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^{31}P NMR Spectra of Rod Outer Segment and Sarcoplasmic Reticulum Membranes Show No Evidence of Immobilized Components due to Lipid–Protein Interactions†

Jeffrey F. Ellena, Robert D. Pates, and Michael F. Brown*
Department of Chemistry and Biophysics Program, University of Virginia, Charlottesville, Virginia 22901
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ABSTRACT: ^{31}P NMR studies of rod outer segment (ROS) and sarcoplasmic reticulum (SR) membranes have been performed under conditions where broad and narrow spectral components can be clearly resolved. Control studies of an anhydrous, solid powder of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), as well as aqueous binary mixtures of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), demonstrate clearly that broad spectral components can be detected. For the codispersions of DSPC and DOPC in the mixed-phase region at 22 °C, the ^{31}P NMR spectra consist of a superposition of a broad component and a narrow, axially symmetric component, due to coexisting solid and liquid-crystalline domains, which are in slow exchange on the ^{31}P NMR time scale. The ^{31}P NMR spectra of the native ROS and SR membranes, however, consist of only a narrow component, to within experimental error, indicating that most or all of the phospholipids are in the liquid-crystalline (L_α) phase at 22 °C. The above conclusions are in agreement with many, but not all, previous studies [see, e.g., Yeagle, P. L. (1982) *Biophys. J. 37*, 227–239]. It is estimated that at most 10% of the phospholipids in the ROS and SR membranes could give rise to broad ^{31}P NMR spectral components, similar to those seen for anhydrous or solid-phase lipids, corresponding to ~7 phospholipids/rhodopsin molecule and ~11 phospholipids/Ca^{2+}-ATPase molecule, respectively.

The effects of intrinsic membrane proteins on the structural and dynamic properties of membrane lipids have been studied by various spectroscopic techniques, including fluorescence depolarization (Wolber & Hudson, 1982), spin-label electron paramagnetic resonance (EPR) (Ellena et al., 1983; Pates et al., 1985b; Marsh, 1985),[1] and nuclear magnetic resonance (NMR) (Seelig & Seelig, 1980; Brown et al., 1982; Davis, 1983). Spin-label EPR studies of different membrane systems have shown that under the appropriate conditions two spectral components can be resolved (Jost et al., 1973a,b; Marsh, 1985). One component has been attributed to the lipids in van der Waals contact with the hydrophobic surfaces of intrinsic membrane protein molecules, and the other component to bulk lipids not in direct steric contact with membrane proteins. Most NMR studies of membrane systems have yielded somewhat different results, however, in that only a single spectral component is usually detected. One explanation for the discrepancy is that the motional rates necessary to produce averaging of the spectra are different in the two cases (Brown et al., 1977; Seelig & Seelig, 1980; Paddy et al., 1981). That is, since the NMR frequencies are less than those in EPR spectroscopy, the distinct components seen with spin-label EPR could be averaged over the much longer NMR time scale and only a single component observed (Brown et al., 1977). Several EPR spin-label studies have further suggested that there may be some selectivity at the membrane lipid-protein interface (Brotherus et al., 1981; Knowles et al., 1981; Ellena et al., 1983; Brophy et al., 1984; Esmann et al., 1985). Also, in some cases there may be a small number of lipids that are tightly bound to high-affinity sites of intrinsic membrane proteins (Robinson, 1982). Nonetheless, most current opinion favors the view that the motional rates and amplitudes of the lipids in steric contact with intrinsic membrane proteins are not greatly different from those of lipids in the bulk bilayer phase of the membrane (Sefcik et al., 1983).

Reports of additional NMR spectral components due to membrane protein associated lipids have appeared, however

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[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; M_r, molecular weight; NMR, nuclear magnetic resonance; ROS, rod outer segment; SR, sarcoplasmic reticulum; $\Delta\sigma$, chemical shift anisotropy; τ_c, correlation time; T_M, gel to liquid-crystalline phase transition temperature; X, mole fraction.

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[cf. Dahlquist et al. (1977), Paddy et al. (1981), and Yeagle (1982)]. In a recent series of communications, it has been suggested that intrinsic membrane proteins are able to cause motional restriction or immobilization of lipids on the NMR time scale, leading to broad ^{31}P NMR spectral components or to a reduction of the lipid resonance intensity due to the presence of an unobserved broad spectral component (Yeagle & Romans, 1981; Yeagle, 1982, 1984; Albert et al., 1982, 1985; Albert & Yeagle, 1983; Selinsky & Yeagle, 1984, 1985). The two systems studied in greatest detail are the rod outer segment (ROS) disk membranes from the bovine retina and the sarcoplasmic reticulum (SR) membranes from rabbit skeletal muscle. Since the above reports have remained largely unverified, we have performed additional studies employing solid-state NMR methods and we communicate here some of our preliminary findings.

Experimental Procedures

Preparation of Phospholipid Samples. Phospholipids were synthesized by the method of Mason et al. (1981) or were purchased from Avanti Polar Lipids (Birmingham, AL). Anhydrous 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was prepared as described by Herzfeld et al. (1978). The DPPC was dried overnight under a vacuum of 10^{-6} torr at 85 °C to constant weight, and the tube was then sealed. Binary mixtures of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were prepared by mixing aliquots of the phospholipids in $CHCl_3$, followed by evaporation of the solvent and exposure to a vacuum of 0.05 torr overnight. The DSPC/DOPC mixtures were then hydrated with 20 mM MOPS buffer, pH 7.0, dispersed by vortexing the samples, and transferred to flat-bottomed NMR tubes.

Preparation of Rod Outer Segment and Sarcoplasmic Reticulum Membranes. Bovine ROS membranes were prepared from frozen retinas (Lawson Co., Lincoln, NE) as described (Papermaster, 1982). All work was conducted under dim red light (Kodak Safelight filter no. 1, 15-W bulb). All manipulations used argon-saturated buffers and were performed under an argon atmosphere to minimize lipid peroxidative damage. The purified ROS membranes typically had A_{280}/A_{500} ratios of 2.4 to 2.7 before regeneration [cf. Raubach et al. (1974)], were 10% bleached, and were 95% regenerable after bleaching followed by addition of 11-*cis*-retinal. The lipid/protein ratio and the phospholipid head group and fatty acyl chain composition were determined by using standard techniques and were in agreement with published values (Stone et al., 1979; Miljanich et al., 1979). The ROS membranes were washed and exchanged by centrifugation into 20 mM HEPES buffer, pH 7.0, pelleted in a Sorvall T865.1 rotor at an average relative centrifugal force of 300000*g* for 4 h, and transferred to NMR tubes. The ROS membrane samples were characterized before and after NMR spectroscopy as described above and were not found to have changed significantly during the course of the experiments. Sarcoplasmic reticulum (SR) membranes were isolated from rabbit back and hind leg white (fast) skeletal muscles, provided by the laboratory of Dr. Michael J. Peach at the University of Virginia, according to the method of Fernandez et al. (1980). Argon-saturated buffers were used to reduce lipid peroxidation. The light SR membrane fraction at the 35/40% (w/v) interface of the sucrose density gradients was harvested, and the membranes were washed and exchanged by centrifugation into 20 mM HEPES buffer, pH 7.0. The Ca^{2+}-activated ATPase activity of the SR membrane preparations was measured with a coupled enzyme assay (Barnett, 1970) and was found to be in the range reported by other groups (Nakamura & Ohnishi, 1975; Moore et al., 1981). The lipid/protein ratio and the phospholipid head group and fatty acyl chain composition of the SR membrane preparations were in agreement with published values (Fiehn & Hasselbach, 1970; Moore et al., 1978; Van Winkle et al., 1982). The SR membranes were pelleted at an average relative centrifugal force of 150000*g* for 2 h in a Sorvall T865.1 rotor and then transferred to NMR tubes. The enzymatic activity of the SR membrane samples was characterized before and after NMR spectroscopy; no significant differences were noted.

Nuclear Magnetic Resonance Spectroscopy. ^{31}P NMR spectra were obtained with a home-built NMR spectrometer, operating at a magnetic field strength of 5.882 T (Larmor frequencies of 250.44 MHz for ^1H and 101.38 MHz for ^{31}P), located in the laboratory of Dr. Dennis A. Torchia at the National Institutes of Health in Bethesda, MD. The sample temperature for all spectra was 22 ± 1 °C. A single 90° pulse (4 μs) was applied, and the free induction decays were acquired in quadrature beginning 10 μs after the end of the pulse. A total of 250–3500 free induction decays were typically averaged and Fourier transformed, Butterworth filters were used with a spectral width of ±50 kHz (dwell time = 10 μs), and 4096 data points were collected. A coherent ^1H decoupling field of strength 40 kHz was gated on during the acquisition. All free induction decays were left-shifted one point, and a 180° first-order phase correction (corresponding to the Nyquist frequency) was applied to compensate for this operation. No additional first-order phase correction was applied. Thus, the time between the middle of the 90° pulse and the beginning of the final free induction decay (i.e., the effective dead time) was 22 μs. The left shift and the 180° first-order phase correction yielded ^{31}P NMR spectra with flat base lines over the spectral region of interest, and eliminated the possibility of subjective adjustment of the base line through first-order phase correction. All free induction decays were zero-filled to a final size of 8192 points, and a 100–Hz exponential line broadening was applied before Fourier transformation. The spin-lattice (T_1) relaxation time of the anhydrous, solid DPPC powder was measured by the progressive saturation technique; an approximate value of 3 ± 0.5 s was obtained with little detectable anisotropy. A recycle time of 12 s was employed for the anhydrous DPPC sample. Recycle times of 8 s were employed for the phospholipid bilayer samples and the membrane samples. Therefore, according to the Bloch equations, and assuming an upper limit of 3 s for the ^{31}P spin–lattice relaxation times of the membrane phospholipids (Yeagle et al., 1975; McLaughlin et al., 1981; Seelig et al., 1981; Tamm & Seelig, 1983), all ^{31}P NMR spectral components will be ≥93% of their equilibrium intensities (Carrington & McLachlan, 1967).

Results

A ^{31}P NMR spectrum of an anhydrous, solid powder sample of DPPC, obtained with ^1H decoupling at 22 °C, is shown in Figure 1a. The control spectrum suggests that the spectrometer used for this study is capable of obtaining relatively undistorted ^{31}P NMR spectra of phospholipids in the rigid limit, where little molecular motion occurs (Griffin, 1976). Significant intensity is observed over the spectral range of −110 to +140 ppm, relative to 85% H_3PO_4, beyond which the base line is flat to within experimental error. The three principal values of the rigid-lattice or static chemical shift tensor of the phosphodiester moiety can be evaluated directly from the spectrum (Mehring, 1983) and are in agreement with the reported values of $\sigma_{11} = -98$ ppm, $\sigma_{22} = -34$ ppm, and $\sigma_{33} =$

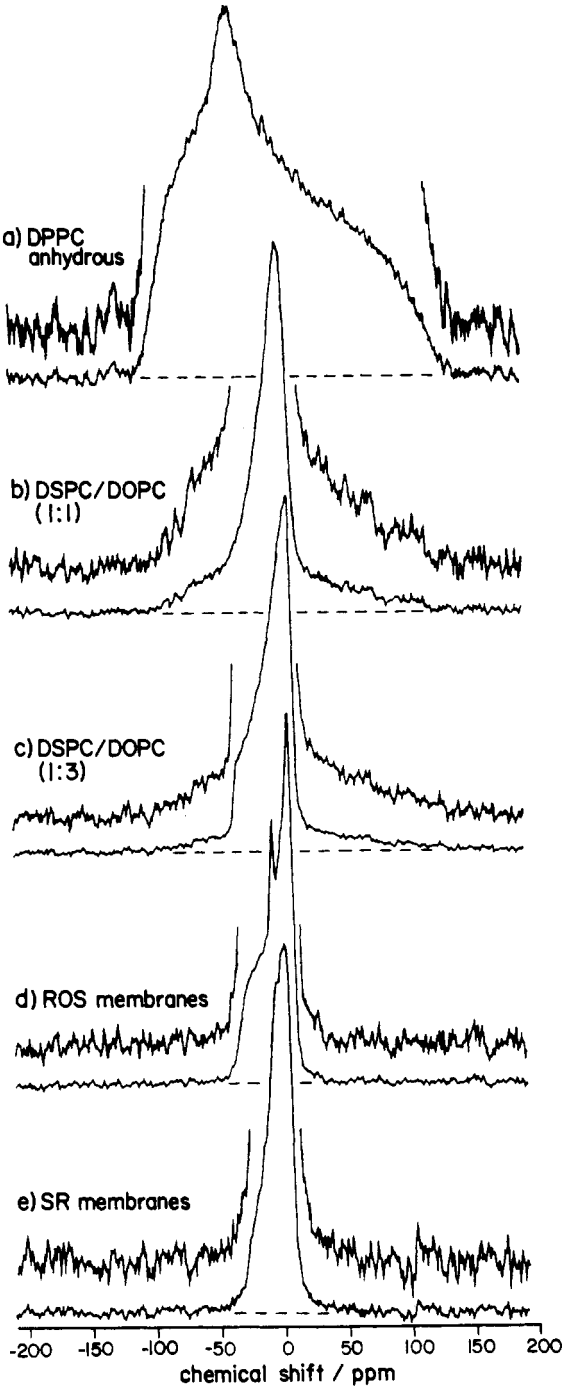

FIGURE 1: ^{1}H-decoupled ^{31}P NMR spectra obtained at 22 °C with a single 90° pulse of 4-μs duration. The chemical shift scale is relative to 85% H_3PO_4. A linear, frequency-dependent phase correction representing a 180° shift across the spectral width was applied (see text). The vertical scale has been expanded by a factor of 3 to either side of the spectra to illustrate the flat base lines and the presence or absence of an underlying component. (a) Anhydrous DPPC powder. (b) Aqueous dispersion of a binary mixture containing DSPC and DOPC at a mole ratio of X_{DSPC}/X_{DOPC} = 1.0 and ~70 wt % H_2O. (c) Aqueous dispersion of a binary mixture containing DSPC and DOPC at a mole ratio of X_{DSPC}/X_{DOPC} = 0.33 and ~70 wt % H_2O. (d) Native unbleached ROS membranes containing ~70 wt % H_2O. (e) Native SR membranes containing ~70 wt % H_2O.

+134 ppm (Herzfeld et al., 1978). Since the principal values of the rigid-lattice chemical shift tensor do not depend greatly on the nature of the polar head group (Herzfeld et al., 1978) or on the degree of hydration (Griffin, 1976), the spectrum of the anhydrous DPPC powder is indicative of the maximum chemical shift range over which spectral intensity is to be expected for pure phospholipid bilayers.

As a model for the type of situation that might be encountered in native biomembranes, parts b and c of Figure 1 show additional, control ^{31}P NMR spectra of hydrated binary mixtures of DSPC and DOPC, containing ~70 wt % H_2O, at mole ratios of X_{DSPC}/X_{DOPC} = 1.0 and 0.33 (1:1 DSPC/DOPC and 1:3 DSPC/DOPC, respectively). It is well-known that mixtures of phospholipids that have a large difference in their gel to liquid-crystalline phase transition temperatures (T_M) tend to exhibit monotectic behavior, in which solid- (gel-) phase solutions are not formed to a significant degree [cf. Ladbrooke and Chapman (1969), Mabrey and Sturtevant (1976), and Sklar et al. (1979b)]. This appears to be the case for the binary mixtures of DSPC and DOPC, for which the values of T_M are 58 and −22 °C, respectively (ΔT_M = 80 °C) (Phillips et al., 1970; De Kruyff et al., 1973). Two components are clearly seen in the ^{31}P NMR spectra of the DSPC/DOPC mixtures, with relative proportions that depend on the sample composition. The relatively narrow component, with an axially symmetric, residual chemical shift tensor [$\Delta\sigma \cong -50$ ppm; cf. Seelig (1978)], is indicative of a liquid-crystalline phase arising from the lower melting DOPC component. The underlying broad component is consistent with the presence of a solid or gel phase comprised mainly of DSPC at the temperature employed (22 °C). An increase in the intensity of the broad component with increasing mole fraction of DSPC is observed; compare parts b and c of Figure 1. The width of the broad component is similar to that of the anhydrous DPPC powder; i.e., spectral intensity is observed over the approximate range of −100 to +120 ppm, beyond which the base line is flat to within experimental error. The presence of two distinct components suggests that the exchange rate of phospholipids between the solid-phase and liquid-crystalline-phase environments is slow relative to the difference in their residual chemical shift tensors ($\Delta\nu_{ex} < 10^4$ s^{-1}).[2] To our knowledge, a detailed phase diagram for the DSPC/DOPC system is not available [cf. Phillips et al. (1970) and De Kruyff et al. (1973)]. However, the estimated areas of the broad components in Figure 1b,c correspond to the presence of 40% solid-phase lipid in the binary mixture with X_{DSPC}/X_{DOPC} = 1.0 and 25% solid-phase lipid in the mixture with X_{DSPC}/X_{DOPC} = 0.33 at 22 °C. These values are in good agreement with the sample composition and expected monotectic behavior, assuming solid- (gel-) phase immiscibility [cf. Sklar et al. (1979b)]. Thus it can be concluded that as little as 25% solid-phase lipid can be easily detected (cf. Figure 1c). We estimate that ~10% or less solid-phase lipid could be seen under the conditions of our experiments.

Parts d and e of Figure 1 show ^{31}P NMR spectra of bovine retinal ROS membranes and SR membranes from rabbit skeletal muscle (light fraction), respectively. In contrast to the two-component ^{31}P NMR spectra in Figure 1b,c, only a relatively narrow, axially symmetric component is observed in the ^{31}P NMR spectra of these biological membranes at 22 °C. There is little or no resonance intensity in regions outside of the relatively narrow component, e.g., in the −110 to −50 and +30 to +140 ppm regions, where significant intensity is observed for the anhydrous DPPC and multilamellar DSPC/DOPC mixtures (Figure 1a–c). Rather, the ^{31}P NMR spectra of the ROS membranes and SR membranes are indicative of phospholipid bilayers in the liquid-crystalline (L_α)

[2] Estimated by assuming a difference of (~100 ppm)(101.4 MHz) $\cong 10^4$ Hz for the perpendicular bilayer orientation. A lower limit to the lifetime of a phospholipid in the solid or liquid-crystalline domains can thus be estimated to be $\tau_{ex} > (2\pi\Delta\nu_{ex})^{-1} \cong 10^{-5}$ s.

state (Seelig, 1978). For the ROS membranes, the apparent residual chemical shift anisotropy $\Delta\sigma \cong -40$ ppm, in agreement with previous results (Deese et al., 1981a; Mollevanger & De Grip, 1984; Albert et al., 1984). For the case of the SR membranes, the apparent residual chemical shift anisotropy $\Delta\sigma \cong -40$ ppm. ^{31}P NMR spectra of the corresponding dispersions of total extracted ROS and SR lipids (not shown) were very similar to those of the native membranes [cf. Davis and Inesi (1972), Deese et al. (1981a), McLaughlin et al. (1981), Brown et al. (1982), Mollevanger and De Grip (1984), and Albert et al. (1984)].

Discussion

Our results indicate that the presence of intrinsic membrane proteins has a relatively minor influence on the ^{31}P NMR spectra of the phospholipid constituents of the native ROS and SR membranes (Deese et al., 1981a; McLaughlin et al., 1981). For the case of the ROS disk membranes, the visual pigment rhodopsin ($M_r \cong 41\,000$) is believed to constitute >95% of the intrinsic membrane protein (Krebs & Kühn, 1977). Approximately 75 ± 1 phospholipids are present per rhodopsin molecule in bovine ROS disk membranes (Miljanich, 1978; Stubbs & Litman, 1978). Membranes derived from the light fraction of vertebrate muscle sarcoplasmic reticulum (Meissner, 1975) likewise contain >90% of their total intrinsic membrane protein as the Ca^{2+}-ATPase ($M_r \cong 119\,000$), which is responsible for the active transport of calcium [cf. Fleischer et al. (1979) and Herbette et al. (1981)]. Roughly 113 ± 6 phospholipids are present per Ca^{2+}-ATPase molecule, which may be present as a dimer in the membrane [cf. Napolitano et al. (1983)]. In each of the above cases, a single molecular species comprises the bulk of the intrinsic membrane protein, and is a major structural as well as a major functional component of the membrane.

The relatively undistorted spectrum obtained for the dry DPPC powder control (Figure 1a) evinces that the spectrometer used in this study is capable of *directly* observing broad ^{31}P NMR spectral components. This is due to the relatively uniform radio-frequency excitation over a large spectral width and the efficient decoupling of ^{31}P–^{1}H magnetic dipolar interactions. The DSPC/DOPC mixtures (Figure 1b,c) can be viewed as approximate models for the situation that may or may not exist in protein-containing membranes.[3] Two components were expected in the DSPC/DOPC ^{31}P NMR spectra because they were recorded at a temperature where DOPC is in the liquid-crystalline phase and DSPC is predominantly in the solid phase (22 °C). The width of the broad component of the DSPC/DOPC spectra (Figure 1b,c) is similar to that of the rigid-limit ^{31}P NMR spectrum of the dry DPPC powder (Figure 1a). This indicates that the solid-phase (DSPC) component has a line shape that is considerably broader than the sharp, liquid-crystalline component [cf. Campbell et al. (1979)]. In the ^{31}P NMR spectrum of the sample containing a mole ratio of $X_{DSPC}/X_{DOPC} = 0.33$, the amplitude ratio of the broad to sharp components is small due to the difference in component widths; nevertheless, one can see clearly the broad component. A similar broad component is *not* evident in the biological membrane spectra to within experimental error (Figure 1d,e). On the basis of a comparison with the $X_{DSPC}/X_{DOPC} = 0.33$ spectrum, we estimate that at most ~10% of the total spectral intensity of the ROS and SR membrane ^{31}P NMR spectra could be due to a broad component. Ten percent of the total phospholipids would represent an upper limit of about 7 phospholipids per rhodopsin for the ROS membranes and about 11 phospholipids per Ca^{2+}-ATPase for the SR membranes (light fraction). These values are considerably less than the estimates of 24 ± 2 and 25 ± 2 for the numbers of lipids that can be accommodated about the intramembranous perimeters of rhodopsin and Ca^{2+}-ATPase, respectively (Watts et al., 1979; Marsh, 1985; Pates et al., 1985b). Protein–protein contacts could reduce these figures, however. Several other ^{31}P NMR experiments on ROS and SR membranes have been performed, including variation of temperature and ionic strength, measurement of spectral intensity vs. phosphorus content, and attempted selective saturation of an unobservable, broad component (not shown). These studies have yielded no evidence for the presence of observable or unobservable, broad spectral components (Pates et al., 1985a; Ellena et al., 1986).

The above results employing ^{31}P NMR are consistent with many earlier investigations, which suggest that most or all of the lipids in the ROS or SR membranes are in the liquid-crystalline state at the temperature employed (22 °C) (Chabre, 1975; Davis et al., 1976; Sklar et al., 1979a; McLaughlin et al., 1981; Brown et al., 1982; Miljanich et al., 1985). The observation of characteristic, axially symmetric ^{31}P NMR spectra for the ROS and SR membranes, with little or no broad component, differs from the results of spin-label EPR studies but as noted earlier may be explainable in terms of the spectroscopic time scales of the two techniques. For the case of spin-label EPR studies of membranes, two distinct spectral components have been observed in a variety of different systems. It has been concluded that the exchange of spin-labeled lipids between the two environments is relatively slow on the EPR time scale, i.e., with respect to the difference in their effective hyperfine and g tensors in frequency units, such that the spectrum of the fluid component appears very similar to that of the corresponding spin-labeled lipid dispersions in the L_α phase. The latter can then be used as basis spectra for subtractions and double integration of the EPR spectra of the spin-labeled membranes, to a first approximation [cf. Jost et al. (1973a,b), Knowles et al. (1979), Ellena et al. (1983), Marsh (1985), Esmann et al. (1985), Pates et al. (1985b), and Lange et al. (1985)]. In this manner it has been shown that the relative area of the additional broad component depends on the lipid/protein ratio, and that it appears correlated with the number of lipids necessary to form a single shell about the perimeter of an individual, intrinsic membrane protein molecule (Jost et al., 1973a,b; Nakamura & Ohnishi, 1975; Watts et al., 1979; Thomas et al., 1982; Silvius et al., 1984). Thus, the lipid–protein interactions detected by spin-label EPR appear to be of a relatively short-range nature (Marsh, 1985). The shape of the broad component obtained by spectral subtraction indicates that its motion is restricted, approaching that of an "immobilized" spectrum on the EPR time scale (defined by the rigid-limit hyperfine and g tensor anisotropies of the nitroxide moiety) or falling within the "slow-motional" regime (1×10^{-9} s $< \tau_c < 3 \times 10^{-7}$ s) (Freed, 1976; Schreier et al., 1978). Depending on the system and the temperature, estimates of the effective rotational correlation time τ_c of the broad EPR spin-label component are in the range of about 10–50 ns (Esmann et al., 1985; Pates et al., 1985b), suggesting that the rate of the segmental motions of the spin-labeled lipids is

[3] We do not intend to suggest that the solid phase of the DSPC/DOPC mixtures is a precise model for those phospholipids in steric contact with, or bound to, intrinsic membrane proteins. However, we do believe that analysis of the two-component phospholipid spectra can be useful in putting limits on the amount of a broad component that can be observed in the presence of a narrow component, and thereby on the relative amounts of broad and narrow components that may be present in biological membranes.

reduced at the protein interface. Using EPR techniques, it has also been possible to estimate the rate of exchange of spin-labeled lipids on and off the surface of several intrinsic membrane proteins. For the case of rhodopsin-containing membranes, a value of $\sim 10^7$ s^{-1} has been obtained (Davoust et al., 1983; Marsh, 1985; Pates et al., 1985b). This estimate is close to the diffusion-controlled limit for phospholipids in pure bilayers (Devaux & McConnell, 1972; Lindblom et al., 1981), as suggested previously (Brown et al., 1976, 1977).

For the case of ^{31}P NMR, the static chemical shift anisotropy of the ^{31}P nucleus (or quadrupolar interaction for the case of ^2H NMR) is much smaller in magnitude than the anisotropies of the rigid-limit hyperfine or g tensor principal values of the nitroxide spin-labels [cf. Libertini and Griffith (1970)]. Thus, motions with rates in excess of the nuclear interaction strength in frequency units ($\sim$5–50 kHz for ^{31}P NMR at typical magnetic field strengths to 170 kHz for ^2H NMR), but less than the corresponding effective, rigid-limit anisotropies of the spin-labels ($\sim 10^2$ MHz for X-band spectrometers), could lead to motional averaging of the NMR spectra but not the EPR spectra. Only a single, motionally averaged spectral component is seen in studies of protein-containing membranes, e.g., in ^2H NMR studies of the acyl chain region (Seelig & Seelig, 1978; Rice et al., 1979a,b; Deese et al., 1981b; Seelig et al., 1981; Paddy et al., 1981; Bienvenue et al., 1982) or ^2H and ^{31}P NMR studies of the polar head group region (Seelig & Seelig, 1978; Rice et al., 1979a,b; Seelig et al., 1981; Deese et al., 1981a; McLaughlin et al., 1981; Tamm & Seelig, 1983; this work). Moreover, little influence of protein on the NMR spectra of lipids in membranes appears evident; the main effect appears to be a slight line broadening together with a small decrease in the absolute magnitude of the residual chemical shift anisotropy for ^{31}P NMR, or the residual quadrupolar interaction for ^2H NMR. The above results could be due to macroscopic, motional averaging of the NMR line shapes of protein-containing membrane samples [cf. Campbell et al. (1979)]. It thus appears as if the motional rates of most of those lipids interacting with intrinsic membrane proteins are altered only slightly and that the corresponding motional amplitudes are affected to only a rather small extent; i.e., any changes in the degree of segmental ordering are small [cf. also Brown et al. (1982) and Deese et al. (1983)]. In like fashion, the ^1H NMR spectral line shapes of sonicated vesicles of native ROS membranes (Brown et al., 1976, 1977) and SR membranes (Deese et al., 1982a) are influenced only slightly by the presence of rhodopsin or Ca^{2+}-ATPase, respectively. For the case of the ROS membranes (Brown et al., 1976, 1977, 1982), it has been suggested that (i) the presence of rhodopsin leads to only small changes in pairwise additive, *intra*molecular ^1H dipolar interactions, and thus the segmental ordering of the lipid molecules in contact with the protein surface, and (ii) the lateral diffusive exchange of lipids on and off rhodopsin may approach that of bilayers in the L_α phase to account for the averaging of *inter*molecular ^1H dipolar interactions. While some reinterpretation of these earlier ^1H NMR studies is needed [cf. Brown et al. (1982) and Deese et al. (1982b)], the broad outlines have been supported by subsequent investigations employing ^2H NMR (Deese et al., 1981b; Bienvenue et al., 1982) and spin-label EPR (Watts et al., 1979; Davoust et al., 1983; Marsh, 1985; Pates et al., 1985b). Thus, the observation of a single component in the ^{31}P or ^2H NMR spectra of protein-containing membrane samples is not inconsistent with the presence of two components in the corresponding spin-label EPR spectra—i.e., due to the different time scales of the two techniques (Brown et al., 1977, 1982).

From the above, then, it appears that the majority of NMR and spin-label EPR studies of lipid–protein interactions in membranes agree in that the rates of relatively high frequency segmental motions of those lipids in steric contact with proteins may be reduced, but that the boundary lipids may be in relatively free, translational diffusive exchange with bulk lipids [cf. Brown et al. (1976, 1977) and Marsh (1985)]. It is difficult, however, to reconcile the observations of Yeagle and collaborators (Yeagle & Romans, 1981; Yeagle, 1982, 1984; Albert et al., 1982, 1985; Albert & Yeagle, 1983; Selinsky & Yeagle, 1984, 1985) with the above picture, and since we have been unable to reproduce their results, some questions must remain. The reasons for the discrepancies are not clear at present. Differences between membrane preparations and between spectrometers and spectral acquisition conditions could be important, although no obvious inconsistencies based on comparison of experimental materials and methods are evident (P. L. Yeagle, personal communication).

In summary, we have paid careful attention to the biochemical composition and function of the ROS and SR membranes, the spectrometer capabilities, and the spectral acquisition parameters. Under these conditions we have found no evidence for the presence of separate, broad ^{31}P NMR spectral components, due to immobilization of lipids by intrinsic membrane proteins. Only a single, liquid-crystalline (L_α) phase of the membrane lipids is detected near physiological temperature, to within experimental error. At present, we must conclude that the spectral properties of no more than 10% of the phospholipids of the native ROS and SR membranes are greatly altered by the presence of intrinsic membrane proteins, such as rhodopsin or Ca^{2+}-ATPase.

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Registry No. DPPC, 63-89-8; DSPC, 816-94-4; DOPC, 4235-95-4; ATPase, 9000-83-3.

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In Vivo Function of *Escherichia coli* Pyruvate Oxidase Specifically Requires a Functional Lipid Binding Site†

Charlotte Grabau* and John E. Cronan, Jr.
Department of Microbiology, University of Illinois, Urbana, Illinois 61801
Received March 26, 1986; Revised Manuscript Received April 21, 1986

ABSTRACT: The pyruvate oxidase of *Escherichia coli* is a peripheral membrane flavoprotein that is dramatically activated by lipids. The enzyme strongly binds to phospholipid vesicles in vitro. In vivo, in addition to enzyme activation, binding is thought to be important to provide access of the enzyme to ubiquinone dissolved in the lipid bilayer. It was unclear if both or either of these attributes is needed for enzyme function in vivo. To differentiate between activation and lipid binding, we have constructed, using recombinant DNA techniques, a mutant gene that produces a truncated protein. The truncated protein lacks the last 24 amino acids of the C-terminus of the oxidase (due to introduction of a translation termination codon) and thus is closely analogous to the activated species produced in vitro by limited chymotrypsin cleavage [Recny, M. A., Grabau, C., Cronan, J. E., Jr., & Hager, L. P. (1985) *J. Biol. Chem. 260*, 14287-14291]. The truncated protein (like the protease-derived species) is fully active in vitro in the absence of lipid, and its activity is not further increased by addition of lipid activators. Moreover, the truncated enzyme fails to bind Triton X-114, a detergent that binds to and activates the wild-type oxidase. Strains producing the truncated protein were devoid of oxidase activity in vivo. This result indicates that binding to membrane lipids is specifically required for function of the oxidase in vivo; activation alone does not suffice.

We have chosen the lipid-activated enzyme *Escherichia coli* pyruvate oxidase as a model to study the activation of enzymes by lipid and the physiological importance of such interactions. Pyruvate oxidase is one of the better characterized lipid-activated enzymes. The oxidase, a peripheral membrane flavoprotein coupled to the electron-transport chain, catalyzes the conversion of pyruvate to acetate and CO_2 (Hager, 1957; Williams & Hager, 1966; Koland et al., 1984). The enzyme is composed of four identical subunits (M_r 62 000), each of which contains a tightly bound FAD[1] molecule and a loosely bound TPP[1] molecule (Koland et al., 1984; O'Brien et al., 1976; Grabau and Cronan, unpublished experiments). In vitro, the enzyme utilizes artificial electron acceptors such as ferricyanide. Pyruvate oxidase displays a dramatic activation by both monomeric and aggregated amphiphiles (Cunningham & Hager, 1971; Blake et al., 1978). The addition of any of a wide variety of lipids or detergents results in a 20-25-fold increase in the enzyme specific activity (Russell et al., 1977a,b; Recny & Hager, 1983) accompanied by a tight association of the activator with the protein (Russell et al., 1977b).

Pyruvate oxidase can also be activated by limited proteolysis of the enzyme when incubated with pyruvate and TPP. Proteolytic activation (the degree of activation is similar to that given by phospholipids) involves the clipping of a small M_r 2600 peptide from the C-terminus of the protein. Proteolytic treatment in the absence of pyruvate and TPP results in enzyme inactivation due to cleavage at a different site (producing a M_r ca. 51 000 subunit and a M_r ca. 11 000 peptide) (Russell et al., 1977a; Recny & Hager, 1983). Both activation phenomena are dependent on the presence of pyruvate and TPP. In the presence of substrate and cofactor, pyruvate oxidase undergoes a conformational change that exposes both the lipid binding site and the proteolytic cleavage site. The two activation phenomena are mutually exclusive.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; TPP, thiamin pyrophosphate; kbp, kilobase pairs; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

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