Phosphatidylcholine Exchange between the Boundary Lipid and Bilayer Domains in Cytochrome Oxidase Containing Membranes†

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ABSTRACT: A phospholipid spin label, 16-doxylphosphatidylcholine, is employed in a study of lipid–protein interactions in cytochrome oxidase containing membranes. Two methods are used to label the membranous cytochrome oxidase: dispersion in cholate with subsequent detergent removal, and fusion with vesicles of the pure phospholipid label in the absence of detergent. A fraction of the label is immobilized, which is calculated to fall in the range of 0.17–0.21 mg of phospholipid/mg of protein (0.15–0.19 after correction for lipids not extracted by chloroform–methanol). This narrow range of values is independent of methods of labeling, protein isolation, and lipid depletion within experimental error. When labeling by fusion is utilized, the patches of pure phosphatidylcholine spin label diffuse in the plane of the bilayer, become diluted, and demonstrate exchange with bound phospholipid. These observations are evidence that boundary lipid, as reflected by the partitioning of the phosphatidylcholine label, is in equilibrium with adjacent bilayer regions and that it consists of a relatively constant amount of phospholipid associated with the hydrophobic portion of the protein.

Many membrane proteins penetrate deeply into or completely through the phospholipid bilayer. It has been postulated, based on spin labeling data, that the hydrophobic surfaces of the integral membrane proteins are solvated by an immobilized layer of lipid (Jost et al., 1973a). Although this remains a working hypothesis, additional reports are accumulating that are consistent with the idea of a boundary layer or annulus (Warren et al., 1974; Stier and Sackmann, 1973; Traüble and Overath, 1973; Nakamura and Ohnishi, 1975; Brisson et al., 1975).

In the initial work on cytochrome oxidase in which the immobilized boundary lipid was experimentally identified, fatty acid spin labels were used to probe the lipid–protein interface, with the reservation that this required the assumption that the fatty acid molecule mimics the behavior of a phospholipid molecule (Jost et al., 1973a,b; Griffith et al., 1974). Lipid labels with some aqueous solubility (e.g., the fatty acid labels) are often labels of choice for labeling membranes since aqueous suspensions of membranes will pick up the label from a dry film, and the lipid labels readily intercalate in the bilayer. Phospholipid spin labels, while more closely resembling the native molecules, require fusion of spin label vesicles with membrane vesicles, use of the appropriate phospholipid exchange protein, or dispersal of the membrane in detergent in order to introduce the label with subsequent removal of the detergent.

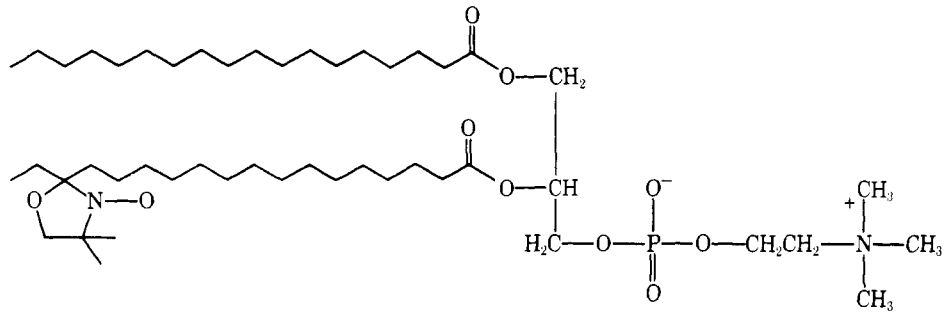
In this study the assumptions in our earlier studies of boundary lipid are tested using the phosopholipid spin label derived from 16-doxylstearic acid to determine the distribution of this phospholipid analogue in the membrane lipid domains. In addition, the earlier tentative interpretation that exchange occurs between boundary lipid and bilayer is more rigorously tested by fusing labeled phospholipid vesicles with membranous cytochrome oxidase and analyzing the equilibrium distribution of phosphatidylcholine in the major lipid domains.

Materials and Methods

All solvents were reagent grade. The phospholipid spin label 16-doxylphosphatidylcholine was synthesized from 16-doxylstearate (Syva) by standard methods (Selinger and Lapidot, 1966; Hubbell and McConnell, 1971). The stock solutions were routinely purified on Silica Gel G and stored at 5 mg/mL in ethanol at −20 °C. For the fusion experiments, the phospholipid spin label was repurified to assure freedom from possible contaminating 16-doxylstearic acid since slow hydrolysis of the phosphatidylcholine spin label is sometimes observed after prolonged storage. Cholic acid (Aldrich) was purified by recrystallization from ethanol and the salt formed by titrating with NaOH.

A lipid-deficient preparation of cytochrome oxidase, prepared by the method of Yu et al. (1975) and supplied to us by Drs. Tsoo E. King and Chang-An Yu, contained 11 nmol of heme *a*/mg of protein, 1.2 μg of total lipid phosphorus/mg of protein, and a specific activity of <0.05 nmol of O_2 uptake (nmol of heme *a*)$^{-1}$ s^{-1}. When reconstituted with mitochondrial lipids, this activity increased to 18–20 nmol of O_2 (nmol of heme *a*)$^{-1}$ s^{-1}, a level very similar to lipid-rich preparations (Yu et al., 1975). Aliquots of this preparation were stored at 20 mg/mL in 1% Emasol 1130–50 mM phosphate buffer, pH 7.4, in liquid nitrogen, and there was no apparent loss of the restorable activity during the time of storage. Immediately before use the Emasol was removed from the cytochrome oxidase by suspending in 1% cholate–50 mM phosphate buffer, bringing to 37% ammonium sulfate saturation at pH 7.4, centrifuging, and resuspending the pellet in 50 mM phosphate–1% cholate. A few of the experiments were performed with a sample of cytochrome oxidase prepared by the method of Sun et al. (1968) and provided by Dr. R. A. Capaldi. This sample contained 0.50 mg of lipid phosphorus/mg of protein and had approximately the same activity as the cholate preparation under the same assay conditions (Kuboyama et al., 1972). Mitochondrial lipids were extracted from an aqueous homogenate of beef heart mitochondrial paste using chloroform and methanol ratios as given by Bligh and Dyer (1959) and were stored under nitrogen at 30 mg/mL in chloroform–methanol at −20 °C. Mitochondrial lipids prepared in this

† From the Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received December 12, 1976.* This study was supported by United States Public Health Service Grant CA 10337 and United States Public Health Service RCDA Grant CA 23359 (to O.H.G.).



fashion contain a small amount of protein (~8% w/w).

To ensure reproducible aliquots for lipid phosphorus and protein determinations, a portion of the recovered ESR[1] sample was solubilized in 1% sodium dodecyl sulfate. The presence of this detergent does not interfere with the lipid extraction procedure and phosphorus or protein determinations. Protein was determined by the method of Lowry et al. (1951) as modified by Miller (1959), using Pentex fatty acid poor bovine serum albumin (fraction V), with $\epsilon_{279}^{1\%} = 6.67$ (Sterman and Foster, 1956) as the standard. Lipid phosphorus was measured by the method of Lowry and Tinsley (1974) using adenosine 5′-phosphate, disodium salt (P-L Biochemicals) as the phosphorus standard. For lipid phosphorus measurements, the detergent solution was extracted with chloroform–methanol (Bligh and Dyer, 1959). This extraction fails to remove all of the lipid phosphorus from cytochrome oxidase, similar to the results obtained by Awasthi et al. (1971). These workers found that addition of base was necessary to recover all lipid phosphorus, so total lipid phosphorous was determined by including ammonium hydroxide in the chloroform–methanol extraction. Two-dimensional thin-layer chromatography using the solvent systems of Parsons and Patton (1967) was used to characterize the phospholipids that were nonextractable with neutral chloroform–methanol, and to monitor the composition of the mitochondral lipids associated with the cytochrome oxidase. Aliquots of the ESR samples to be used for thin-layer chromatography were taken before sodium dodecyl sulfate solubilization for total lipid and protein determinations since sodium dodecyl sulfate interferes with the visualization of the chromatogram when using sulfuric acid spraying and charring. Activity was measured by oxygen uptake as described by Kuboyama et al. (1972).

Preparation of Experimental Samples. For the labeling conditions involving cholate, a mixture of mitochondrial lipids and 20–75 μg of phosphatidylcholine spin label was dried under nitrogen to form a thin film on the bottom of a small vial. The amount of mitochondrial lipids added ranged from 0.25 to 2.25 mg, depending on the desired final lipid-to-protein ratio. A dispersion of 5 mg of cytochrome oxidase in ~0.4 mL of 1% sodium cholate (50 mM phosphate buffer, pH 7.4) was added to the lipid film and mixed by mild bath sonication on ice for 5 min. The mixture of protein and lipid in buffered cholate solution was exhaustively dialyzed (18 h, 4 changes, with stirring) in the cold room (4–6 °C) against 10 mM Tris buffer, pH 7.4, to remove cholate. The sample was concentrated and any free lipid removed by layering on 10% sucrose, 10 mM Tris, pH 7.4, and centrifuging at 58 000*g* at 4 °C for 2–3 h. Using [^{14}C]cholate the estimated residual cholate after dialysis and centrifugation is less than 30 μg of cholate/mg of protein. The samples were recovered after the ESR experiment and a portion suspended in ~1 mL of 1% sodium dodecyl sulfate for the protein and lipid phosphorus determinations.

For the labeling method involving fusion (cf. Grant and McConnell, 1973), 0.15 mL of cytochrome oxidase (3 mg of protein) in 10 mM Tris-HCl buffer (pH 7.4) was mixed with 80 μL of a bath sonicated aqueous dispersion of 16-doxylphosphatidylcholine (0.24 mg of spin label/mL) and made up to a final volume of 1 mL with buffer (10 mM Tris-HCl–5% glycerol–2.5 mM sodium citrate–50 mM sodium phosphate, pH 7.4). This mixture was incubated 5 h at 37 °C and stored at −20 °C for 3 days. Before the final ESR data were taken, any excess spin label was removed by centrifuging the sample through 10% sucrose. Only very subtle spectral changes were seen when comparing spectra before and after centrifugation through sucrose, which is an indication that fusion was fairly complete under these conditions.

ESR spectra were recorded on a Varian E-9 spectrometer interfaced with a 32K Varian 620/L100 computer to digitize the data. Most of the experiments were run with a Varian Field/Frequency Lock used in the lock mode so that the spectra were automatically in register. Data analysis followed the procedures of Klopfenstein et al. (1972), but additional criteria for baseline adjustment and subtraction endpoints were introduced. Any systematic deviation of the baseline (i.e., average over first 5 G compared with average over the last 5 G) of more than 1% of the centerline height was deemed indicative of the presence of visually undetectable spin–spin exchange and the sample discarded. Such criteria are necessary for quantitative data treatment since undetected line broadening due to spin exchange contributes to quantitative errors. ESR instrument settings: microwave power, 5 mW; scan time, 8–16 min; scan range, 100 G; filter time constant, ≤0.3 s; modulation amplitude, ≤1 G.

Results

The ESR spectral line shapes are strongly dependent on the phospholipid-to-protein ratios in membrane samples. Figure 1a illustrates a typical spectrum of a cytochrome oxidase sample of intermediate lipid content. We analyze this composite spectrum in terms of the contribution of a bound component representing lipid association in the absence of bilayer (Figure 1b). Spectral deconvolution to determine the fraction of the bound component is performed by subtracting spectrum b from spectrum a, titrating to an endpoint that requires there be no negative inflections in the baseline at amplifications of at least 32-fold. This procedure yields the fraction of the phospholipid spin label that is immobilized, $\chi_b = N_b/N_t$. In this example $\chi_b = 0.58$. This procedure also provides the difference spectrum c which is characteristic of fluid bilayer, although there is some residual influence of the protein. This effect is seen in the line width of the difference spectrum, where slightly broader lines are seen at lower lipid/protein ratios.

[1] Abbreviations used: ESR, electron spin resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

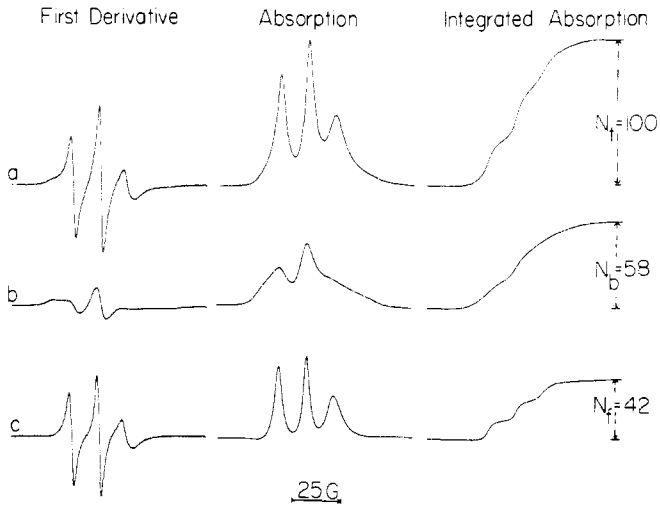


FIGURE 1: Typical ESR spectral analysis of 16-doxylphosphatidylcholine spin label in cytochrome oxidase membranes. (a) Experimental spectrum: cytochrome oxidase vesicles containing 0.32 mg of phospholipid/mg of protein. (b) Bound line shape: lipid-poor cytochrome oxidase containing 0.11 mg of phospholipid/mg of protein. (c) Difference spectrum: experimental minus bound component. Integration of the experimental first derivative spectrum (first column) yields the absorption spectrum (second column). A second integration (third column) gives the relative absorption of each first derivative spectrum and is proportional to the amount of spin label present. All spectra in each column are normalized to the relative absorption, but between columns scaling down by a constant is necessary for plotting. N_t, N_b, and N_f are the relative numbers in arbitrary units of the total, bound, and fluid spin labels, respectively.

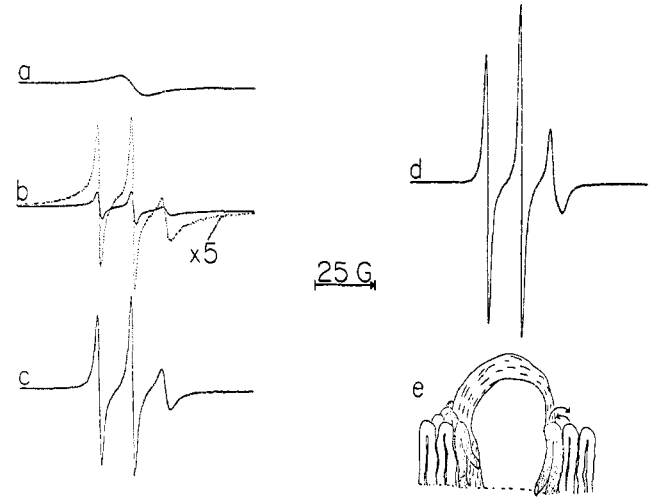


FIGURE 2: Fusion of 16-doxylphosphatidylcholine spin label vesicles with cytochrome oxidase vesicles of high lipid content (0.50 mg of phospholipid/mg of protein). The ESR spectra are (a) spin label vesicles of 16-doxylphosphatidylcholine in buffer, (b) 2.5 h after addition of sample a to unlabeled cytochrome oxidase and incubation at 37 °C, (c) sample b after further incubation and discontinuous sucrose gradient centrifugation (see Materials and Methods), (d) the same spin label in mitochondrial lipid vesicles without cytochrome oxidase. All spectra were recorded at 24 °C and are normalized to reflect the same integrated absorption. Spectrum b is also shown amplified five times (dotted lines) to make the line shape easier to see. The diagrammatic sketch (e) is of a protein embedded in the phospholipid bilayer. Boundary lipid (shaded) is immobilized on the hydrophobic surfaces of the protein complex, but exchanges with the adjoining fluid bilayer, as indicated by the arrows.

Since the line width in the bilayer (i.e., difference spectrum) is a function of motion, the average motion appears to increase as the extent of the bilayer increases, although this assumes that the distribution of the spin label in the bilayer is independent of the extent of the bilayer. Bilayer that contains very little protein (~8% w/w) gives spectrum 2d that, when scaled proportional to spectrum c, has 1.7 times the center line height of spectrum c. That is, the *average* bilayer motion is somewhat higher in pure lipids than is seen when protein is present, even with the boundary lipid component removed (Jost et al., 1973b).

A number of different samples containing the phospholipid spin label were analyzed by this procedure and the results are summarized in Table I. The samples differ in isolation procedures, reassociation with phospholipids, and labeling procedures as indicated. There are two cytochrome oxidase preparations (I and II). Preparation I is the lipid-poor preparation of Yu et al. (1975) and the small amount of natural phospholipids associated with this preparation is largely diphosphatidylglycerol, similar to that seen by Awasthi et al. (1971). For labeling these lipid-poor samples, we have found it necessary to add some mitochondrial lipid, even when only the bound spectrum was to be obtained (Table I), in order to completely eliminate spin exchange effects in the final spectrum. Preparation II is the lipid-rich preparation of Sun et al. (1968). The major phospholipids are phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol from the inner mitochondrial membrane.

There are essentially two labeling methods. The first uses dispersion in detergent to solubilize the lipids, and the detergent is then removed by dialysis. Dialysis under these conditions also removes some phospholipid (on the order of 20–30% of the total phospholipid when the lipid content is high), resulting in some reduction of lipid level. The second method utilizes fusion of aqueous phospholipid spin label vesicles with lipid-rich cytochrome oxidase in the absence of detergent. In both methods any remaining free lipid vesicles were removed by centrifugation through sucrose. The results of the spectral analysis are given in the fifth column of Table I. The ESR data are then combined with data from chemical analyses to calculate the amount of bound lipid.

Representative ESR spectra from the fusion labeling experiments are shown in Figure 2. The top spectrum (Figure 2a) is of pure phospholipid spin label vesicles in buffer. Electron-electron spin exchange in high local concentrations of spin label causes this well-known broad line shape (Jost and Griffith, 1971; Devaux and McConnell, 1972). This control sample was carried through the same incubation conditions as the cytochrome oxidase samples and there was no change in line shape or evidence of signal decay. In the parallel experiment these spin label vesicles are incubated with lipid-rich cytochrome oxidase in the absence of detergent. Midway through the incubation spectrum b (Figure 2) was obtained. The three lines, with a line shape and coupling constant characteristic of bilayer, are combined with the exchange-broadened line of undiluted spin labels. This broad line shape contributes a large fraction of the absorption as shown by the line height. This spectrum multiplied by five is shown with dotted lines, so that the line shape is more clearly evident. Upon completion of incubation (see Materials and Methods), the spectrum is visually indistinguishable from Figure 2c. However, the actual spectrum c was recorded after an additional step of sucrose density centrifugation to remove residual free phospholipid vesicles. For comparative purposes, the dilute phospholipid spin label in vesicles of mitochondrial lipids is shown in Figure 2d, scaled to show the same spin label concentration as spectrum Figure 2c. This control (Figure 2d) contains about 8% bound component. This value probably represents a combination of label bound by the chloroform–methanol-soluble protein contami-

TABLE I: Fraction of Phosphatidylcholine Spin Label Immobilized in Different Cytochrome Oxidase Preparations.[a]

Initial lipid[b]	Lipid added[c]	Labeling method	Final lipid[d] (C_t)	Fraction immobilized[e] (χ_b)	Bound lipid[f] $C_b = \chi_b C_t$	Bound lipid (corr for nonextractable lipid) $C_b = \chi_b(C_t - 0.04)$
(1) 0.04 (I)	0.28	Cholate	0.31	0.58	0.18	0.16
(2) 0.04 (I)	0.37	Cholate	0.32	0.53	0.17	0.15
(3) 0.04 (I)	0.44	Cholate	0.35	0.51	0.18	0.16
(4) 0.50 (II)	0.006	Fusion	0.59	0.32	0.19	0.18
(5) 0.50 (II)	0.01	Cholate	0.36	0.58	0.21	0.19
(6) 0.04 (I)	0.06	Cholate	0.10	1.0		
(7) 0.11 (II)	0.01	Cholate	0.11	1.0		

[a] All lipid data are expressed as mg of phospholipid/mg of protein, assuming an average phospholipid molecular weight of 775. [b] Initial phospholipid content of cytochrome oxidase before adding labeled and unlabeled phospholipid (I, lipid-poor preparation of Yu et al., 1975; II, lipid-rich preparation of Sun et al., 1968). [c] Phospholipid added, including phospholipid spin label (mg of phospholipid/mg of protein). Samples 1-3 and 6, the added lipids were mitochondrial lipids plus spin label; samples 4, 5, and 7, only the spin label was added. [d] Lipid phosphorus and protein determined on aliquots of the sodium dodecyl sulfate solubilized ESR sample. [e] Obtained by spectral subtraction and integration as in Figure 1; all values are estimated to be within ±10%. [f] C_t = total mg of phospholipid/mg of protein obtained by chemical analysis on the ESR sample; χ_b = fraction of immobilized spin label; C_b = calculated amount of immobilized lipid/mg of protein.

nants and the inevitable uncertainty in the precise endpoint. Spectral analysis of Figure 2c indicates it is composite, composed of 32% of the immobilized component (Figure 1b) and 68% fluid bilayer (see Table I, line 4). There is no detectable residual line broadening from spin exchange in the final line shape from the cytochrome oxidase labeled by fusion.

Discussion

Several conclusions can be reached on the basis of these data. First, it is apparent that the *phosphatidylcholine* spin label is reporting a second lipid environment when the protein is present. Second, the approximate amount of this immobilized lipid remains constant with respect to the protein (over a twofold variation in total lipid/protein ratio). In addition, the actual amount represented by this bound component is on the same order as that seen with the fatty acid spin label. The essential data are given in the next to last column of Table I. The amount of immobilized lipid ranges from 0.17 to 0.21 mg of phospholipid/mg of protein. This is a very narrow range considering the variety of preparations, sample manipulations, and labeling conditions employed. The amount of boundary lipid measured appears to be relatively independent of several variables. It is in the same range whether lipid depletion is achieved by acetone extraction (Jost et al., 1973a) or cholate treatment, or whether the final lipid level is reached by lipid depletion or lipid restoration, providing the final sample contains more than ~0.2 mg of phospholipid/mg of protein. In the present study the amount of boundary lipid is roughly the same for cytochrome oxidase that is prepared using cholate (Yu et al., 1975) or Tritons (Sun et al., 1968) as the detergent, and it is similar for the two labeling methods, dispersion in cholate or fusion in the absence of detergent. The values here are similar to the range found using the 16-doxylstearic acid spin label, which was 0.17-0.23 (uncorrected for nonextractable lipid, Jost et al., 1973a). These observations help establish the validity of the interpretation of earlier work employing fatty acid labels, i.e., that bound lipid consists of a relatively constant amount of lipid associated with the hydrophobic surfaces of the protein.

The last column of Table I shows the bound lipid when the total lipid is adjusted for lipid that is not extracted by the usual chloroform-methanol procedure. It was reported by Awasthi et al. (1971) that some residual phospholipid remained associated with cytochrome oxidase after repeated extraction and could be extracted only by using chloroform-methanol made basic with ammonium hydroxide. We have also confirmed their observation that this relatively nonextractable lipid is largely diphosphatidylglycerol. Adjusted values for the bound lipid (last column, Table I) are based on the assumption that this tightly held lipid fraction (~0.04 mg of phospholipid/mg of protein) is not exchangeable with the bilayer and may even be a form of captive lipid, i.e., integral to the structure of the complex (Griffith and Jost, 1977). These adjusted values of boundary lipid are somewhat lower, but the essential conclusions are the same, that is, that the protein immobilizes a relatively constant amount of phospholipid at the protein-lipid interface, as indicated diagramatically in Figure 2e. The binding constants are unknown, and if boundary phospholipids of different structure (either side chain or polar head group) have slightly different binding constants, the phosphatidylcholine spin label may well not be reporting all binding sites. The value we report for immobilized phospholipid necessarily depends on the assumption that the phosphatidylcholine spin label is reflecting the behavior of naturally occurring phospholipid.

The experiment involving fusion of phospholipid spin label vesicles with the detergent-free cytochrome oxidase vesicles provides further insight into the exchange of lipids between boundary lipid and fluid bilayer regions. The previous data are consistent with the interpretation that the boundary and bilayer regions are adjacent and that exchange occurs between them. However, because of the monomeric water solubility of the fatty acid spin label, the previous data did not rule out the possibility that the lipid binding sites were surrounded by protein, and the label exchanged between the bound regions and the bilayer via the aqueous solvent. The monomeric solubility of the phospholipid spin label is negligible. Labeling by fusion produces an instantaneous large local concentration of spin label. Only by lateral diffusion and exchange can this label become diluted, occupy boundary sites, give the equilibrium spectrum shown in Figure 2c, and give the data (Table I) showing immobilized and bilayer components. The final calculated amount of bound lipid in the fusion experiments is consistent with the values obtained using detergent labeling. All of these data are in agreement that the immobilized lipid is at the lipid-protein interface. These observations exclude

the possibility that the bound lipid reflects protein regions accessible only via the aqueous phase. It also helps establish that boundary lipid (as reflected by phosphatidylcholine partitioning between the two lipid domains) is associated with the hydrophobic surfaces of the protein and that the immobilized lipid is in equilibrium with the adjacent bilayer regions. The exchange between boundary and bilayer lipid, however, must be slow on the ESR time scale to yield the composite spectrum observed.

Acknowledgments

We are pleased to acknowledge the expert technical assistance of Debra McMillen and useful discussions with Dr. Jaakko Brotherus. We thank Drs. Tsoo E. King and Chang-An Yu for the cholate prepared cytochrome oxidase and Dr. Roderick A. Capaldi for the Triton prepared cytochrome oxidase.

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