Nuclear Magnetic Resonance Studies of Lipid–Protein Interactions. A Model of the Dynamics and Energetics of Phosphatidylcholine Bilayers That Contain Cytochrome *c* Oxidase†

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ABSTRACT: Reconstituted membrane systems of synthetic phosphatidylcholines and the integral membrane enzyme cytochrome *c* oxidase were prepared in order to conduct nuclear magnetic resonance studies of lipid–protein interactions. These lipids, labeled with a geminate difluoro group on the 1-position hydrocarbon chain, were combined with the enzyme to give active lipid–protein particles with a well-defined ratio of lipid to protein. The fluorine magnetic resonance spectra of a series of preparations with different lipid/protein ratios suggest that the hydrocarbon chain mobility of the lipid is substantially reduced with increasing amounts of protein. The fluorine spectra of a single lipid–protein preparation show a dramatic increase in the number of the more mobile lipid chains with increasing temperature. The results suggest that the enzyme orders the lipid bilayer well beyond those lipids in direct contact with the protein surface, and that the amount of the lipid restricted by the enzyme is dependent upon temperature. The exchange of lipid between the restricted and the more mobile lipid environments most probably does not occur over the time scale measurable by the magnetic resonance techniques, about 10^{-3} s.

A widely accepted model of biological membranes describes a fluid-mosaic lipid bilayer as the basic structural unit of the membrane, which contains globular proteins capable of performing a variety of enzymatic functions (Singer, 1974). Such a model implies that any enzyme which resides in the bilayer must have lipid associated with the protein surface. If the enzyme spans the membrane, then the lipid encircles the protein surface in the bilayer configuration. This bilayer of lipid in direct contact with the surface of the protein has been called either boundary lipid for the case of cytochrome *c* oxidase (Jost et al., 1973), or annular lipid for the case of sarcoplasmic reticulum Ca-ATPase (Hesketh et al., 1976). Both groups have determined the amount of lipid in the boundary layer, and find that it is approximately that amount of lipid required to obtain optimal enzymatic activity. Electron spin resonance studies (Jost et al., 1973) of preparations of lipid and cytochrome *c* oxidase show that the chain mobility of the boundary lipid is considerably restricted compared to the surrounding free lipid, whereas the free lipid behaves similarly to lipid in bilayers without protein. Other studies such as those of retinal rod preparations indicate a more uniform immobilization of the hydrocarbon chains, suggesting that the microscopic lipid environments are not as clearly defined (Hong and Hubbell, 1972; Stubbs et al., 1976).

We wish to make further investigations to determine the amount of lipid affected by the introduction of an integral membrane protein into the lipid bilayer. We are also interested in the rate of exchange of lipid between those microscopic environments of restricted and nonrestricted hydrocarbon chain motion. Finally, in order to gain a qualitative understanding of the energetics of lipid–protein interactions, we wish to examine the way in which temperature regulates the magnitude of the lipid–protein interactions in a bilayer. One problem with the spectroscopic studies to date is that the time frame of the analysis is very fast, preventing the observation of slower rate processes. Nuclear magnetic resonance (NMR)[1] techniques allow for the study of motional properties of the lipid on a much slower time scale, one which corresponds to the turnover rates of many enzymes.

We have applied the NMR method to a reconstituted lipid–cytochrome *c* oxidase system. Accordingly, we have synthesized well-defined phospholipids which contain fluorine or deuterium nuclei at specific positions on one hydrocarbon chain of a phosphatidylchlorine (PC) molecule. Cytochrome *c* oxidase has been shown to completely span the lipid bilayer (Eytan et al., 1975; Henderson et al., 1977), and can be reconstituted with lipid to give active lipid–protein complexes (Yu et al., 1975; Vik and Capaldi, 1977). This communication reports the fluorine and deuterium NMR properties of these PC–cytochrome *c* oxidase preparations, and discusses the dynamics and energetics of the lipid–protein interactions that are revealed by the spectroscopic results.

Experimental Procedure

Synthesis of Fluorine-Containing Lipids. The lipids used in this study were obtained using standard synthetic procedures. Accordingly, methyl 12-oxostearate and methyl 7-oxopalmitate were synthesized by methods found in the literature (Waggoner et al., 1969; Hunig et al., 1957). The ketone of each compound was converted to a geminate difluoro group by using a commercially available fluorinating reagent (Fluoreze-M, PCR, Inc.) following the procedure of Mathey and Bensoam (1971). After washing this reaction mixture with water, the dichloromethane layer was dried ($MgSO_4$) and the

† From the Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received June 27, 1977.* This work was supported by grants from the National Science Foundation (BMS-75-10422 and PCM-74 00957-A02) and an award to F.W.D. from the Alfred P. Sloan Foundation. K.J.L. is a U.S. Public Health Service Predoctoral Trainee (GM-00715).

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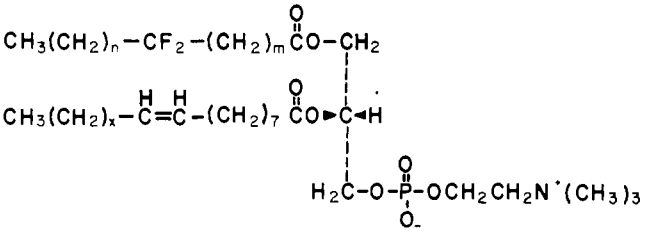
[1] Abbreviations used are: PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; lyso-PC, 1-acyl-*sn*-glycero-3-phosphorylcholine; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; rf, radiofrequency; T_1, nuclear spin-lattice relaxation time; T_2, nuclear spin-spin relaxation time; T_c, lipid solid-fluid phase transition temperature.

solvent was removed. Since a substantial portion of the ketone does not react, the remaining ketone was reduced with $NaBH_4$ in absolute ethanol. The resulting mixture of hydroxyl- and fluoro-containing fatty acid esters could then be neatly separated by silica gel chromatography using petroleum ether solvent. Following this purification step, the fluorinated fatty acid esters were saponified to the corresponding fatty acids.

The saturated PC's, 1,2-(12′,12′-difluorostearoyl)-PC and 1,2-(7′,7′-difluoropalmitoyl)-PC, were prepared by combining the appropriate fatty acid anhydride with free L-α-glycerophosphorylcholine according to the method of Cubero Robles and van den Berg (1969). The free glycerophosphorylcholine was prepared from the cadmium chloride complex (Sigma) by washing the compound through a column of Amberlite MB-1 ion-exchange resin in water, with approximately 20 g of resin per g of compound. The phosphatidylcholines were purified on silica gel as described in the literature method, but it was found that the isolated product contained small amounts of lyso-PC and fatty acid. As a result, the lipids were purified to homogeneity by LH-20 Sephadex chromatography on a 1.5 × 90 cm column, operating at 37 °C with 95% EtOH solvent.

The saturated PC's were hydrolyzed to the corresponding 1-acyl lyso-PC following the procedure of Wells and Hanahan (1969). In a typical preparation, 100 mg of dry lipid was suspended in 10 mL of 95:5 diethyl ether–methanol. To this mixture was added 100 μg of phospholipase A from *Crotalus durissus terrificus* (Sigma) in a 0.2-mL solution of 0.22 M NaCl, 0.02 M $CaCl_2$, and 1 mM EDTA, pH 6. After the reaction, the solvents were removed and the lyso-PC was separated from the fatty acid by LH-20 chromatography (25 °C, 95% EtOH solvent). Metal cations were removed by washing the lyso-PC-containing column fractions through another column containing 5 g of Amberlite MB-1 ion-exchange resin in 95% EtOH solvent.

The lyso-PC compounds were acylated with an unsaturated fatty acid by the anhydride method described above to give the final products: 1-(7′,7′-difluoropalmitoyl)-2-palmitoleoyl-PC (I) and 1-(12′,12′-difluorostearoyl)-2-oleoyl-PC (II).



I: m=5, n=8, x=5

II: m=10, n=5, x=7

The details of the synthesis of 16,16,16-trideuteriopalmitic acid will be discussed elsewhere (Dahlquist et al., 1977). The synthesis of 1-(16′,16′,16′-trideuteriopalmitoyl)-2-palmitoleoyl-PC was accomplished by exactly the method described for the synthesis of the fluorine-containing PC's.

Isolation of Cytochrome c Oxidase. Cytochrome *c* oxidase was purified from beef heart mitochondria by the method described by Capaldi and Hayashi (1972). Enzyme activity was measured by following the rate of oxidation of ferrocytochrome *c* at 550 nm, in a solution containing 0.5% Tween-80 detergent (Smith, 1955; Vanneste et al., 1974). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Heme concentration was measured by the difference spectrum at 603–630 nm of the reduced heme in 1% Triton X-100 (Briggs and Capaldi, 1977).

Preparation of Lipid–Protein Particles. In a typical preparation, 5 mg of lipid (6 μmol) in ethanol solution was dried onto a pear-shaped flask, dried in vacuo, then suspended in 0.5 mL of a buffer containing 20 mg/mL sodium cholate (twice recrystallized from 70% EtOH), 0.090 M NaCl, and 0.020 M Tris-HCl, pH 7.4. A variable amount of cytochrome *c* oxidase (2–20 mg) was suspended in 0.5 mL of the same buffer. The lipid and protein solutions were then combined and mixed by vortexing.

The lipid–protein mixture was dialyzed at 4 °C against four 0.5-L washes of 0.090 M NaCl and 0.020 M Tris-HCl (pH 7.4) over a 24-h period. The turbid solution which results was made clear with about five 30-s sonications using a Branson probe-type sonicator. The sample was cooled with an ice bath during sonication. The solution was then layered onto a 10-mL 10–60% (w/v) sucrose gradient buffered with 0.090 M NaCl and 0.020 M Tris-HCl (pH 7.4). The sample was centrifuged at 75 000*g* for 18 h at 4 °C. The gradient was collected in 0.5-mL fractions, and the average sucrose density of each fraction was recorded by refractometry. The three fractions containing the most lipid–protein particles were pooled, and a D_2O solution in the same buffer was added to give approximately 20% D_2O. This sample was then used for enzyme assay and NMR measurements. Lipid–protein determinations and electron microscopy were performed after the sucrose was removed by dialysis against the buffer described above. To obtain the lipid/protein ratio (mg/mg), lipid phosphorus was assayed by the method of Ames and Dubin (1960), and the protein was determined by the method of Lowry et al. (1951).

To determine the effect of the labeled lipids on enzyme activity, lipid–protein complexes (5:1 mg/mg lipid to protein) were diluted to 2.5 μg/mL in assay buffer (2 mL final volume) containing 50 mM sodium phosphate, 25 μM cytochrome *c*, and 30 mM ascorbate (pH 7.4) and the activity was measured at 25 °C with an oxygen electrode (Yellow Spring Co.). Corrections were made for oxygen consumption in the absence of cytochrome *c* oxidase (typically 25 nmol of O min^{-1}). Activities were determined as described by Vanneste et al. (1974) and are reported as a percentage of the activity obtained with a concurrently assayed sample of asolectin–cytochrome *c* oxidase complex.

Instrumentation. Fluorine NMR measurements were carried out on a Varian XL-100-15 spectrometer operating at 94.1 MHz, which was locked to the D_2O in the sample. Spectra were acquired in the Fourier transform mode with the aid of a Varian 620/i minicomputer. Proton decoupling was done for all experiments by irradiating with 5 W of 100-MHz rf power using the spectrometer's spin decoupler. Reflected decoupler power was dissipated by attaching a 100-MHz isolater (Microwave Associates, Inc.) to the high power output of the decoupler.

Deuterium magnetic resonance spectra were obtained using a modified Bruker SXP-90 spectrometer in the laboratory of D. Myer Bloom at the University of British Columbia. All spectra were acquired using a solid echo pulse technique in order to overcome the line-shape distortions encountered when observing deuterium spectra.

Electron micrographs were obtained on a Hitachi HS-7S transmission electron microscope. Samples were prepared by first placing a drop of a solution containing latex particles (Pelco, Inc.) 0.109 (±0.027) μm in diameter onto a 200 mesh carbon-stabilized parlodian grid. After 1 min, this solution was drawn off and a drop of sample applied. After 1 min, excess

TABLE I: Sucrose Density Centrifugation of Lipid-Cytochrome *c* Oxidase Particles.

Lipid/protein ratio[a] (mg/mg)	Position on sucrose gradient (% sucrose) (w/v)
2.3 ± 0.2	15.9-23.9
1.6 ± 0.2	20.3-27.9
1.2 ± 0.2	24.2-30.8
0.68 ± 0.1	30.7-37.1
0.38 ± 0.05	39.2-45.4
0.19 ± 0.05	>60[b]

[a] Lipid: 1-(7′,7′-difluoropalmitoyl)-2-palmitoleoyl-PC. [b] Sample pelleted at bottom of gradient.

TABLE II: Activity of Cytochrome *c* Oxidase Following Recombination with Various Lipids, Relative to Asolectin.

Lipid	Act. (%)[a]
Asolectin	100
1-Palmitoyl-2-oleoyl-PC	95
Dimyristoyl-PC	56
1-(7′,7′-Difluoropalmitoyl)-2-palmitoleoyl-PC	73
1-(16′,16′,16′-Trideuteriopalmitoyl)-2-palmitoleoyl-PC	76

[a] Activities are expressed in percent relative to a concurrently assayed sample of asolectin and enzyme.

solution was drawn off and the grid was negatively stained with a drop of 1% ammonium molybdate (pH 7). Excess stain was drawn off and the grid was allowed to dry.

Differential scanning calorimetry was done using a calorimeter of the design of Privalov et al. (1975). Normally, samples of 0.5 mg/mL lipid in the NaCl-Tris buffer described above were scanned at a heating rate of 1.0 °C/min.

Results and Discussion

Properties of the Fluorinated Lipid. It is our experience that the fluorine-containing phosphatidylcholines synthesized for our experiments are highly similar to the corresponding PC without fluorine. The fully saturated, fluorinated lipids form lipid bilayers, which when sonicated become small, single bilayer vesicles, impermeable to metal cations (Longmuir and Dahlquist, 1976).

Differential scanning calorimetry measurements were conducted in order to obtain the solid-fluid phase transition temperature, T_c, of the two fully saturated lipids: 1,2-(12′,12′-difluorostearoyl)-PC and 1,2-(7′,7′-difluoropalmitoyl)-PC. The T_c of 48.8 °C for the fluorinated, distearoyl compound is approximately 6 °C less than the T_c measured for distearoyl-PC by Mabrey and Sturtevant (1976). The T_c of the fluorinated dipalmitoyl-PC is 31.5 °C, which is about 10 °C lower than the T_c of 41.4 °C of dipalmitoyl-PC. The greater thermodynamic change is induced by fluorines closer to the head group of the PC molecule. [We have also examined 1,2-(7′-,7′-difluorostearoyl)-PC and find that it too has a T_c 10 °C lower than the unlabeled distearoyl-PC.] When the fluorines are located farther away from the head group, the change in transition temperature is less.

The transition temperature is a function of both the enthalpy and the entropy of the phase change. At the midpoint of the transition, $\Delta G = 0$, and $T = T_c = \Delta H/\Delta S$. Since temperatures are referenced to the Kelvin scale a drop of 10 °C in the T_c upon introduction of fluorines into the 7′ position of the hydrocarbon chain represents only a 3% change in $\Delta H/\Delta S$.

The NMR studies reported here need to be performed at temperatures above the phase transition, yet at temperatures low enough to ensure the stability of the enzyme for a period of several hours. For this reason, lipids with one unsaturated chain and one saturated, fluorine-containing chain were prepared. The transition temperatures of all the unsaturated lipids used in this study are below 0 °C.

Properties of Fluorinated Lipid-Cytochrome c Oxidase Particles. We find that phosphatidylcholine and cytochrome *c* oxidase can be successfully combined to yield enzymatically active particles, and that the ratio of PC to enzyme can be controlled to a considerable degree. These particles are made by incubating the lipid and the enzyme separately with cholate buffer, combining the two solutions, and dialyzing to remove the cholate. Since some aggregation of the particles occurs during dialysis, it is necessary to sonicate the solution prior to centrifugation.

Sucrose density centrifugation of the sonicated lipid-protein particles serves two purposes. First, the density gradient separates any unincorporated lipid from the lipid-protein complexes, because the free lipid remains at the top of the gradient. Second, by observing the spread of particles over the gradient, we obtain an indication of the distribution of lipid/protein ratios resulting from a given preparation. While the bulk of the preparation bands in a small region, lipid-protein particles are found to range over about one-fifth of the entire gradient. In order to obtain enough sample for subsequent experiments, the three most concentrated 0.5-mL fractions from the 10-mL gradient were combined for use in lipid-protein determination, enzyme assay, electron microscopy, and NMR spectroscopy. Table I lists the lipid/protein ratios in milligrams/milligram for a series of such preparations, and the corresponding region of the density gradient from which each sample was collected.

The incorporation of fluorine or deuterium into the hydrocarbon chains of PC has little effect upon the enzymatic activity of cytochrome *c* oxidase. Table II compares the activities of various PC-cytochrome *c* oxidase complexes and an asolectin-cytochrome *c* oxidase complex. Any difference induced by the fluorine or deuterium nuclei is not detectable within the experimental error of the technique, and is certainly far smaller than the changes in activity that arise from changes in chain length and the degree of unsaturation (Vik and Capaldi, 1977).

Electron micrographs were obtained for a variety of lipid-protein preparations in order to determine the size distribution of the resulting particles. The top of Figure 1 shows a micrograph of vesicles composed of an unsaturated, fluorinated lipid (containing no protein) referenced to a latex sphere 0.11 μm in diameter. The distribution of particle sizes is definitely bimodal, with a large proportion of vesicles approximately 150 Å in diameter. The remainder of the particles range from 300 to 500 Å. Incorporation of enzyme eliminates the formation of the vesicles of the smaller size, as is shown in the center of Figure 1. With this lipid-protein preparation (1.2:1 mg/mg), the average vesicle size is approximately 350 Å, and ranges from about 250 to 500 Å in diameter. The size dependence of these preparations is similar to that observed by Ruben et al. (1976) in their studies of phospholipid vesicles with and without cytochrome *c* oxidase.

Increasing the protein content of the vesicles increases the average vesicle size somewhat. The bottom photograph of Figure 1 shows a representative micrograph for a preparation of vesicles with a low lipid/protein ratio (0.38 mg/mg). The

average vesicle size is approximately 500 Å, and ranges from 300 to 800 Å in diameter.

Fluorine NMR Studies of 1-(7′,7′-Difluoropalmitoyl)-2-palmitoleoyl-PC–Cytochrome c Oxidase Particles. The NMR experiments were performed with the anticipation that an integral membrane protein such as cytochrome *c* oxidase organizes the lipid into one or more lipid environments, which are identifiable by their different hydrocarbon chain mobilities. In order to assess the various hydrocarbon chain motions in the lipid–protein particles, we examine the line widths arising from the fluorine NMR spectra of the fluorinated lipids used in the lipid–protein preparations. It is generally true that the more restricted the motion of a magnetically sensitive nucleus on a molecule, the shorter the nuclear spin–spin (T_2) relaxation time, and hence the larger the line width of the observed NMR absorption spectrum. This predicts that the NMR line widths resulting from motionally restricted lipids are substantially larger than those resulting from the free lipid. The actual relationship between NMR line widths and hydrocarbon chain motions is a complex one, since the rates of chain motion are not uniform over all directions in space. The effect of this anisotropy of motion on NMR line widths has been treated by other workers for the case of hydrocarbon chains in a lipid bilayer, but is still a matter of some controversy (Lichtenburg et al., 1975; Stockton et al., 1976). We shall restrict our interpretation to only the qualitative conclusion that the slower and/or more restricted the hydrocarbon chain motions, the broader the observed NMR resonance.

We shall interpret the broad components of the NMR spectra as resonances arising from hydrocarbon chains in a restricted environment which result from the interaction of cytochrome *c* oxidase with the lipid molecules. Similarly, the sharper components observed in the spectra will be considered to be due to fatty acid chains in a freer lipid environment. For convenience, we will call the lipids showing hindered hydrocarbon chain motions "restricted lipid", and the more mobile carbon chains "free lipid". However, these categories in no way mean that the "restricted" lipid is only that portion of the bilayer in direct contact with the surface of the protein. Nor do we wish to imply that "free" lipid behaves, in the NMR experiments, in the same way as lipid in bilayers without protein.

Figure 2 presents the fluorine magnetic resonance spectra of various preparations of 1-(7′,7′-difluoropalmitoyl)-2-palmitoleoyl-PC and cytochrome *c* oxidase, reconstituted to give enzymatically active particles at specific ratios of lipid to protein. All spectra were obtained at a sample temperature of 24 °C, well above the phase transition temperature for this lipid. There is little change in line width over the higher values of lipid/protein ratio, where the observed line width is approximately 100 Hz. As the preparations tend toward lower ratios of lipid to protein (less than 1:1 mg/mg), the apparent line width increases dramatically. For the NMR spectrum of the preparation containing the lowest lipid/protein ratio, we observe a line width of some 1200 Hz. This spectrum with the largest line width arises only from lipid in direct contact with the protein, since the lipid/protein ratio of this preparation is nearly equal to that where the lipid is entirely in the boundary layer. This line width is about ten times larger than is observed at high lipid contents, and is about 30 times larger than the line width observed for the free lipid alone. These results appear to demonstrate the anticipated line-width increase displayed by lipid under increasing influence by the protein.

The line-width data suggest that a given preparation of lipid and cytochrome *c* oxidase contains at least two classes of lipid: a free lipid environment which dominates the observed NMR spectrum at high ratios of lipid to protein, and a restricted lipid environment which becomes evident at the lower lipid/protein ratios. Alternatively, it is possible that the large variations in observed fluorine NMR line widths arise from the increase in average vesicle diameter that occurs when cytochrome *c* oxidase is introduced in increasing amounts into the lipid bilayer. This size increase slows the tumbling rate of the particles and, in the absence of other averaging mechanisms, leads to a less complete averaging of the chemical-shift anisotropy and dipolar interactions experienced by the fluorine nuclei. A similar argument could apply if translational diffusion controlled line width. The larger the vesicle size, the less effective translational diffusion becomes as a mechanism for averaging the orientations of the PC molecules over all angles relative to the laboratory magnetic field. However, we have found that the largest

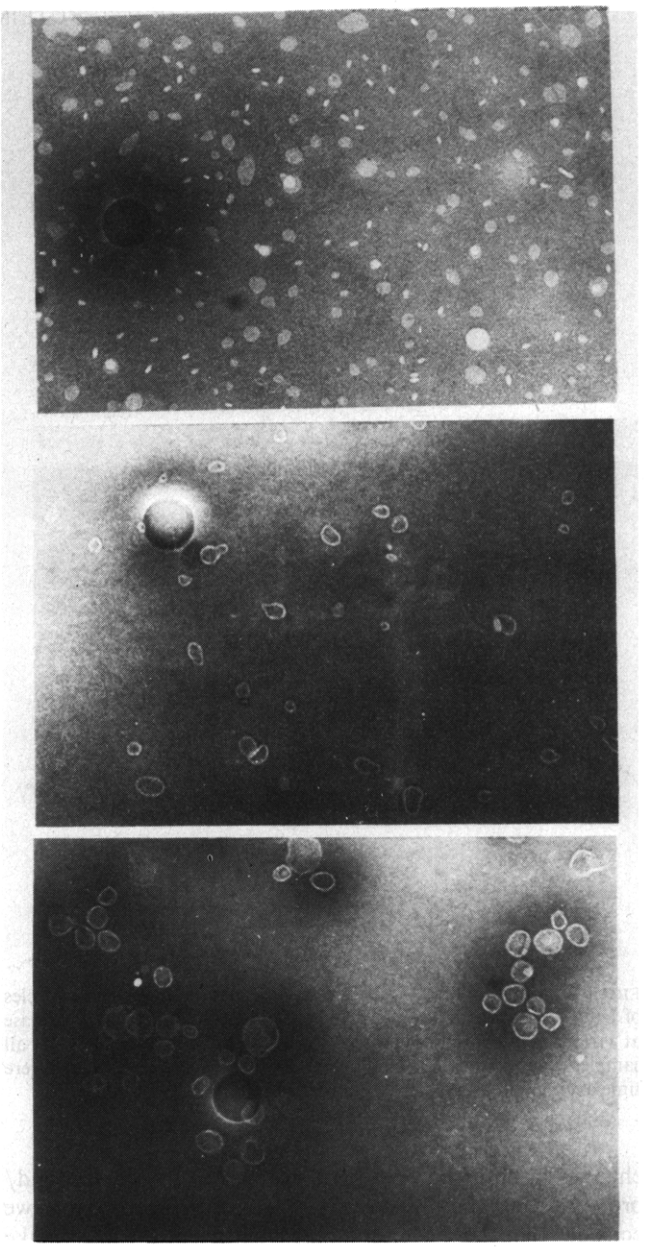


FIGURE 1: Electron micrographs of sonicated vesicles of lipid and lipid–protein complexes. All micrographs contain at least one latex sphere $\simeq 0.11$ μm in diameter: (top) sonicated vesicles of 1-(12′,12′-difluorostearoyl)-2-oleoyl-PC; (center) sonicated vesicles of 1-(7′,7′-difluoropalmitoyl)-2-palmitoleoyl-PC and cytochrome *c* oxidase at a lipid/protein ratio of 1.2:1 mg/mg; (bottom) the same lipid–protein components at a ratio of 0.38:1 mg/mg.

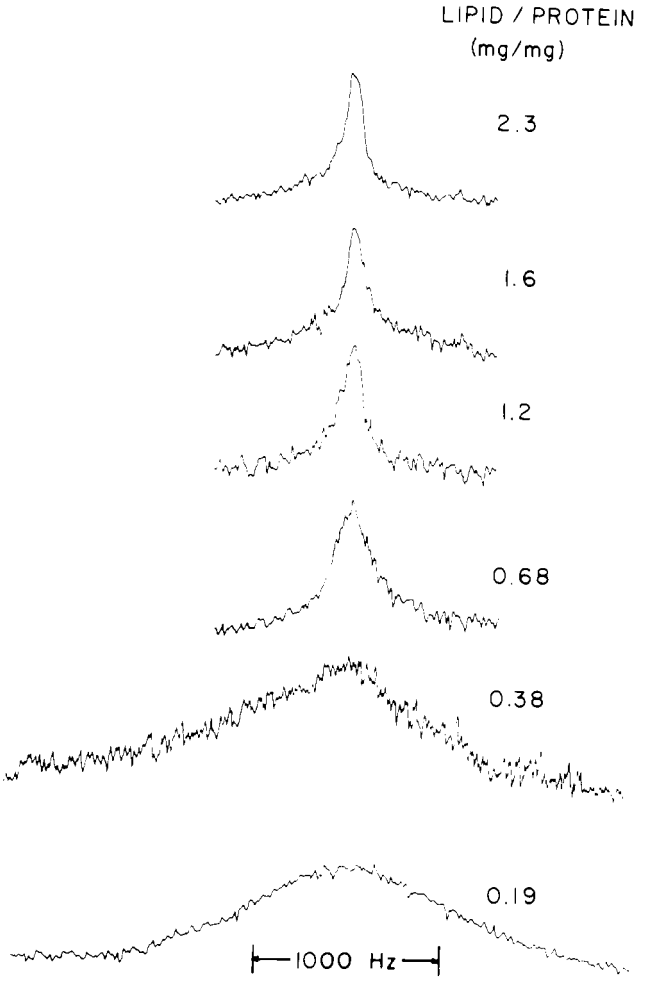


FIGURE 2: Fluorine NMR spectra of sonicated lipid-protein particles of 1-(7',7'-difluoropalmitoyl)-2-palmitoleoyl-PC and cytochrome *c* oxidase at various ratios of lipid to protein. Probe temperature was 24 °C for all samples. Spectra were accumulated at a repetition rate of 0.4 s, and were time averaged for anywhere from 6 to 24 h.

changes in observed line width occur over a range of lipid/protein ratios where vesicle size changes are small. Thus, we consider that these averaging mechanisms at the most make minor contributions to the line widths seen in Figure 2. Instead we are led to suggest that the line-width changes observed as a function of lipid/protein ratio arise because the fluorine probe distinguishes between free lipid environments and those lipid environments that interact strongly with cytochrome *c* oxidase.

One major purpose of the studies presented here is to obtain information about the *rate* at which the more restricted lipids exchange with the free lipid. Nuclear magnetic resonance techniques are suitable for such studies because of the long lifetime of the excited nuclear spin. This longer lifetime allows for the potential observation of chemical rate processes which cannot be detected by most other spectroscopic techniques. The rate information we wish to determine here is whether the exchange of restricted lipid with free lipid is fast or slow relative to the signal lifetime (T_2 relaxation time) of the excited fluorine nuclear spin. Considering that the line widths we observe in the lipid-protein preparations are on the order of several hundred hertz (T_2 in the millisecond range), fast exchange conditions would prevail if the bulk of the restricted lipid exchanged with the free lipid within 10^{-3} s.

If fast chemical exchange is occurring between the lipid environments, one should observe for a given lipid-protein preparation a single resonance whose line width is the weighted average of the line widths characteristic of the free and restricted lipid environments. A second possibility is that the lipid does not exchange between the lipid environments within the T_2 relaxation time of the observed NMR signal. Under such slow exchange conditions, one should observe the resonances of the free and restricted states directly. In practice, the spectra such as those in Figure 2 do not offer the possibility of resolving the environments. For one reason, the chemical-shift differences among the resonances of the various lipid environments are probably far smaller than the line widths of the resonances themselves, giving an observed NMR absorption pattern that is the sum of several resonances. For another, it is difficult to distinguish a broad resonance from the baseline when the spectrum is dominated by NMR signals from more mobile lipids.

This latter problem of determining the broad line widths in the presence of sharp ones is further accentuated by a delay in data acquisition inherent in the NMR system. In the XL-100 spectrometer system, data acquisition does not begin until approximately 400 μs following the radiofrequency excitation pulse. This means that the signal of a broad resonance (short T_2 relaxation time) decays significantly between the time of the rf pulse and the start of data acquisition. With a resonance 1200 Hz in line width (T_2 = 0.26 ms), as was seen with a lipid-protein preparation of 0.19 mg/mg, the signal decays to 22% of its original value before the start of data acquisition. For a resonance with a line width of 100 Hz (T_2 = 3.2 ms), as is seen at the higher lipid/protein ratios, 88% of the signal intensity remains at the start of acquisition. For a spectrum containing broad and relatively sharp resonances, the signal intensity of the broad lines is far less than would be expected from the relative amount of lipid in that restricted environment. It is our expectation that under slow exchange conditions, the signals we observe come predominantly from fluorine nuclei from the most mobile lipid environment present in a given sample.

Fluorine NMR Studies of 1-(12',12'-Difluorostearoyl)-2-oleoyl-PC–Cytochrome c Oxidase Particles. To test the fast and slow exchange models of lipid-protein interactions, an NMR study at various temperatures was carried out on another lipid-protein preparation. Accordingly, lipid-protein particles were prepared composed of 1-(12',12'-difluorostearoyl)-2-oleoyl-PC and cytochrome *c* oxidase in a ratio of 1.6:1 mg/mg. The fluorine magnetic resonance spectra of this preparation were acquired at a series of temperatures above the phase transition temperature of this lipid, and are shown in Figure 3. All spectrometer settings, as well as all computer-controlled parameters for acquiring the spectra, were kept constant for all the temperatures examined. Also, sonicated vesicles of 1-(12',12'-difluorostearoyl)-2-oleoyl-PC were prepared without enzyme and examined by NMR over the same temperature range. The fluorine NMR spectra obtained from this pure phospholipid sample are also shown in Figure 3.

The fluorine NMR spectra of the vesicles without enzyme display two resonances. These two resolved resonances are due to environmental differences between the inner and outer monolayers of the single-bilayer vesicles (Longmuir and Dahlquist, 1976). Incorporation of enzyme into the bilayer to give lipid/protein ratios of 2:1 or less invariably gives rise to fluorine NMR spectra which show only one resonance. The enzyme, at these ratios of lipid to protein, seems to diminish the differences between the inner and outer monolayers to such an extent that we can no longer detect them spectroscopically. At very high lipid/protein ratios (>10:1 mg/mg), we do see

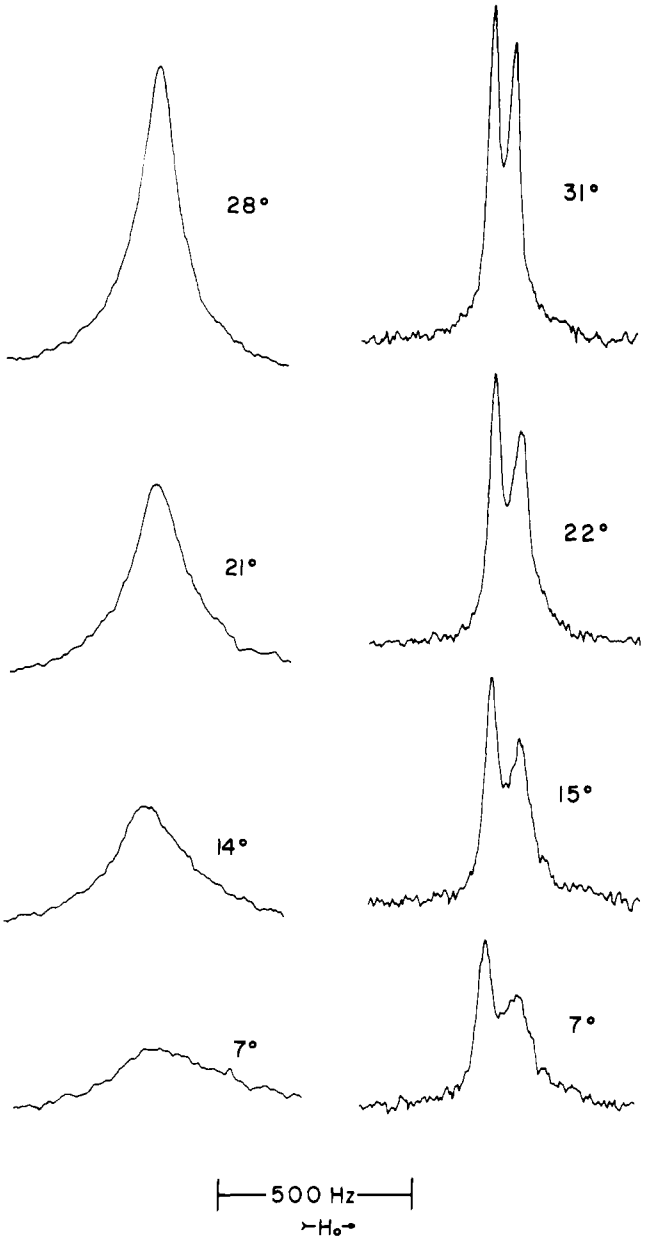


FIGURE 3: (Left) Fluorine NMR spectra of sonicated lipid-protein particles of 1-(12′,12′-difluorostearoyl)-2-oleoyl-PC and cytochrome *c* oxidase at various temperatures. The lipid/protein ratio was 1.6:1 mg/mg. All spectra represent the time average of 16 000 transients, acquired at intervals of 1.5 s. The spectra are scaled to reflect the actual increases in signal amplitude observed with increasing temperature. (Right) Fluorine NMR spectra of sonicated vesicles of 1-(12′,12′-difluorostearoyl)-2-oleoyl-PC at various temperatures. All Fourier transform spectra are the time average of 2000 transients, acquired at intervals of 3.0 s.

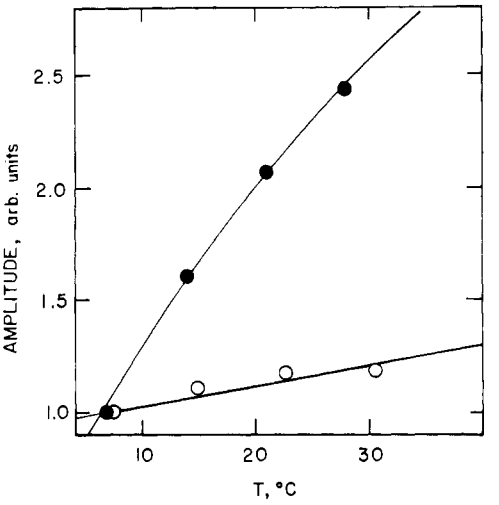


FIGURE 4: (Closed circles) Integrated intensity of the fluorine NMR spectra of Figure 3 of the sample containing cytochrome *c* oxidase. The integrals are normalized to the value obtained for the sample at 7 °C. (Open circles) Integrated intensity of the fluorine NMR spectra of Figure 3 of the sample without cytochrome *c* oxidase. The integrals are normalized to the value obtained for the sample at 7 °C. The integrals were obtained by cutting and weighing the spectra, and with a planimeter.

the inner and outer monolayer resonances, but with a greatly diminished chemical-shift difference between the resonances (K. J. Longmuir and F. W. Dahlquist, unpublished observation).

In the vesicles with enzyme, we see a dramatic increase in signal amplitude as the temperature is raised. Bilayers without protein do not display a large increase in fluorine NMR signal with temperature. The integrated signal amplitudes of the spectra of Figure 3 are shown graphically in Figure 4. Quantitatively, the signal strength of the enzyme-containing sample increases by a factor of 2.4 from 7 to 28 °C. Less than a 20% increase is noted in bilayers without enzyme over the same temperature range, and may be due to the experimental error introduced by the low signal-to-noise ratio obtainable in these experiments.

Provided that the nuclear spins are allowed to return to equilibrium following each rf excitation pulse (T_1 relaxation), this signal amplitude is directly proportional to the number of fluorine nuclei exhibiting line widths sharp enough for our instrumentation to detect. We have measured the T_1 relaxation times of both the above samples and find, for the enzyme-containing sample, the $T_1 = 0.14 \pm 0.01$ s at 14 °C, and for the sample without enzyme, the $T_1 = 0.27 \pm 0.03$ s. In the experiments reported here, the rf pulses were spaced at intervals of 1.5 s for the sample with enzyme and 3.0 s for the sample without enzyme, allowing ample time for the spin system to return to equilibrium following each excitation pulse.

We interpret this dramatic change in signal amplitude to mean that at lower temperatures a larger proportion of the fluorine-containing hydrocarbon chains exist in environments where the chain motion is either slow and/or restricted relative to the free lipid. As a result, at lower temperatures a larger proportion of the fluorine nuclei on these chains gives rise to NMR absorptions with line widths too large to be detected with appreciable amplitude by the instrumentation used in these experiments. At higher temperatures, a portion of these restricted chains assume the properties of the free lipids, and thus contribute to the overall increase in the observed amplitude.

This increase in signal amplitude means that restricted and free lipids coexist in lipid bilayers which contain enzyme, and that the amount of restricted lipid relative to free lipid is strongly dependent upon temperature. This signal increase also indicates that the exchange of lipid between the restricted and free environments in the enzyme-containing bilayers is slow relative to the lifetime of the observed NMR signal. If, instead, lipid were undergoing fast exchange among all environments, one would observe a single resonance whose line width would be a function of the average of all the environments, and whose signal amplitude would not change with temperature because all the fluorine nuclei contribute to the one, observable resonance. However, it is clear that the exchange of lipid is slow enough such that the free and restricted environments give rise to NMR signals characteristic of that environment; hence at

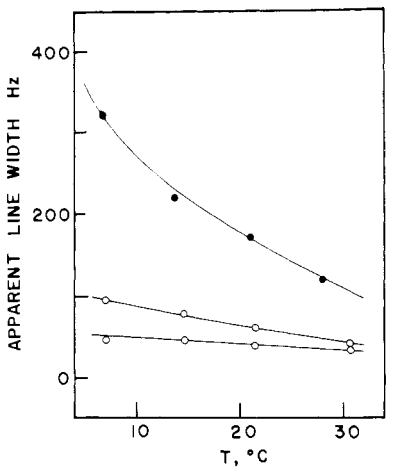


FIGURE 5: Apparent line widths for the spectra of Figure 3: (closed circles) sample with enzyme; (open circles) sample without enzyme. The line widths of the spectra of the enzyme-containing vesicles were obtained by measuring their apparent line widths at half-height. The line widths of the spectra of the vesicles without enzyme were obtained by computer simulation of the spectra and adjusting the line width and peak separation parameters to give the best visual fit. The larger line widths arise from the upfield resonance, corresponding to the outer monolayer of the bilayer. A ratio of 1.2:1 between the intensities of the outer and inner monolayer resonances appears to give the best fit.

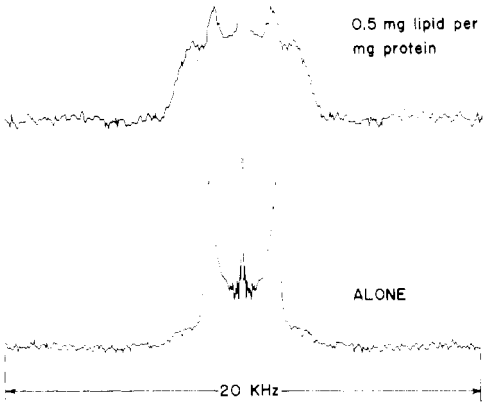


FIGURE 6: Deuterium NMR spectra of a dispersion of 1-(16′,16′,16′-trideuteriopalmitoyl)-2-palmitoleoyl-PC. The lipid concentration for both samples was ≃ 50 mg/mL, and the sample was contained in 10-mm NMR tubes. Pulse repetition rate was 0.4 s. The center resonance arises from natural abundance HDO: (top) lipid and cytochrome *c* oxidase in a ratio of 0.5:1 mg/mg, 4 °C; (bottom) lipid alone, 9 °C.

a given temperature we can only observe those lipid chains which reside in the freer lipid environments.

The apparent line widths also change as a function of temperature, as is shown in Figure 5, which indicates a sharpening of the observed line width as the temperature is raised. An interpretation of this line-width change is not easy to make. As one possibility, this NMR spectrum of the free lipid in the enzyme-containing bilayer may be a composite of several resonances with somewhat different line widths, and perhaps with slightly different chemical shifts. Such an NMR spectrum would result from a range of fluid lipid environments which exchange slowly among themselves. On the other hand, the freer lipid environments may intermix so rapidly that fast exchange conditions prevail and we observe in the NMR one resonance which contains information about the average fluidity of the free lipid. We have not yet established which is the case. Whatever the conditions from which the observed resonance arises, we can make a general statement that the protein orders even the free lipid far more than is found in vesicles without protein, and in a strongly temperature-dependent fashion. The fact that the fluorine NMR line widths decrease with increasing temperature also indicates that the free lipid line widths are in no way influenced by chemical exchange processes with the more restricted lipid environments. [If the line widths were influenced by slow chemical exchange processes, increasing the temperature would cause an increase in the observed line width (Dwek, 1973).] Thus, it is likely that the fastest possible exchange rate of lipid is on the order of 10^2 s^{-1}.

Deuterium NMR Studies of 1-(16′,16′,16′-Trideuteriopalmitoyl)-2-palmitoleoyl-PC-Cytochrome c Oxidase Particles. Deuterium magnetic resonance studies have recently been done with deuterated lipid–cytochrome *c* oxidase particles, and confirm the results discussed with the fluorine NMR studies. The details of these experiments have been published elsewhere (Dahlquist et al., 1977). For this deuterium experiment, lipid–protein particles were made consisting of 1-(16′,16′,16′-trideuteriopalmitoyl)-2-palmitoleoyl-PC in a lipid to protein ratio of 0.5:1 mg/mg. Whereas the fluorine magnetic resonance studies were done with sonicated preparations, these deuterium NMR studies were performed with samples of unsonicated dispersions.

Usually, a deuterium NMR spectrum of a dispersion of a lipid deuterated at a single position is characterized by a powder pattern spectrum showing two peaks separated by a frequency defined as the residual quadrupole splitting. This splitting arises from incomplete averaging of the carbon-deuterium bond over all possible orientations with respect to the applied field (Seelig and Seelig, 1974; Stockton et al., 1976). For the lipids used in our work, the larger the magnitude of the observed quadrupole splitting, the less complete the averaging of this motion over all directions. An example of this resonance absorption pattern is shown in Figure 6, which contains a spectrum of a dispersion of 1-(16′,16′,16′-trideuteriopalmitoyl)-2-palmitoleoyl-PC at 9 °C. The spectrum can be characterized by only one quadrupole splitting constant (2.8 kHz), and therefore indicates that the lipid, as seen by the behavior of the terminal methyl group of the hydrocarbon chain, is present in one lipid environment.

Figure 6 also displays the deuterium spectrum of lipid–protein particles of 1-(16′,16′,16′-trideuteriopalmitoyl)-2-palmitoleoyl-PC and cytochrome *c* oxidase at 4 °C. The spectrum is composed of a powder pattern characterized by at least two quadrupole splitting constants (2.6 and 4.6 kHz), which suggests that the enzyme affects the lipid bilayer to produce at least two classes of lipid which vary substantially by their hydrocarbon chain mobility.

Since these two lipid environments are directly observable in the deuterium spectra of these enzyme-containing bilayers, the rate of exchange of lipid between the environments is slower than the difference in residual splitting constants for the two states in radians/second (about 10^4 s^{-1}). Otherwise, we would have observed a spectrum characterized by a single residual quadrupole splitting parameter, which would have reflected the average of the restricted lipid and free lipid mobilities. Dahlquist et al. (1977) have also observed with deuterium NMR that the amount of restricted lipid relative to free lipid decreases with increasing temperature, indicating that the extent of hydrocarbon chain order induced by the enzyme is a phenomenon strongly dependent upon temperature.

Conclusions

Our experiments are intended to gain further information

about the properties assumed by a phospholipid bilayer when it contains an integral membrane protein. Accordingly, we find that the nuclear magnetic resonance studies on well-defined, reconstituted lipid–protein systems can yield a variety of data about membrane structure. First, the extent to which a membrane-bound enzyme restricts the hydrocarbon chain motions in the surrounding lipid can be observed. Second, the technique provides information about the rate of exchange of lipid between the more fluid and the more restricted hydrocarbon environments, both of which are present in an enzyme-containing bilayer. Third, by examining the effect of temperature upon the hydrocarbon chain mobilities of these bilayers, one can better understand the energetics which maintain the lipid–protein interactions in these model membrane systems.

Properties of the Lipid with Fluorine and Deuterium Lables. The model membrane chosen for these experiments consists of a well-defined, fluid phosphatidylcholine, and the enzyme cytochrome *c* oxidase. In all cases, the nuclear magnetic label, whether it be fluorine or deuterium, is present at one specific position on the hydrocarbon chain of the lipid. This labeled, saturated hydrocarbon chain is attached to the 1 position of the glycerol backbone, and the 2 position is occupied by the 9,10-cis unsaturated fatty acid with the same number of carbon atoms as the labeled fatty acid chain. Specific labeling of the lipid molecule ensures that the fluorine or deuterium nuclear magnetic resonance signals arise from nuclei at only one chemical position of the molecule. Therefore, if more than one type of NMR signal is seen with a given sample, the differences must be due solely to differences in the surrounding microscopic environment.

Placing the NMR label on the endogenous lipid avoids any problem that might arise from partitioning of an exogenous lipid probe preferentially into one of several lipid environments. For example, if the NMR label were present on a fatty acid, one would have to be concerned with the partitioning of the exogenous probe among the various phospholipid environments that are found in bilayers that contain integral membrane protein. In addition, the exogenous lipid probe may itself influence the bilayer properties by increasing the amount of that lipid environment which it prefers to reside in. Labeling the endogenous lipid avoids perturbations to the state of the bilayer.

The effect of the fluorine nuclei upon membrane structure is small. The fluorinated lipids form lipid bilayers whose thermodynamic properties, as judged by scanning calorimetry measurements of the phase transition temperature, are slightly different from the corresponding, unlabeled PC. Although these changes in phase transition temperature are the most sensitive indications of perturbations in bilayer structure, possible changes in enzymatic activity are more biologically relevant indicators of perturbations that are caused by the labeling of the PC. We have found that the PC's labeled with fluorine or deuterium do not alter the enzymatic activity of cytochrome *c* oxidase to any measurable degree when compared with the unlabeled PC analogues. Finally, other workers have found the fluorine-containing fatty acids to be biologically viable molecules, as they are readily assimilated into fatty acid auxotrophs of *Escherichia coli* (C. Ho, personal communication).

Reconstitution of Cytochrome c Oxidase with Labeled PC. The reconstitution procedures used with the fluorinated PC's yield small, lipid–protein particles with a nearly homogeneous lipid/protein ratio. The 9,10-cis unsaturated fatty acid in the PC of these particles ensures that the bilayer that is formed is above its solid–fluid phase transition temperature for all the sample temperatures examined in this study. Cytochrome *c* oxidase spans the bilayer (Henderson et al., 1977), suggesting that each monolayer contains about the same amount of protein surface area.

The size of the particles varies with the protein content of the bilayer. It is evident that the incorporation of any enzyme at all causes a marked increase in the size of the sonicated vesicles. None of the protein-containing particles is smaller than 300 Å, and the average vesicle size is 350 Å. Increasing the amount of protein relative to lipid causes a smaller increase in particle size, such that even at low lipid to protein ratios of approximately 0.4:1 (mg/mg), the preparations yield well-defined, nonaggregated particles of ~500 Å in diameter.

The dramatic changes in NMR line width observed over the range of lipid/protein ratios studied in our experiments cannot be sufficiently explained by the small increases in vesicle size. If vesicle tumbling alone were responsible for the line-width changes, one might expect the line widths to vary according to the ratio of the third power of the average vesicle diameter. Thus, for the two vesicle populations reported in this study, the line-width increase should be roughly $(500\ Å/350\ Å)^3$, or 2.9. The observed line width changes instead by a factor of six between a lipid/protein ratio of 1.2 and 0.4 (mg/mg). The role of vesicle size on the observed NMR line widths is still a matter of some controversy. For example, Sheetz and Chan (1972) report very little dependence of vesicle size upon observed line width. Thus, the calculated factor of 2.9 for the two preparations observed here is almost surely the maximum contribution vesicle tumbling can make to the observed line-width changes. As a result, it seems clear that the protein itself is a major determinant of the observed NMR line widths.

Characterization of Phospholipid Environments in Enzyme-Containing Bilayers. The nuclear magnetic resonance approach allows us to draw a number of conclusions regarding the interactions between phospholipid and cytochrome *c* oxidase.

(1) There exists more than one lipid environment in enzyme-containing bilayers. The spectra obtained with either fluorinated or deuterated lipid clearly show the simultaneous existence of at least two lipid environments which are distinguishable by the differences in their hydrocarbon chain motion. We have called the less mobile class of lipid "restricted" lipid, and it is that class of lipid which interacts strongly with the enzyme. The more mobile lipid class we have called "free" lipid, but its motion is not identical with lipid in bilayers without enzyme.

(2) The exchange of lipid between the free and restricted environments is slow. We have found that the temperature dependence of the observed fluorine NMR signal amplitude of a given sample is not consistent with a model of fast exchange of lipid between two discrete lipid environments. The deuterium magnetic resonance results also contain distinct NMR absorption patterns for a least two lipid environments in the multilayer system. If the lipid were rapidly exchanging between the various lipid environments, one would have observed an absorption pattern characterized by a single quadrupole splitting parameter whose value would be between those actually observed for the sample. Both arguments suggest exchange rates slower than 10^4–10^3/s.

(3) The amount of restricted lipid is temperature dependent. The studies of the NMR properties of both fluorinated lipid in sonicated, protein-containing vesicles and the deuterated lipid in unsonicated, protein-containing multilamellar structures (Dahlquist et al., 1977) demonstrate that the enzyme can influence the mobility of a larger amount of the lipid at lower temperatures. As the temperature is raised, more of the lipid

becomes spectroscopically indistinguishable from the free lipid.

Energy Differences among Lipid Environments. As long as significant amounts of the restricted and free lipids are present in the bilayer simultaneously, we can observe the equilibria that exist among them. The relative amounts of lipid in these environments can be changed and then restored to their original values by small changes in temperature away from and back to the original temperature, suggesting that the states observed meet the thermodynamic requirement of reversibility. At the temperatures studied, the ratios of lipid in the free and restricted environments are of the order of magnitude of one, meaning that the free-energy differences among the various environments are of the order of RT (a few kilocalories/mole). Since the equilibria are sensitive to temperature, there are clearly enthalpy differences as well among the more restricted and the less restricted states. Although an apparent enthalpy change could be determined by examining the relationship between the various equilibria and the temperature, the interpretation of such numbers would be difficult because we do not know the cooperativity of a transition between a more restricted lipid state and a less restricted one. The enthalpy change suggested by the temperature dependence of the equilibrium refers to the entire cooperative unit of hydrocarbon chains. The number of phospholipid molecules in that unit is under investigation, but not yet known.

Model of Lipid–Protein Interactions. The experimental data allow for the development of a more sophisticated model of lipid–protein interactions than those models of membrane behavior which suggests that an integral membrane protein diffuses rapidly through the plane of the fluid lipid bilayer. If indeed such fast lateral migration takes place, then the enzyme carries with it *at least* one boundary layer of lipid, if not more, the amount being highly dependent upon temperature. The length of time that this lipid remains associated with the enzyme, before being replaced by another lipid, is at least as long as the time required for a typical enzymatic reaction to occur. For example, the turnover number for cytochrome *c* oxidase is between 300 and 400 s^{-1} (Smith and Camerino, 1963). Thus, the composition of lipid around the enzyme remains the same during the time course of an enzymatic reaction. If certain lipids are required by the enzyme for the expression of optimal enzymatic activity, the enzyme is clearly capable of retaining these lipids for a long period of time relative to the time of the reaction process.

Several parameters are required to describe the amount of lipid retained by an enzyme in this slowly exchanging, restricted hydrocarbon chain environment. First, one must specify the temperature at which the measurements of the extent of the lipid–protein interactions are obtained. This is because an integral membrane protein can restrict the mobility of a greater amount of lipid at a lower temperature. The type of lipid used in the experiment must also be specified, since it appears that the longer the hydrocarbon chain length, the greater the amount of restricted lipid at a given temperature, and thus the smaller the overall fluidity of the membrane. Clearly, the "fluidity" of a biological membrane cannot be determined only by analyzing the types of hydrocarbon chains on the extracted lipid, although it is an important factor. Consideration must be given to the amount, and type, of intergral membrane protein, as the protein plays a key role in determining not only the dynamics of the lipid closely associated with it, but the dynamics of the lipid throughout the entire biological membrane.

We can now propose a model for the interaction of phospholipid with mitrochondrial cytochrome *c* oxidase which necessarily includes at least four different classes of lipid within the bilayer.

(I) Nonexchanging Lipid. It has been shown that preparations of cytochrome *c* oxidase always contain some phospholipid molecules which copurify with the enzyme (Awasthi et al., 1971). Robinson and Capaldi (1977) have shown that these six–ten molecules of phospholipid (per cytochrome *c* oxidase–heme aa_3 complex) are not removable by a variety of detergents. This lipid is also not exchangeable with a large molar excess of PC (Vik and Capaldi, 1977) and is either very tightly bound, or shielded from access by the bulk lipid. The labeled PC used in our reconstitution experiments does not access these nonexchanging lipid sites.

(II) Boundary Lipid. The boundary lipid (or annular lipid) refers to the remainder of the lipid in direct contact with the protein surface. So far we have not spectroscopically resolved the boundary layer from a larger component of lipid which also shows restricted mobility, and whose quantity is temperature dependent.

(III) Motionally Perturbed Lipid. This lipid is associated with the solvation layers of lipid extending several layers away from the boundary lipid. The motionally perturbed lipid and the boundary lipid together account for the slowly exchanging class of restricted lipid we observe in the NMR experiments. It is the motionally perturbed lipid which melts into the free lipid with increasing temperature. Our results indicate that at temperatures 10–20 °C above the lipid phase transition temperature, this class corresponds to about two or three solvation layers of lipid encircling the boundary lipid.

(IV) Free Lipid. The remainder of the bilayer, those regions of lipid many solvation layers away from the protein surface, is termed free lipid. However, it is clear that even this lipid is influenced by the presence of the protein and shows some change in hydrocarbon chain mobility when compared with bilayers without protein. The ratio of lipid to protein in real biological membranes is low enough such that the amount of free lipid is small. It is therefore unlikely that the properties of free lipid are ever very important for determining biochemical function unless substantial lateral phase separation occurs which would segregate bulk lipid from aggregated lipid–protein complexes.

Acknowledgments

We thank Dr. David C. Muchmore for the synthesis of the deuterated PC; Drs. Myer Bloom and James Davis of the University of British Columbia for their very helpful discussion; Steven Vik for performing the oxygen electrode assays of cytochrome *c* oxidase; and Jeanne Sweetland for the preparation of cytochrome *c* oxidase.

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Lipid Requirements for Cytochrome *c* Oxidase Activity†

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ABSTRACT: Cytochrome *c* oxidase depleted of endogenous lipid by detergent exchange has been reconstituted into vesicles with synthetic lipids of known head group and fatty acid composition and enzymic activities have been measured. No evidence for head group specificity was found. However, the enzyme does require the fluid environment provided by unsaturated fatty acids. The state of dispersion of the enzyme was found to affect the activities regenerated in reconstitution studies. The highest activities were obtained using lysolecithin containing an oleoyl fatty acid as the lipid component.

Cytochrome *c* oxidase, the terminal member of the respiratory chain, is a multi-subunit enzyme (Briggs et al., 1975; Downer et al., 1976), containing two hemes and two copper atoms (Kuboyama et al., 1972; Yonetani, 1961). The protein spans the mitochondrial inner membrane (Hackenbrock and Hammon, 1975; Schneider et al., 1972; Henderson et al., 1977) and probably exists as a dimer or four-heme complex (Robinson and Capaldi, 1977; Briggs and Capaldi, 1977; Henderson et al., 1977).

After isolation from the membrane, cytochrome *c* oxidase requires phospholipids or certain other amphiphiles for activity. The importance of different lipid head groups for activity has been examined in several laboratories (Brierley and Merola, 1962; Awasthi et al., 1971; Yu et al., 1975) but the results have been variable for reasons which were discussed earlier (Robinson and Capaldi, 1977). Our recent studies on the interaction of detergents with cytochrome *c* oxidase have provided evidence that the activity of this enzyme is sensitive to the fatty acid composition of bound amphiphile (Robinson and Capaldi, 1977). Here, we have measured the activity of cytochrome *c* oxidase in the presence of synthetic phospholipids of known head group and fatty acid composition. Our results indicate that cytochrome *c* oxidase requires phospholipids with unsaturated fatty acids for optimal activity. If a fluid lipid environment is provided, the enzyme is apparently insensitive to the phospholipid head group.

Materials and Methods

Cytochrome *c* oxidase was isolated from beef heart mitochondria as described by Capaldi and Hayashi (1972). Asolectin (95% soy phosphatides) was obtained from Associated Concentrates and used without further purification. Mitochondrial lipids were isolated from beef heart mitochondria as described by Rouser and Fleischer (1967). The following

† From the Department of Biology and the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received May 19, 1977.* This investigation was supported by National Science Foundation Grant No. PCM 74-00957 and Institutional Grant No. GM 00715 from the National Institutes of Health.