different sites for appreciable lengths of time. Most of the available EPR spin-label experiments indicate that two distinct sets of phospholipid orientational order parameters exist. One interpretation of this result is that one order parameter corresponds to phospholipid sites which are on the surface of individual proteins; the lipids at these sites have been given the names of "boundary" (Jost et al., 1973a) or "annular" (Warren et al., 1974) lipids. A recent, alternate interpretation is that these spin-labeled lipids are trapped between proteins (Chapman et al., 1977, 1979; Favre et al., 1979; Davoust et al., 1979, 1980). The other order parameter is interpreted to correspond to the spectrum of spin-labeled "free" or "bulk" lipids which give EPR spectra similar to those obtained for the spin-label in pure lipid systems. It is, therefore, of interest to consider the NMR characteristics of a system having two sites a and b with orientational order parameters S_a and S_b, respectively, and splittings $S_a\Omega_Q$ and $S_b\Omega_Q$ for a coupling constant Ω_Q. The general theory of motional narrowing predicts that when the time of exchange τ_{ex} between a and b is very long, i.e., $|S_a - S_b|\Omega_Q\tau_{ex} \gg 1$, the NMR spectrum is a superposition of the a and b spectra, while a short exchange time, $|S_a - S_b|\Omega_Q\tau_{ex} \ll 1$, leads to a spectrum characterized by the average order parameter:

$$S = (\chi_a S_a + \chi_b S_b) \tag{6}$$

where χ_a and χ_b are the equilibrium occupation probabilities (the mole fractions) for the two sites.

In the case of EPR spin-labels, the anisotropic hyperfine interaction responsible for the difference in splitting between the two sites has a coupling constant $\Omega_{hyp} \simeq 10^8$ s^{-1} (Marsh et al., 1978). The coupling constant for ^{2}H NMR in phospholipids is $\Omega_Q = {}^3/_4(e^2qQ/\hbar) \simeq 2\pi(126\text{ kHz}) \simeq 8 \times 10^5$ s^{-1}. Therefore, the observed qualitative differences between EPR spin-labels and ^{2}H NMR in reconstituted membranes containing cytochrome *c* oxidase, which we will describe in the next section and which have previously been found by other workers (Oldfield et al., 1978; Seelig & Seelig, 1978; Kang et al., 1979a; Rice et al., 1979a), can, in principle, be explained in terms of the inequalities $\Omega_{hyp} \gg \tau_{ex}^{-1} \gg \Omega_Q$ being satisfied. Since these inequalities have definite implications with respect to the deuterium nuclear spin relaxation properties, we have derived a general form for the correlation function $G(t)$ of a two-site system in the Appendix which can be used to calculate the nuclear spin relaxation properties in terms of Ω_Q, S_a, S_b, χ_a, χ_b, τ_{ex}, and the correlation times τ_a and τ_b in each of the two sites. The well-known expressions for the spin–lattice relaxation time T_1 and the relaxation time T_2 (in our case T_{2e}) for quadrupolar interactions are given by (Abragam, 1961)

$$1/T_1 = {}^1/_{15}\Omega_Q^2[j(\omega_0) + 4j(2\omega_0)] \tag{7}$$

and

$$1/T_2 = {}^1/_{90}\Omega_Q^2[9j(0) + 15j(\omega_0) + 6j(2\omega_0)] \tag{8}$$

where for exponential correlation functions such as those derived in the Appendix the reduced spectral densities are given for the two site model by

$$j(\omega) = \sum_{\alpha=a,b} \chi_\alpha \left[(1 - S_\alpha^2)\frac{2\tau_\alpha}{1 + \omega^2\tau_\alpha^2} + (S_\alpha - \bar{S})^2\frac{2\tau_{ex}}{1 + \omega^2\tau_{ex}^2} \right] \tag{9}$$

It may be seen from eq 7–9 that in the short correlation time limit $\omega_0\tau_c \ll 1$ for $\tau_c = \tau_a$, τ_b, or τ_{ex}, $T_1 = T_2$, but that if any of the correlation times do not satisfy the short correlation time limit, T_2 is less than T_1. If the intensity factor $1 - S_\alpha^2$ or $(S_\alpha - \bar{S})^2$ for a term in the long correlation time limit, i.e., $\omega_0\tau_c \gg 1$, is reasonably large, then the inequality $T_2 \ll T_1$ is obtained.

For a hierarchy (in time) of N motions characterized by an index $\mu = 1, 2, ..., N$ such that the $\mu = 1$ motion reduces the order parameter within a given state α from $S_\alpha^{(0)} = 1$ to $S_\alpha^{(1)}$, the $\mu = 2$ motion from $S_\alpha^{(1)}$ to $S_\alpha^{(2)}$, etc., the result of eq 9 can be generalized by replacing the first term in the square brackets as follows:

$$(1 - S_\alpha^2)\frac{2\tau_\alpha}{1 + \omega^2\tau_\alpha^2} \rightarrow \sum_{\mu=1}^{N}(S_\alpha^{(\mu-1)^2} - S_\alpha^{(\mu)^2})\frac{2\tau_\alpha^{(\mu)}}{1 + \omega^2\tau_\alpha^{(\mu)^2}} \tag{10}$$

where $\tau_\alpha^{(\mu)}$ is the correlation time of the μth motion and we identify $S_\alpha^{(N)}$ with S_α in (9). This generalization is of special interest in dealing with fast internal molecular motions such as the rotations of a CD_3 group about its axis of symmetry.

In order to analyze the relaxation rate in a two-component mixture made up of a fraction of χ_f of free lipid and a fraction χ_p of protein-associated lipid, it is convenient to assume that the hierarchy of correlation times may be divided into those which satisfy the correlation time limit $\omega_0\tau_\alpha^{(\mu)} \ll 1$, leading to effective order parameters S_α', and much longer ones, $\omega_0\tau_\alpha^{(\mu+1)} \gg 1$, etc. The latter correlation times are assumed to be so long that they contribute to $1/T_{2e}$ via $j(0)$ in (8) but do not contribute appreciably to $j(\omega_0)$. In that case, assuming that motions slower than the exchange rate do not modify the order parameter, we write

$$1/T_{2e} = (1/T_1) + (1/T_2') \tag{11}$$

where

$$\frac{1}{T_2'} = \chi_f\frac{1}{T_{2f}'} + \chi_p\frac{1}{T_{2p}'} + \frac{\Omega_Q^2}{5}\chi_f\chi_p(S_p - S_f)^2\tau_{ex} \tag{12}$$

and

$$\frac{1}{T_{2\alpha}'} = \frac{\Omega_Q^2}{5}\sum_{\mu=\mu_0+1}^{N}(S_\alpha^{(\mu-1)^2} - S_\alpha^{(\mu)^2})\tau_\alpha^{(\mu)} \tag{13}$$

where S_p and S_f are the other parameters for protein-associated and free lipids, respectively. This relationship implies that under fast exchange conditions the overall relaxation rate is the population average of the free and protein surface values plus an extra term which has to do with the exchange process. A similar expression for spin–lattice relaxation, T_1, would lack the long correlation time terms, including the exchange term, since they would not be expected to generate large fluctuations at the Larmor frequency.

Results

^{2}H NMR Spectra of (CD$_3$-P)(PO)PC Alone. Representative ^{2}H NMR spectra of (CD$_3$-P)(PO)PC over the temperature range 8 to −68 °C are shown in Figure 2. The spectra at high temperatures show the sharp Pake doublet characteristic of the ^{2}H NMR powder pattern of fluid-phase phospholipids. The observed residual quadrupolar splittings seen in the spectra of the fluid phase change from about 2 to 4 kHz. At low temperatures, the powder pattern spectra show relatively featureless broad peaks with intensity extending over about 40 kHz. At temperatures near the gel- to fluid-phase transition of about −15 °C, the spectra show both the broad gel-phase spectrum and the sharper fluid-phase spectrum. It is important to note that we can easily detect broad spectral features of the gel-phase spectrum even in the presence of the

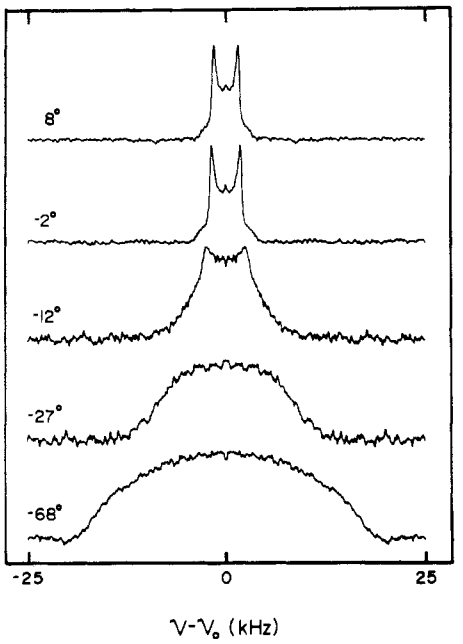

FIGURE 2: ^2H NMR spectra at 23.3 MHz of $(CD_3\text{-}P)(PO)PC$ in excess buffer at 8, −2, −12, −27, and −68 °C. Spectra result from 5000 or 10 000 acquisitions at a rate of 4 s^{-1}, with τ = 60 μs above and 30 μs below the phase transition.

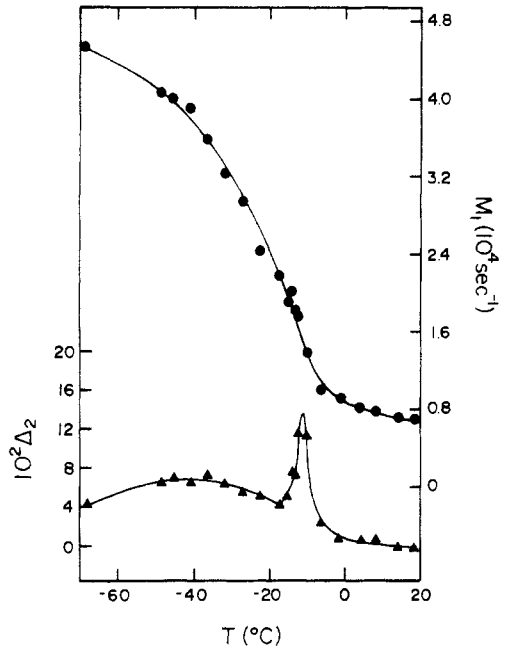

FIGURE 3: Temperature dependence of the first moment (M_1) and mean squared width of distribution of order parameters (Δ_2) for $(CD_3\text{-}P)(PO)PC$.

sharper fluid-phase spectrum.

These qualitative changes in the spectra of the lipid can be characterized quantitatively by using the method of the moments. Figure 3 shows the temperature dependence of the values of the first moment as derived from the spectra in Figure 2 and additional spectra. This shows a rather gradual transition from large values of the first moment at low temperatures to much smaller values above the phase transition temperature. The temperature dependence of the mean squared fractional spread in order parameter, Δ_2, is also plotted in Figure 3. The sharp maximum in Δ_2 observed in the vicinity of −12 °C results from the coexistence of both gel- and fluid-phase spectra near this temperature. This provides a sensitive method for examination of the phase transition of

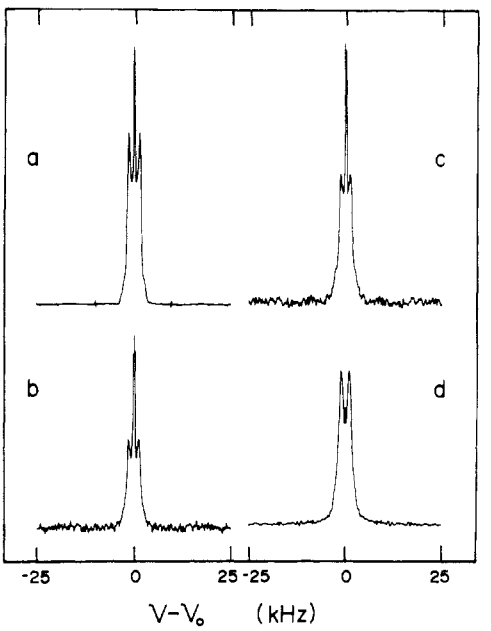

FIGURE 4: ^2H NMR spectra at 34.4 HMz at 0 °C for $(CD_3\text{-}P)(PO)PC$ alone and with three concentrations of cytochrome *c* oxidase: (a) $(CD_3\text{-}P)(PO)PC$ alone, (b) L/P = 0.88, (c) L/P = 0.37, and (d) L/P = 0.18. Spectra result from approximately 40 000 acquisitions at a rate of 2 s^{-1}, with τ = 60 μs.

$(CD_3\text{-}P)(PO)PC$ and indicates it occurs near −12 °C under these conditions.

^2H NMR Spectra of $(CD_3\text{-}P)(PO)PC$/Cytochrome c Oxidase Reconstituents. The most striking feature of the ^2H NMR spectra of the $(CD_3\text{-}P)(PO)PC$/cytochrome *c* oxidase reconstituents is that, at relatively high temperatures ($T \geq -5$ °C), the average orientational order of the methyl deuterons in the saturated chain of $(CD_3\text{-}P)(PO)PC$ is unaffected by the protein over the complete range of protein concentrations studied ($0.098 \leq L/P \leq 0.88$). This is illustrated in Figure 4 which shows the ^2H NMR spectra for the lipid alone and with three different concentrations of cytochrome *c* oxidase at 0 °C.

Below −10 °C the spectra show a marked dependence on protein concentration and are qualitatively different from those at higher temperatures. This may be seen from the spectra shown in Figure 5, which were obtained at −15 °C. Although there is a strong variation of spectral shape with protein concentration at these lower temperatures, the lack of well-defined peaks in these spectra precludes the measurement of an apparent quadrupolar splitting.

A quantitative picture of these temperature and protein concentration effects on orientational order is provided by the spectral moments. The first moment, which is proportional to the mean orientational order parameter, is plotted vs. temperature in Figure 6 for $(CD_3\text{-}P)(PO)PC$ alone and for samples containing various concentrations of cytochrome *c* oxidase. It is seen that only for temperatures below −5 °C is there an observable dependence of average orientational order on protein concentration. At temperatures less than −10 °C, the protein-dependent spectral changes are directly proportional to the amount of protein in the sample. This is shown in Figure 7, where the average orientational order is plotted vs. the protein to lipid weight ratio, P/L, at −20, −15, and −10 °C. The plots are linear until P/L has a value of about 5, where the orientational order no longer varies strongly with P/L. Presumably the break at $P/L \simeq 5$ occurs because at that protein concentration or higher, all lipid is in contact with the surface of the protein; a lipid to protein weight ratio (L/P) of about 0.2 is the often-quoted value for the amount of lipid

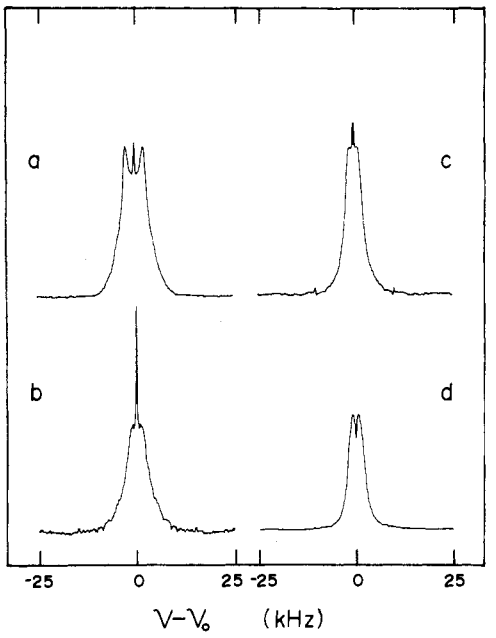

FIGURE 5: ^2H NMR spectra at 34.4 MHz at −15 °C for $(CD_3$-P)(PO)PC alone and with three concentrations of cytochrome *c* oxidase: (a) $(CD_3$-P)(PO)PC alone, (b) $L/P = 0.88$, (c) $L/P = 0.37$, and (d) $L/P = 0.18$. Spectra result from approximately 40 000 acquisitions at a rate of 2 s^{-1}, with $\tau = 60$ μs.

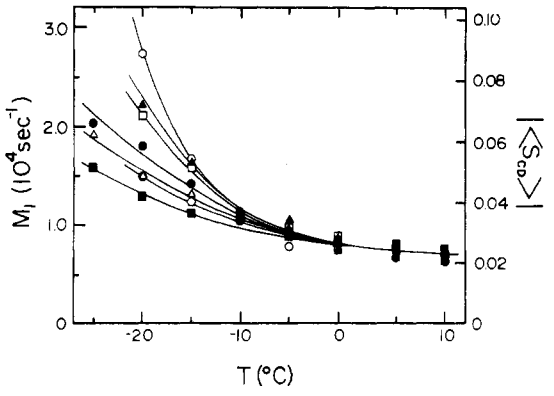

FIGURE 6: First moment and average orientational order parameter as a function of temperature for $(CD_3$-P)(PO)PC alone and six concentrations of cytochrome *c* oxidase: (○) $(CD_3$-P)(PO)PC alone, (▲) $L/P = 0.88$, (□) $L/P = 0.53$, (●) $L/P = 0.37$, (△) $L/P = 0.24$, (■) $L/P = 0.18$, and (○) $L/P = 0.098$.

necessary to coat the protein surface of cytochrome *c* oxidase (Jost et al., 1973a).

The temperature dependence of the effect of protein on the average orientational order would appear to coincide roughly with the marked broadening of the spectra (compare Figures 4 and 5). More careful comparisons of spectra with and without protein show that some broadening remains at temperatures where protein has no effect on the average orientational order (see Figure 4). A quantitative measure of this spectral broadening is provided by Δ_2 (see Materials and Methods). While the data contain considerable scatter (data not shown), it is clear that Δ_2 increases with increasing protein concentration at constant temperature, even at high temperatures (10 °C) where no effect of protein concentration on the average orientational order parameter is observed. At any one temperature, the value of Δ_2 is 2–3-fold greater in the sample with $L/P = 0.098$ than the value for the lipid alone.

EPR Spectra of $(CD_3$-P)(PO)PC/Cytochrome c Oxidase Reconstituents. Representative EPR spectra at 9.5 GHz of the fatty acid spin-label 14-doxylstearate diffused into the

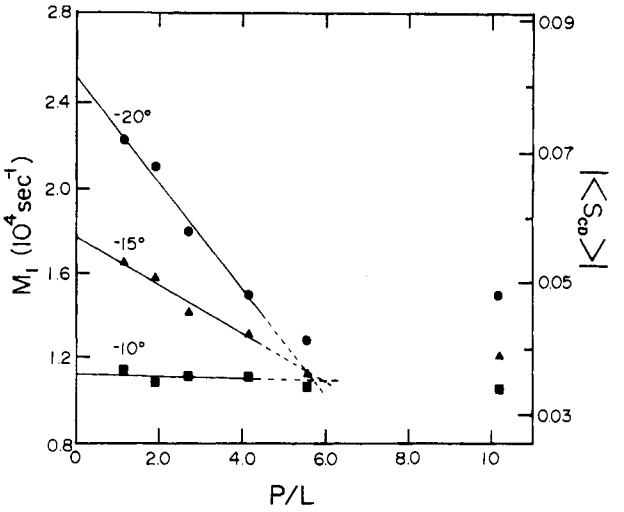

FIGURE 7: First moment and average orientational order parameter as a function of protein to lipid weight ratio at three temperatures: (●) −20 °C, (▲) −15 °C, and (■) −10 °C.

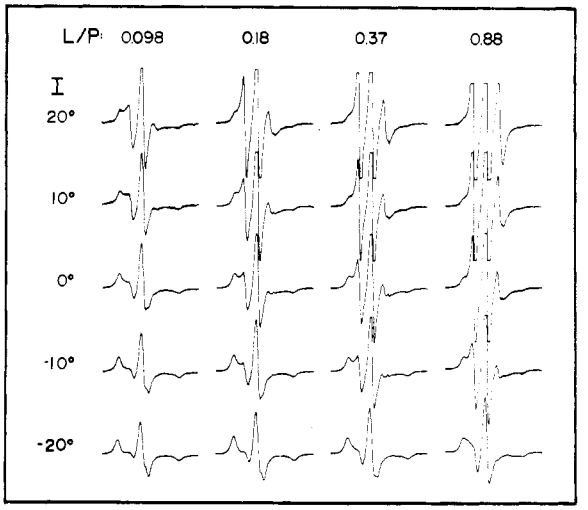

FIGURE 8: EPR derivative spectra at 9.5 GHz plotted to constant integrated intensity (double integral) at 20, 10, 0, −10, and −20 °C and at four lipid to protein weight ratios: $L/P = 0.098$, 0.18, 0.37, and 0.88.

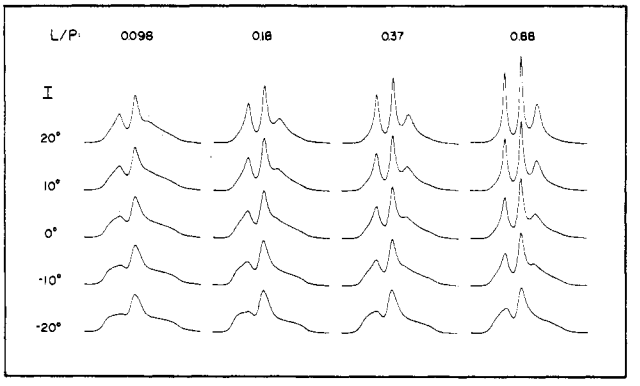

FIGURE 9: EPR absorption spectra at 9.5 GHz plotted to constant integrated intensity at 20, 10, 0, −10, and −20 °C and at four lipid to protein weight ratios: $L/P = 0.098$, 0.18, 0.37, and 0.88.

identical samples used to obtain the ^2H NMR spectra are shown in Figure 8. As is customary in EPR spectroscopy, the derivatives of the absorption spectra are shown here. They are plotted to constant integrated intensity, i.e., constant double integral of the derivative spectra shown in Figure 8, to aid in comparisons between spectra. We also show the absorption

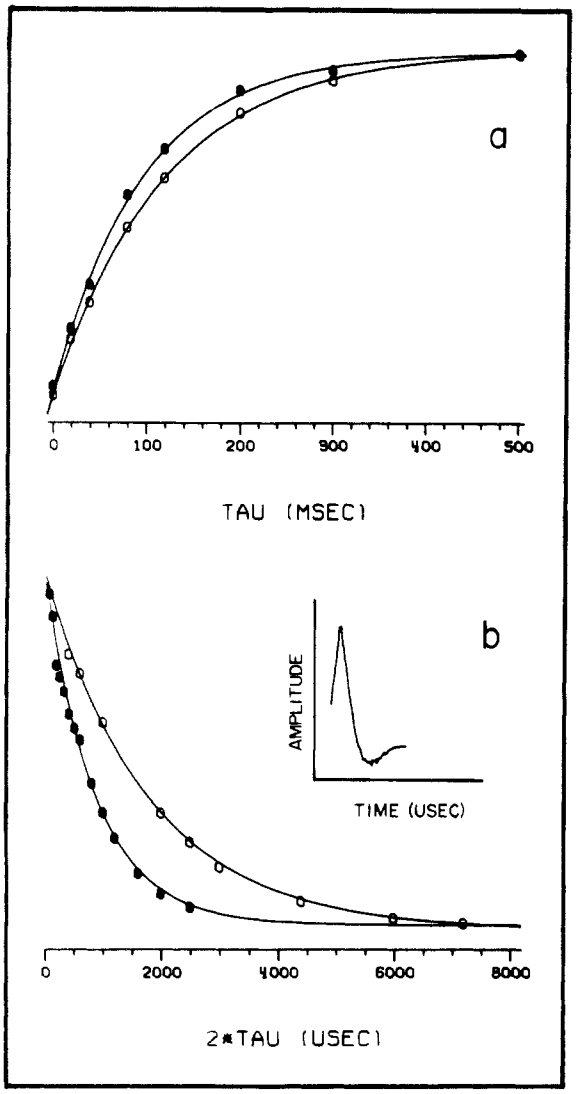

FIGURE 10: Least-squares fits of a single exponential to T_1 (a) and T_{2e} (b) data from (CD_3-P)(PO)PC alone (open circles) and at L/P = 0.44 (solid circles). The best-fit T_1 values are 120 ● 5 and 96 ± 5 ms for (CD_3-P)(PO)PC alone and the reconstituent, respectively. Best-fit T_{2e} values are 1799 ± 60 and 892 ± 60 μs, respectively. Inset shows a typical quadrupolar echo from a P/L = 0.18 sample at −15 °C, showing no distinct sharp spike near the echo maximum.

signal in Figure 9 for comparison to the standard ^2H NMR presentation.

Qualitatively, the EPR derivative spectra show that at any one temperature, the usual variation of the fraction of motionally restricted component with protein concentration is observed. It would also appear that the fraction of motionally restricted component within any one sample decreases with increasing temperature. This decreasing amount of motionally restricted component with increasing temperature is observed in all the samples, even those containing an amount of phospholipid almost half of (0.098 mg/mg) or roughly equal to (0.18 mg/mg) that generally considered necessary to cover the surface of cytochrome *c* oxidase.

Some qualitative features of the individual EPR *absorption* spectra offer a more general interpretation that has obvious analogies to the ^2H NMR spectra. These EPR spectra, shown in Figure 9, are EPR powder patterns analogous to ^2H NMR powder patterns. It is clear that the widths of the powder pattern spectra increase with increasing protein concentration. It is also apparent that increases in temperature cause a substantial narrowing of the absorption envelope for all these samples. When these powder patterns are analyzed by using the two-state model, there appears to be a clear decrease in the amount of broad component present at higher temperatures and lower protein concentrations. In a more general type of analysis, however, the observed spectra are consistent with a range of spin-label order parameters. This distribution of order parameters has an average value and a width that decrease with increasing temperature and decreasing protein concentration. Such an analysis is similar to the way in which we have analyzed the ^2H NMR spectra. Further, though this type of analysis when applied to EPR spectra may be complicated by line-width effects, it is not limited to a model-dependent partitioning of the spectrum between various motional classes of sites.

^2H NMR Relaxation Measurements. A limited number of relaxation time measurements have been made at −5 °C, as shown in Figure 10. In all of the spin–lattice (T_1) relaxation measurements, the relaxation varied exponentially with time, within experimental error, and could be characterized by relaxation times of 120 ± 5 and 96 ± 5 ms for samples containing lipid alone and L/P = 0.44, respectively. The decay of the quadrupolar echo (T_{2e}) at −5 °C was also exponential and characterized by relaxation times of 1799 ± 60 and 892 ± 60 μs, respectively, for the two samples.

Upper Limits on the Amount of Any Second ^2H NMR Spectral Component with Much Higher Orientational Order. We have not been able to find any evidence for spectra corresponding to two distinct motional environments, such as those we reported earlier (Dahlquist et al., 1977) for temperatures above 0 °C. The previous two-component spectrum at 2 °C had a first moment $M_1 = 1.14 \times 10^4$ s^{-1}, while the recent spectrum for the corresponding lipid to protein ratio at 0 °C has a first moment $M_1 = 0.91 \times 10^4$ s^{-1}. Thus, two sets of spectra, though they appear to be qualitatively different, have similar mean orientational order parameters.[2]

In view of the fact that an appreciable fraction of the EPR spectra for these systems arises from orientationally restricted hydrocarbon chains, it is of interest to determine quantitatively the fraction of the ^2H NMR signal which could arise from strongly immobilized chains. For a rotating methyl group on a strongly immobilized hydrocarbon chain, the quadrupolar splitting of the powder spectrum is approximately 37 kHz (Valic et al., 1979), which is approximately 10 times the average splitting of the spectra shown in Figure 4. It is clear that no large spectral component is observable having a splitting close to 37 kHz. The simplest and most sensitive way of establishing an upper limit for such a hypothetical broad

[2] It is important to recognize that even in our original work (Dahlquist et al., 1977), the spectral component of greater orientational order did not have an order parameter characteristic of strongly restricted motion. The order parameter of about 0.03 observed for this component was still much smaller than the order parameter of 0.33 which results when the only molecular motion is the rotation of the terminal methyl group. Thus, despite the fact that the order parameter of the more motionally restricted component was about twice that of the other component, it was still characteristic of a high degree of molecular motion. In fact, the first moments of the two-component spectra of our previous study are quite similar to those obtained from the single-component spectra reported here in the high-temperature region. The conditions producing our original, two-component ^2H NMR spectra remain unknown. However, Swanson et al. (1980) have recently shown that cytochrome *c* oxidase preparations not passed through a gel exclusion column before incorporation into cholate dialysis vesicles can form apparent protein aggregates in the vesicles. Our only procedural modification in the present sample preparation from what we had done previously includes a gel filtration step in which we find that a variable amount (generally less than 10%) of the cytochrome *c* oxidase elutes in the void volume. This material presumably represents large cytochrome *c* oxidase aggregates. The presence of such aggregates offers a plausible explanation for our original observation of two ^2H NMR spectral components with small orientational order.

component is to examine the behavior of the echo near its maximum (see, e.g., the inset to Figure 10b). The peak amplitude of the echo is proportional to the integrated intensity of the ^2H NMR spectrum since the echo signal and spectrum are Fourier transforms of each other. If the spectrum consists of two components whose average order parameters differ by an order of magnitude from each other, the high-frequency component will manifest itself as a narrow spike (in the time domain) superimposed in the broader echo signal arising from the narrow spectral component. An examination of quadrupolar echo signals such as that shown in the insert to Figure 10b, which are recorded by using a large enough bandwidth to accept the broadest signal, indicates that no sharp signal distinguishable from noise can be observed. We have tried a second approach as well. After the Fourier transform is calculated from the echo in Figure 10b to obtain the spectrum in Figure 5d, all of the data (signal and/or noise) at frequencies greater than approximately 15 kHz were replaced by zeros. The inverse transform was then calculated and subtracted from the original echo. There was no observable signal in the resulting difference echo. The ratio of the amplitude of the original echo to the amplitude of the noise in the difference echo was 30:1. Hence, we can set an upper limit of about 3% to the amount of broad component in this sample containing roughly the amount of lipid ($L/P = 0.18$) generally considered necessary to cover the surface of cytochrome *c* oxidase.

Discussion

In this section, we examine more closely what quantitative conclusions can be drawn from our results about the influence of cytochrome *c* oxidase on the motions of the fatty acid chains of the $(CD_3\text{-}P)(PO)PC$ molecules and on the mean association time of the lipid–protein complexes. Qualitatively, the EPR spectra indicate that phospholipid molecules in the presence of protein have at least two sites with measurably different degrees of motional restriction over the entire temperature range studied. However, the ^2H NMR spectra can each be characterized in terms of a relatively narrow distribution of orientational order parameters at all temperatures studied.

Models which reconcile this qualitative discrepancy between the ^2H NMR and EPR data have been proposed by Seelig & Seelig (1978) and Oldfield and co-workers (Oldfield et al., 1978; Kang et al., 1979a; Rice et al., 1979a,b). These models propose exchange between lipids on and off the protein surface at a rate slow on the EPR time scale but fast on the ^2H NMR time scale. Here we consider whether the ^2H NMR spectra support such a two-state model and whether the observed ^2H NMR relaxation times are compatible with such an exchange rate between the two states.

For a two-component system containing mole fractions of "free lipid", χ_f, and "protein-associated lipid", χ_p, the observed value of M_1 is given by

$$M_1 = M_{1,f} + (M_{1,p} - M_{1,f})\chi_p \qquad (14)$$

where $M_{1,f}$ and $M_{1,p}$ are the first moments of the free and protein-associated components, respectively. Thus a plot of M_1 vs. χ_p should be linear over its range of 0 to 1. A quantity proportional to χ_p and easily measured is the protein to lipid weight ratio P/L:

$$\chi_p = \frac{N(\text{moles of protein})}{\text{moles of lipid}} = N\left(\frac{M_L}{M_p}\right)\left(\frac{P}{L}\right) = \left(\frac{L}{P}\right)_{\text{surface}}\left(\frac{P}{L}\right) \qquad (15)$$

where N is a fixed number (by assumption) of lipid sites per protein molecule and M_L and M_P are the molecular weights of the lipid and protein, respectively. A plot of M_1 vs. P/L should be linear until there is not enough lipid to occupy all the sites on the protein surface. At this point ($\chi_p = 1$), one would expect a break in the plot, with M_1 remaining constant at $M_1 = M_{1,p}$ as P/L is increased further. The position of this break on the P/L axis represents the amount of protein-associated lipid, while its M_1 value represents the first moment of the spectrum of the protein-associated lipid. The M_1 axis intercept of the linear portion of the plot gives the value of the first moment for the free lipid.

Such plots have been presented in Figure 7 for data at −20, −15, and −10 °C. As can be seen in the figure, (1) the −20 and −15 °C plots are quite linear at low protein to lipid ratio, (2) the M_1 intercepts are within 8% of the free lipid values, and (3) both plots have a break at P/L roughly between 4 and 6. Within this region of P/L, the two lines intersect each other and the temperature-invariant −10 °C line at $P/L = 5.7 \pm 0.2$. The intersection of the three straight lines in Figure 7 implies that the number of protein-associated lipids per protein molecule is independent of temperature below −10 °C and gives $(L/P)_{\text{surface}} = 0.18$. This value is close to the value obtained from previous EPR studies with cytochrome *c* oxidase (Jost et al., 1973a) and is the same as the value obtained from our own EPR measurements on these samples at −20 °C, as described below. While the agreement in Figure 7 between a two-state model and experiment does not prove this model, it does indicate that this model adequately describes the average orientational order parameter of the system. It should be emphasized that this fit to a two-state model applies only to the low-temperature region where the pure phospholipid would be in or near the gel phase.

It is important to recognize that at temperatures below −5 °C, the average orientational order of the lipid in the presence of protein is less than that for the lipid alone, as is shown in Figure 6. For example, from the breakpoint in the data of Figure 7, the value of the first moment of the protein surface spectrum is $1.1 \times 10^4\ s^{-1}$. This compares to the free lipid value at −15 °C of $1.7 \times 10^4\ s^{-1}$. However, this result appears to be in direct conflict with the EPR data on the same samples. The spectra in Figures 8 and 9 clearly show an increase in the orientational order of some of the lipid in the presence of protein. This increase in the EPR orientational order can be treated more quantitatively by using a two-state model [see, e.g., Jost et al. (1973a)]. The exact values obtained for the amount of free and surface lipid depend to some extent on the assumptions made about the spectra characterizing the two environments. At temperatures near the phase-transition temperature of the pure lipid, one obtains a value near 0.2 mg of lipid per mg of protein for the amount of protein-associated lipid. At higher temperatures, the average order of the EPR spectrum clearly decreases. This could be the result of a decrease in the *amount* of protein-associated lipid or the result of a temperature-dependent decrease in the *orientational order* of the protein-associated lipid. There appears to be no objective criterion for separating these effects. Different EPR workers apply different criteria to assign the protein associated spectrum. In one case (Davoust et al., 1979), this generates a temperature-dependent amount of protein-associated component, while another laboratory finds a temperature-independent amount with a temperature-dependent protein-associated lipid spectrum (Brotherus et al., 1980). Either interpretation suggests that the effect of protein is to increase the average orientational order at any one temperature, with the magnitude of this increased orientational order decreasing with increasing temperature. Hence, protein concentration and temperature

modulate the order parameters in both the EPR and ^2H NMR spectra, but the sign of the effect is opposite for the two techniques. This apparent discrepancy can be examined in relation to the ^2H NMR *relaxation* measurements.

In analyzing the relaxation time measurements, we first note that the value of $T_1 \simeq 0.12$ s for the free lipid can be accounted for by the rotation of the CD_3 group about its symmetry axis, though some additional rapid motions may also make appreciable contributions. The CD_3 rotation gives $S_f^{(1)} = {}^1/_3$ so that $1 - (S_f^{(1)})^2 = {}^8/_9$ of the spectral density of the fluctuating quadrapolar interactions is associated with it (see eq 7, 9, and 10 and identify sites a and b with f and p, respectively). For $\tau_f^{(1)} = 2 \times 10^{-11}$ s, which is the expected order of magnitude of the correlation time for CD_3 rotation near 0 °C in systems such as this (Jeffrey et al., 1979), this motion can account *completely* for the observed value of T_1. If $\tau_f^{(1)}$ is actually shorter than this value, then other motions which modulate part of the remaining one-ninth of the spectral density also contribute to T_1. We have shown that T_1 is decreased by about 20% from the free lipid value for the sample with $L/P \simeq 0.44$ in which a little less than half the lipids are believed to be associated with the protein surface. This change in T_1 can tentatively be assigned to a slight slowing down of the CD_3 rotation by the protein–lipid interaction.

Of more interest to us is the decrease of T_{2e} from the free lipid value of 1.8×10^{-3} s by about a factor of two for the $L/P = 0.44$ sample. By use of eq 11, in order to reduce T_{2e} by the observed factor of 2, the protein must induce enough slow motion to contribute $T_2' \simeq 10^{-3}$ s. If the slow motions are characterized by a single correlation time τ_c, their maximum contribution for methyl groups is obtained by putting $S_p^{(1)} = S_p' = {}^1/_3$ in eq 13. Approximating S_p by $S_{1,p}$ and S_f by $S_{1,f}$ in eq 12 ($S_{1,p}$ and $S_{1,f}$ are proportional to $M_{1,p}$ and $M_{1,f}$, respectively, as discussed in reference to Figure 7) and noting that the measured value of $(S_{1,p} - S_{1,f})^2 \ll (S_p')^2$, we obtain

$$\frac{1}{T_2'} \simeq \chi_p \frac{1}{T_{2p}'} \simeq \chi_p \frac{\Omega_Q^2}{5} (S_p')^2 \tau_c \qquad (16)$$

When $T_2' = 9 \times 10^{-4}$ s, $\chi_p = 0.5$, $\Omega_p = 2\pi(1.26 \times 10^5\ s^{-1})$, and $S_p' = {}^1/_3$ are used, eq 16 gives a value of $\tau_c \simeq 2 \times 10^{-7}$ s for the correlation time. This value of τ_c may reflect the time scale of motions on the protein surface and/or it may reflect the time scale of the exchange of lipid between the surface and free lipid. In either case, the order parameter observed after these fluctuations have occurred is reduced from a value of ${}^1/_3$ to a value near 0.

In obtaining this result, we have assumed that there is only one lipid motion on the protein surface sufficiently fast to satisfy $\omega_0 \tau_p^{(\mu)} \ll 1$ or, since $\omega_0 = 2\pi(23.3 \times 10^6\ s^{-1})$, $\tau_p^{(\mu)} \ll 7 \times 10^{-9}$ s. Since the motion corresponds to CD_3 rotation, this assumption is tantamount to assuming that the molecular axis is immobilized for at least 7×10^{-9} s on the surface. We then assumed that all other, slower, fluctuations of deuterium nuclear quadrupolar interactions for lipids associated with the proteins are characterized by a single correlation time τ_c. The value of $\tau_c \simeq 2 \times 10^{-7}$ s is then required to explain the change in T_{2e} due to the presence of the protein. Since the experimental study of T_1 has only been carried out at one frequency, we cannot yet exclude some fast motions of the fatty acyl chains in addition to CD_3 rotations. Of course, such additional fast motions would produce reductions in the apparent value of S_p as determined by the EPR spin-labels and must be severely limited if we insist that the reorientational motions of the nitroxide-labeled fatty acid chains are representative of fatty acid chains in general. Recent fluorescence depolarization results qualitatively agree with the EPR results (Kimmelman et al., 1979; Jahnig, 1979; Heyn, 1979) and reflect motions over the same time scale as the EPR measurements. The presence of any such additional fast motions would reduce S_p' and increase the required value of τ_c in proportion to $(S_p')^{-2}$, as may be seen from eq 16, so that, strictly speaking, 2×10^{-7} s has to be taken as a *lower* limit to τ_c. Since the value of τ_c is much greater than the "averaging time" of the EPR spin-labels which, as discussed under Theory, are characterized by a coupling constant on the order of $10^8\ s^{-1}$, we conclude that the result that $S_{1,p}$ (from ^2H NMR) $\ll S_p$ (from EPR) is indeed *compatible* with the observed ^2H NMR relaxation times.

We now turn to the interpretation of τ_c. *One possibility* is that τ_c is a measure of the time for reorientation of the lipid acyl chains *on* the protein surface. This interpretation requires that $\tau_c \ll \tau_{ex}$, and it *cannot* be ruled out at this time. One physical picture of this reorientation has been proposed by Seelig & Seelig (1978) and, independently, by Oldfield and co-workers (Oldfield et al., 1978; Kang et al., 1979; Rice et al., 1979a,b), who point out that the protein surface with its amino acid side chains is likely to be quite rough so that lipid molecules moving about on the protein surface would cause the necessary reorientation. An alternate, related suggestion by Bloom (1980) is that the protein surface undulates appreciably because of its "squishy" nature and is matched to the fluid mechanical properties of the liquid-crystalline phase of the phospholipid bilayer. There is, however, a *second possibility*, that τ_c is associated with the time of exchange of lipids between the protein surface and the free lipid. When the lipids jump off the protein surface and then back onto the surface, they may return to a state which is different from their original one. This leaves open the possibility that molecular reorientations resulting from the exchange process are responsible for the decrease in ^2H HMR orientational order parameters in the presence of protein. From eq A5 and A6 and assuming that $k_{fp} \simeq k_{pf} \gg k_{pp}$, we obtain the result $\tau_c \simeq 2\tau_{ex}$ which leads to the possibility that $\tau_{ex} \lesssim 10^{-7}$ s. If this interpretation turns out, after further investigation of the relaxation behavior of protein-associated lipids, to be correct, we would conclude that τ_{ex} is not much longer than the time taken for a phospholipid molecule to diffuse away from a protein boundary.[3]

In closing, we remark that the implications of the observed influence of proteins on phospholipid orientational order parameters presented here for the cytochrome *c* oxidase/$(CD_3$-P)(PO)PC system should apply also to the now substantial class of similar studies carried out on reconstituted and bacterial membranes described in many of the papers referenced here. A more precise understanding of the nature of the lipid–protein interaction will be provided by an extension of the study of the dynamical properties of protein-associated lipids by nuclear spin relaxation.

Acknowledgments

We thank David Muchmore for synthesizing the phospholipids used in this study. Special thanks go to Steven Vik and Jeanne Ivy for their patient and gracious advice about the

[3] Recent measurements of the translational diffusion constant of free lipid-like molecules give values of about $4 \times 10^{-8}\ cm^2\ s^{-1}$ (Smith et al., 1979b; Bloom et al., 1978). The time needed to diffuse a distance comparable with a lipid diameter of about 5 Å is about 2×10^{-8} s, but the time required to diffuse a distance of 5 Å from a larger object can be somewhat longer (Berg & Purcell, 1977).

isolation of cytochrome *c* oxidase and for performing the enzyme activity assays.

Appendix

Two-Site Model Correlation Function. Consider a system of molecules having two sites α = a and b where the equilibrium number of molecules at each site is N_α and the total number of molecules $N = N_a + N_b$ is a constant. Suppose that the two sites each have molecular states i = 1, 2, ..., with equilibrium probabilities $\bar{P}_{\alpha i} = N_{\alpha i}/N$, and define $\chi_\alpha = \sum_i \bar{P}_{\alpha i}$ and $P_\alpha = \sum P_{\alpha i}$. Assume that the occupation probabilities satisfy the master equation

$$\frac{dP_{\alpha i}}{dt} = -P_{\alpha i}\sum_{\beta j} W_{\alpha i,\beta j} + \sum_{\beta j} W_{\beta j,\alpha i}P_{\beta j} \qquad (A1)$$

where $W_{\alpha i,\beta j}$ is the probability per unit time that a system in state α,i undergoes a transition to state β,j. Transitions within the states at site α are characterized by a mean lifetime $k_{\alpha\alpha}^{-1}$ and those between states α and $\beta \neq \alpha$ by a mean lifetime $k_{\alpha\beta}^{-1}$.

For simplicity, we assume that the transitions are produced by "strong collisons", i.e., that the transition probabilities are proportional to the equilibrium probabilities of the final states:

$$W_{\alpha i,\beta j} = (k_{\alpha\beta}/\chi_\beta)\bar{P}_{\beta j} \qquad (A2)$$

which satisfies detailed balance in the form $\chi_a k_{ab} = \chi_b k_{ba}$. Using eq A2, we may write eq A1 as

$$dP_{ai}/dt = -(k_{aa} + k_{ab})(P_{ai} - \bar{P}_{ai}) + (k_{aa} - k_{ba})(P_a - \chi_\alpha)(\bar{P}_{ai}/\chi_a) \qquad (A3)$$

The solutions to eq A3 and their counterparts for $P_{\beta j}$ are

$$P_{\alpha i}(t) = \bar{P}_{\alpha i} + (P_{\alpha i}(0) - \bar{P}_{\alpha i})e^{-t/\tau_{ex}} + (\bar{P}_{\alpha i}/\chi_\alpha)P_\alpha(0)(e^{-t/\tau_{ex}} - e^{-t/\tau_\alpha}) \qquad (A4)$$

where τ_{ex} is the correlation time for exchange of molecules between a and b

$$1/\tau_{ex} = k_{ab} + k_{ba} \qquad (A5)$$

and τ_α is a mean lifetime for molecules initially in the state α

$$1/\tau_\alpha = k_{\alpha\alpha} + k_{\alpha\beta} \qquad \beta \neq \alpha \qquad (A6)$$

The three possible types of initial conditions are as follows: case I, molecule initially in state α,i, $P_{\alpha i}(0) = P_\alpha(0) = 1$; case II, molecule initially in state $\alpha,j \neq i$, $P_{\alpha i}(0) = 0$, $P_\alpha(0) = 1$; case III, molecule initially in state $\beta \neq \alpha,j$, $P_{\alpha i}(0) = P_\alpha(0) = 0$.

These results enable us to calculate the conditional probabilities $P_{\alpha i,\beta j}(t)$ that the molecule is in state β,j at time t given that it is in state α,i initially from which the "local field" $\omega(t)$ may be calculated. If the local field has the value $\omega_{\alpha i}$ in the state α,i, its correlation function is given by

$$G(t) = \sum_{\alpha,i}\sum_{\beta,j}\bar{P}_{\alpha,i}P_{\alpha i,\beta j}(t)\omega_{\alpha i}\omega_{\beta j} - (\sum_{\alpha,i}\bar{P}_{\alpha i}\omega_{\alpha i})^2 \qquad (A7)$$

By use of eq A4 and the initial conditions given above, i.e., case I for $P_{\alpha i,\alpha i}(t)$ and case II for $P_{\alpha i,\alpha j}(t)$ where $\beta \neq \alpha$, the correlation function may be written in the form

$$G(t) = \chi_a(\langle\omega_a^2\rangle - \langle\omega_a\rangle^2)e^{-t/\tau_a} + \chi_b(\langle\omega_b^2\rangle - \langle\omega_b^2\rangle)e^{-t/\tau_b} + \chi_a\chi_b(\langle\omega_a\rangle - \langle\omega_b\rangle)^2e^{-t/\tau_{ex}} \qquad (A8)$$

where

$$\langle\omega_{\alpha i}^n\rangle = \frac{\sum_i \bar{P}_{\alpha i}\omega_{\alpha i}^n}{\chi_\alpha} \qquad (A9)$$

is the average of $\omega_{\alpha i}^n$ in the site α.

One case of particular interest is that of local fields associated with quadrupolar interactions which may be written

$$\omega_{\alpha i} = \Omega_Q\left(\frac{3\cos^2\theta_{\alpha i} - 1}{2}\right) \qquad (A10)$$

where $\theta_{\alpha i}$ is the angle between the axis of symmetry of the local electric field gradient and the applied magnetic field and $\Omega_Q = {}^3/_4e^2qQ/\hbar$ is the quadrupolar coupling constant. In general, the correlation function depends on the orientations of the local symmetry axes for the molecular motions and on the local orientational order parameters S_a and S_b for the two sites. We present here only the orientation averaged correlation functions. Assuming an ensemble of randomly oriented bilayers, eq A8 may be written

$$G(t) = {}^1/_5\Omega_Q^2(\chi_a(1 - S_a^2)e^{-t/\tau_a} + \chi_b(1 - S_b^2)e^{-t/\tau_b} + \chi_a\chi_b(S_a - S_b)^2e^{-t/\tau_{ex}}) \qquad (A11)$$

The first two terms in eq (A11) are associated with fluctuations of the local field in each site about the average value *in that site* while the third term is due to fluctuations about the average value for the entire system due to transitions *between* the two sites. This may be seen from the definition of the average order parameter

$$\bar{S} = \chi_a S_a + \chi_b S_b \qquad (A12)$$

and the relationship

$$\chi_a\chi_b(S_a - S_b)^2 = \chi_a(S_a - \bar{S})^2 + \chi_b(S_b - \bar{S})^2 \qquad (A13)$$

Finally, it is of interest to generalize eq A11 to include the possibility of more than two states, i.e., α = a, b, c, ..., and more general mechanisms of molecular motion that are encompassed by eq A1. We suggest as a reasonable empirical generalized form for the correlation function

$$G(t) = {}^1/_5\Omega_Q^2\sum_\alpha \chi_\alpha((1 - S_\alpha^2)g_\alpha(t) + (S_\alpha - \bar{S})^2g_{ex}(t)) \qquad (A14)$$

where $\bar{S} = \sum_\alpha \chi_\alpha S_\alpha$ is the average order parameter for the entire system and the "reduced correlation functions" $g_\alpha(t)$ and $g_{ex}(t)$ have the property that $g(0) = 1$ and $g(t) \to 0$ as $t \to \infty$. Effective correlation times can be defined in terms of the areas under $g(t)$, i.e., $\tau_\alpha = \int_0^\infty g_\alpha(t)\,dt$ and $\tau_{ex} = \int_0^\infty g_{ex}(t)\,dt$, but the actual form of the $g(t)$ can, in principle, be determined empirically from relaxation measurements.

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